Cloning and regulation of the rat bradykinin B_1 receptor gene

by

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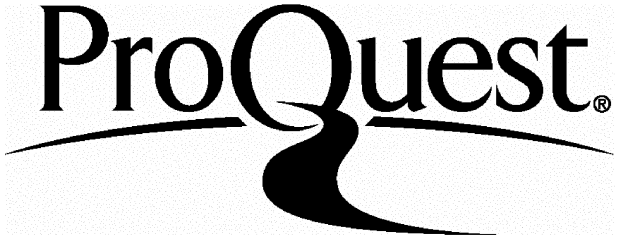

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Abstract

Responses to vasoactive peptides are mediated by the action of two receptors, bradykinin B_1 (BKB_1) and bradykinin B_2 (BKB_2), which belong to the superfamily of G-protein coupled receptors. A role for inducible BKB_1 receptors in pathophysiology has been demonstrated using pharmacological studies in a number of models of tissue injury and in response to specific inducing agents. This study describes the cloning of the rat BKB_1 receptor gene and investigation of the molecular mechanisms involved in the inducible receptor expression.

The first part of this thesis describes the isolation and characterisation of genomic and cDNA clones for the rat BKB_1 receptor. This has allowed detailed analysis of the receptor protein coding sequence and determination of the BKB_1 receptor genomic structure. Primer extension analysis and RACE studies have been used to identify the transcription initiation sites and the 3' cDNA ends and provide evidence for alternative splicing of the BKB_1 receptor transcript. Furthermore, using the rat BKB_1 receptor as a species-specific probe Northern blot analysis has established a distinct expression profile for the receptor mRNA in rat tissues and cultured cell lines.

BKB_1 receptor expression has been examined by Northern blot analysis in JTC-19 cells following the addition of a number of inducing agents. The increase in BKB_1 receptor mRNA levels following the addition of the pro-inflammatory cytokine, IL-1β, and the bacterial endotoxin, LPS, were found to result from an increase in transcription with no change in mRNA stability. Examination of the effects of specifc inhibitors show that multiple signal transduction pathways, including PI 3-kinase activation and NF-κB-dependent and independent pathways are involved in the IL-1β and LPS-mediated BKB_1 receptor transcriptional activation. Reporter gene constructs and electromobility shift assays have been used to delineate important elements involved in the transcriptional regulation of the BKB_1 receptor gene.

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Abbreviations

ACE	Angiotensin converting enzyme
ATF	Activating transcription factor
AP	Activator protein
BAECs	Bovine arterial endothelial cells
BCG	Bacillus Calmette-Guerin
BK	Bradykinin
bp	Base pair
BSA	Bovine serum albumin
cAMP	cyclic adenosine 3' 5'-monophosphate
CBP	CREB binding protein
cDNA	Complementary deoxyribonucleic acid

C/EBP	CCAAT/Enhancer binding protein
cfu	Colony forming unit
CGRP	Calcitonin-gene-related-peptide
COX	Cyclo-oxygenase
CRE	cyclic AMP-response element
CREB	cAMP-response element binding protein
CREM	cAMP-response element modulator
Da	Dalton
DEPC	Diethyl pyrocarbonate
dbcAMP	Dibutyryl cAMP
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRG	Dorsal root ganglia
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycol Glycol-bis(β-aminoethyl Ether)
EMSA	Electromobility shift assay
ERK	Extracellular signal-related kinase
FBS	Foetal bovine serum
G,A,T,C	Guanine, Adenosine, Thymine, Cytosine
G-protein	Guanine nucleotide binding protein
HAT	Histone acetyltransferase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMW	High molecular weight
IFNγ	Interferon gamma
IKK	IκB kinase
IL-1	Interleukin-1
IL-1α	Interleukin-1alpha
IL-1AcP	Interleukin-1 accessory protein

IL-1β	Interleukin-1beta
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IRAK	IL-1 receptor-activated kinase
IP	Inositol triphosphate
JNK	c-Jun N terminal kinase
kb	Kilobase
KID	Kinase inducible domain
LMW	Low molecular weight
LB	Luria Bertani
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAP3K	MAP kinase kinase kinase
MDP	Muramyl-dipeptide
MEK	MAP-kinase /ERK kinase
MEKK	MEK kinase
MMLV	Moloney murine leukemia virus
MOPS	3-[N-morpholino]propanesulphonic acid
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NIK	NF-κB-inducing kinase
NF	Nuclear factor
NF-κB	Nuclear factor-kappaB
ORF	Open reading frame
PEA-3	Polyomavirus enhancer A-binding protein-3
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidinedithiocarbamate
pfu	Plaque forming unit

PI	Phosphatidylinositol
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonyl fluoride
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress activated protein kinase
SCG	Superior cervical ganglia
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SP-1	SV40 protein-1
SSC	Sodium saline-citrate buffer
SV40	Simian virus 40
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
TE	Tris-EDTA buffer
TIP	TNF/IL-1 activated protein
TIS	Transcription initiation site
TLR	Toll-like receptor
TM	Transmembrane
TNF-α	Tumour necrosis factor-alpha
TRAF	TNF receptor-associated factor
Tris	Tris[hydroxyemethyl]aminomethane
UTR	Untranslated region
VSMCs	Vascular smooth muscle cells
v/v	Volume per volume ratio
w/v	Weight per volume ratio

Chapter 1

Introduction

The existence of distinct bradykinin (BK) receptors has been known for over twenty years. These receptors, termed bradykinin B_1 (BKB_1) and bradykinin B_2 (BKB_2), mediate the effects of kinins and form part of the kallikrein-kinin system. However, most of the *in vivo* effects of kinins are mediated by the BKB_2 receptor. It is now apparent that the BKB_1 receptor is up-regulated following certain types of tissue injury and in response to specific inducing reagents. This study investigates the rat BKB_1 receptor; its sequence, gene structure and mechanism of regulation. This chapter will describe the kallikrein-kinin system, with particular emphasis on the classification of the BK receptors. In addition, it will also describe evidence for the *in vitro* and *in vivo* up-regulation of the BKB_1 receptor and discuss the pathophysiological implications of this phenomenon. Furthermore, recent reports characterising the regulatory mechanisms of the human BKB_1 receptor gene are discussed, with an introduction to the possible signal transduction pathways involved.

1.1 Kinins

The first reports relating to the kinin system refer to a substance in rabbit and human urine that caused hypotension in anesthetised dogs (Abelous and Bardier, 1909). This substance believed to be derived from the pancreas, was named kallikrein from the

Greek word for pancreas, kallikreas. In 1937 Werle and colleagues incubated kallikrein with plasma and found a substance capable of contracting the guinea-pig ileum and causing hypotension, this was identified as the peptide kallidin. Snake venom incubated with plasma leads to the formation of a substance causing a slow, delayed contraction of the guinea-pig ileum, this substance was named BK from the Greek for movement, *kinin* and slow, *brady* (Rocha E Silva *et al.,* 1949).

1.1.1 Kinin formation

The kinins (defined as BK-related peptides) are formed by the action of proteolytic enzymes called kallikreins on high molecular weight precursors called kininogens.

1.1.1.1 Kininogens

In mammals three kininogens have been characterised to date: high molecular weight (HMW) kininogen present in the blood, low molecular weight (LMW) kininogen present in the blood and tissues and T-kininogen which is found exclusively in rats. (Habermann, 1963; Suzuki *et al.,* 1967; Jacobsen, 1966; Okamoto & Greenbaum, 1983). In humans and rats HMW-kininogen and LMW-kininogen are generated by alternative splicing of the gene transcript (Kitamura *et al.,* 1983). Two separate genes encode T-kininogens, called T-I and T-II (Enjyoji *et al.,* 1988). The kininogens are single-chain glycoproteins, possessing an amino-terminal heavy chain, a COOH-terminal light chain with a kinin moiety between the two polypeptides that are bridged by a single disulphide loop (Kellermann *et al.,* 1988). The heavy chain, which has the same basic structure in both HMW and LMW kininogen has the ability to inhibit cysteine proteases (Sueyoshi *et al.,* 1985). The light chain of HMW kininogen contains a domain rich in histidine, proline and lysine which binds to damaged endothelial surfaces, crystals and degraded

cartilage products. When plasma kallikrein releases the kinin moiety from HMW kininogen, the light chain enhances clotting through the sequential activation of clotting factors (Kaplan, 1978). In addition, the light chain contains the site that binds plasma kallikrein or clotting factor XI (Sugo *et al.,* 1980). Both the heavy and light chains participate in the binding of HMW kininogen to endothelial cells, platelets and neutrophils (Jiang *et al.,* 1992; Asakura *et al.,* 1992; Meloni *et al.,* 1992; Wachtfogel *et al.,* 1994). The function of the light chains in LMW and T-kininogens remains to be determined.

1.1.1.2 Kallikreins

Plasma and tissue kallikreins exist as large inactive precursors (prekallikreins). Once activated they act on two basic amino acid residues within the kininogen molecules to release either BK or kallidin. Plasma pre-kallikrein is secreted by hepatocytes and circulates in the plasma as a heterodimer complex bound to HMW kininogen (Mandle *et al.,* 1976). Plasma kallikrein releases the nonapeptide BK from HMW kininogen by hydrolysis of Lys-Arg and Arg-Ser bonds. LMW kininogen is a poor substrate for plasma kallikrein, however BK can be generated from this kininogen in the presence of neutrophil elastase (Sato and Nagasawa, 1988). Activation of plasma kallikrein is dependent upon coagulation factor XII (Hageman factor). Activation of factor XII occurs endogenously by contact with collagen, cartilage, basement membrane material or endotoxin (Proud and Kaplan, 1988). Activated factor XII converts plasma pre-kallikrein to kallikrein and subsequently BK is released from HMW kininogen. Kallikrein also has a positive feedback activating factor XII directly.

Tissue kallikrein releases the decapeptide kallidin by hydrolysis of Met-Lys and Arg-Ser bonds in both HMW and LMW kininogen molecules. In rodents however kallidin is not formed. Deoxyribonucleic acid (DNA) sequence analysis of rat and mouse kininogen genes reveals that the amino acid immediately upstream of BK is arginine not lysine, thus preventing the formation of kallidin (Furoto-Kato *et al.,* 1985, Hess *et al.,* 1996). Tissue kallikreins are encoded by a multigene family, the number of functional genes varies between species from 14 in the mouse, to nine in the rat and three in humans. The human tissue kallikreins (hK1 to hK3) are widely distributed throughout the body. The precise mechanism for the activation of tissue kallikreins is unclear, however they can be activated by trypsin, plasmin or plasma kallikrein (Proud and Kaplan, 1988). The three human tissue kallikreins differ in their efficiency of generating biologically active kinins. hK1, the classical tissue kallikrein, is highly effective, whereas hK3 is inactive and hK2 is of intermediate potency (Deperthes *et al.,* 1997). There have been reports that other proteases generated in the inflammatory response may be able to liberate kinins from kininogen: these include mast cell tryptase (Proud *et al.,* 1987), calpains (Higashiyama *et al.,*1986) and eosinophil cationic protein (Venge *et al.,* 1979). The mechanisms of kinin formation by plasma, tissue and cellular pathways are shown in Figure 1.1.

Three enzymes have been described that release T-kinin (Ile-Ser-BK) from T-kininogen these are T-kininogenase (Xiong *et al.,* 1990), endopeptidase K (Damas and Adam, 1985) and acid proteinase (Bedi *et al.,* 1983).

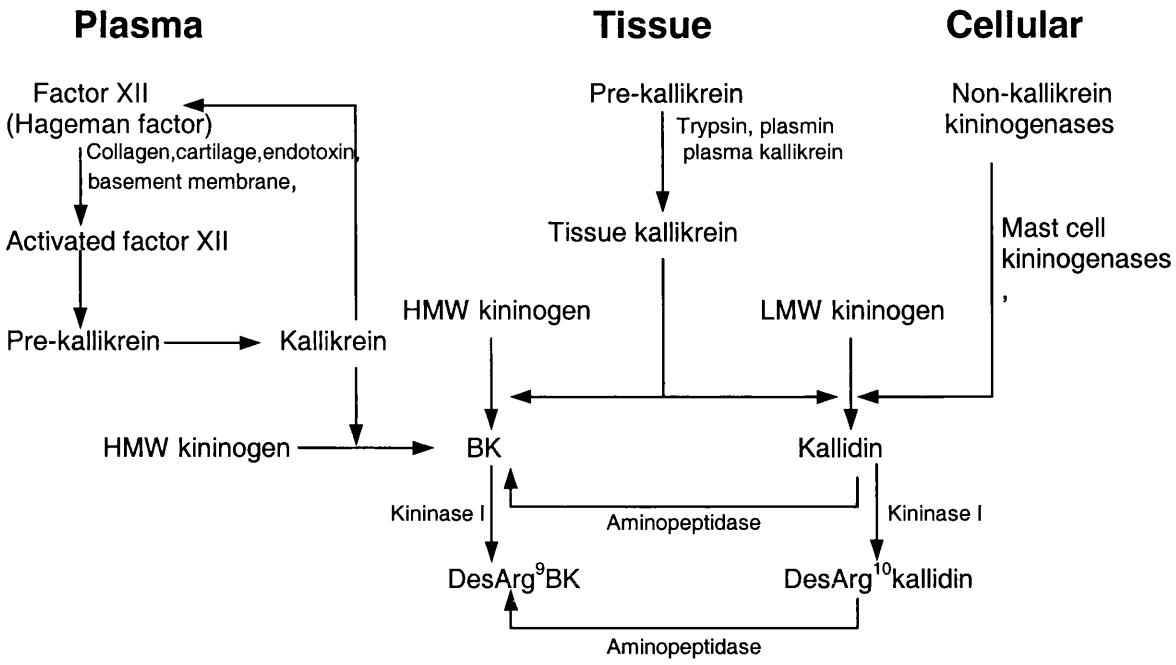


Figure 1.1 Mechanisms of kinin formation via the plasma, tissue and cellular cascades. HMW = high molecular weight; LMW = low molecular weight.

1.1.2 Kinin degradation

The kinin degrading enzymes are called kininases and are divided into two families, kininase I and kininase II.

Kininase I is a family of enzymes consisting of carboxypeptidase N and M (Bhoola *et al.*, 1992). Carboxypeptidase N is synthesised in the liver and accounts for about 90% of the BK degradation. This enzyme removes the C-terminal arginine from the native kinins thereby forming desArg^{9}BK and desArg^{10}kallidin, the BKB_1 receptor agonists. Carboxypeptidase M is predominantly membrane bound and its mechanism of action is the same as carboxypeptidase N.

Kininase II enzymes remove the terminal dipeptide -Phe-Arg from the kinins resulting in the production of inactive fragments. The most abundant kininase II enzyme is

angiotensin converting enzyme (ACE). ACE is predominantly a surface enzyme located on the luminal membrane of endothelial cells which explains the extensive pulmonary inactivation of kinins. The affinity of ACE for desArg^{9}BK is over 100 times lower than the affinity for BK (Ward *et al.,* 1991). Neutral endopeptidase, another kininase II enzyme, cleaves two bonds in BK, the Pro^7-Phe^8 and Gly^4-Phe^5 bond (Zolfaghari *et al.,* 1989).

Other enzymes also play a role in degradation of kinins *in vivo* since BK degradation is only reduced by 50% in rat plasma in the presence of inhibitors to the kininases described above (Ishida *et al.,* 1989a,b). Aminopeptidase P which cleaves the Arg^1-Pro^2 bond may also contribute to inactivation of BK (Ryan *et al.,* 1994; Ward, 1991). Aminopeptidase M, present in plasma hydrolyses kallidin and desArg^{10}kallidin into BK and desArg^{9}BK respectively (Proud *et al.,* 1987). This reaction is pharmacologically neutral for the BKB_2 agonists since BK and kallidin have equivalent potencies at the BKB_2 receptor. However the BKB_1 agonist desArg^{9}BK has much lower affinity for the BKB_1 receptor in some species, in these cases it may represent a relative inactivation of the BKB_1 receptor agonist. The site of action of the kininases is shown in Figure 1.2.

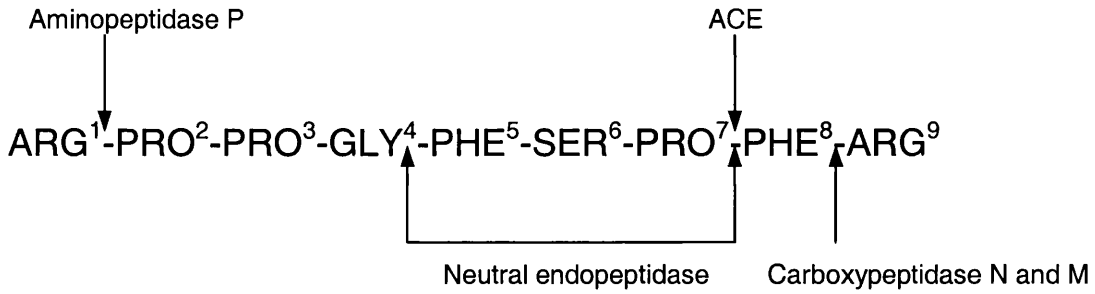


Figure 1.2 Proposed enzymatic cleavage sites for BK. ACE = angiotensin converting enzyme.

1.1.3 Biological levels of the kinins

Accurate determination of biological kinin levels has been difficult to assess due to the difficulty in preventing artifactual generation and degradation of the kinins during extraction. Methods for measurement of kinin levels have used immunological immunoassays (Odya *et al.,* 1983; Raymond *et al.,* 1995; Decarie *et al.,* 1994). Venous blood sampled from humans showed a higher concentration of desArg^{9}BK (204 pg/ml) compared to BK (67 pg/ml) (Odya *et al.,* 1983). Raymond and co-workers (1995) showed an increase in BK and desArg^{9}BK in rabbit plasma activated by kaolin, which was increased in the presence of the ACE inhibitor, enalaprilat. In addition, animals pre-treated with lipopolysaccharide (LPS) exhibited an increase in plasma desArg^{9}BK which was potentiated in the presence of ACE inhibitor. However these measurements may not reflect the tissue kinin levels. Interestingly, immunoreactive kinin levels are increased in carrageenan-induced oedema in rats where a role for the inducible BKB_1 receptors has been proposed (see section 1.4) (Damas *et al.,* 1990; Burch and DeHaas, 1990; Decarie *et al.,* 1996). The maximal concentration for desArg^{9}BK was greater than for BK (Burch and DeHaas, 1990; Decarie *et al.,* 1996) and the half life for desArg^{9}BK is greater than that of BK. Therefore, it has been suggested that desArg^{9}BK accumulates at the site of inflammation (Marceau *et al.,* 1981). It is also possible to measure kinins simply and reproducibly in nasal lavages. As a result, the nasal system has been used to study the mechanisms of kinin formation and metabolism. Using this technique kinin levels have been shown to increase in allergic rhinitis (Dolovich *et al.,* 1970; Proud *et al.,* 1983; Shirasaki *et al.,* 1989), and asthma (Christiansen *et al.,* 1987). Similarly, increased kinin levels have been determined in bronchoalveolar lavage fluid from patients with active pulmonary inflammation (Baumgarten *et al.,* 1992).

1.2 BK receptor classification

The kinins exert their effects by the activation of specific guanine nucleotide binding protein (G-protein) coupled receptors. Two mammalian receptors, BKB_1 and BKB_2 have been characterised using a number of criteria described in the following sections. A BKB_3 receptor in guinea-pig trachea has been proposed (Farmer *et al.,* 1989), there is also evidence for subdivisions of the BKB_2 receptor (Regoli *et al.,* 1994). The actions of kinins include smooth muscle contraction, vasodilation, increased vascular permeability, increased production of eicosanoids and nitric oxide and the induction of pain (Hall 1992; Steranka *et al.,* 1988).

1.2.1 Pharmacological profiles

BK receptors were originally classified according to the relative agonist potencies in isolated smooth muscle preparations, of a series of structurally related peptide analogues of BK (Regoli and Barabe, 1980). The rank order of potency of kinins at the BKB_2 receptor in the rabbit jugular vein is;

$$BK > kallidin > desArg^{10}kallidin > desArg^{9}BK$$

The rank order of potency at the BKB_1 receptor in the rabbit aorta is;

$$desArg^{10}kallidin > desArg^{9}BK > kallidin > BK$$

The receptor type mediating the contraction of the rabbit aorta was termed B_1 since it was more completely defined at the time. BKB_1 receptor pharmacology has been characterised in a number of *in vitro* and *in vivo* pharmacological preparations and cell lines and will be discussed in section 1.3. Recent studies have shown that the relative potencies of some BKB_1 receptor vary between species. Differences are seen between the rodent and human BKB_1 receptors. The mouse BKB_1 receptor has a 2 to 3 fold

selectivity for desArg^{9}BK over desArg^{10}kallidin (Hess *et al.*,1996; Pesquero *et al.*, 1996), the rat BKB_1 receptor has approximately equal affinity for the two ligands (Ni *et al.*, 1998b; Jones *et al.*, 1999). In contrast, the human receptor has a 5000 fold selectivity for desArg^{10}kallidin over desArg^{9}BK (Jones *et al.*, 1999). Since kallidin and its derivative desArg^{10}kallidin are not formed in rodents, the human BKB_1 receptor has evolved relatively greater sensitivity towards the kallidin derivatives than the rat receptor. The biological role of T-kinin is unknown, but studies *in vitro* have shown it to be active at the BKB_2 receptor (Jones *et al.*, 1999). The potency estimates for BK and kallidin at the BKB_1 receptor can be distorted by the presence of arginine carboxypeptidases which result in the production of the desArg metabolites. Most of the reported binding studies to the BKB_1 receptor have used [^{3}H]desArg^{10}kallidin as the agonist ligand. However, the synthetic agonists, Sar-[D-Phe^{8}]desArg^{9}BK and Sar-Tyr-εAhx-Lys-desArg^{9}BK, metabolically stable analogues of desArg^{10}kallidin, have been used in a some studies (Drapeau *et al.*,1991, 1993; Ni *et al.*, 1998b).

The second pharmacological criteria for the classification of the BK receptors was the development of selective and competitive peptide-derived antagonists. The prototype antagonist for the BKB_2 receptor was [D-Phe^{7}]BK (Vavrek and Stewart, 1985). Since then a number of selective BKB_2 receptor antagonists have been developed. One of the most potent of these antagonists is Hoe 140 (Icatibant; DArg-[Hyp^{3}, Thi^{5}, D-Tic^{7}, Oic^{8}]-BK (Hock *et al.*, 1991). Hoe 140 is selective for the BKB_2 receptors, and has been used in many studies to assess the role of kinins in various models of experimental pathology. However, Hoe 140 has been shown to have some affinity for the BKB_1 receptor at high concentrations (Ki 1,000nM) (Bastian *et al.*, 1997). The first nonpeptide kinin antagonists, WIN 64338 and FR 173657 are selective BKB_2 antagonists (Salvino *et al.*,

1993; Sawutz *et al.,* 1994; Aramori *et al.,* 1997; Burgess *et al.,* 1999). The prototype of the BKB_1 receptor antagonists was [Leu^8]desArg^{9}BK (Regoli *et al.,* 1977). Substitution of Phe^8 in desArg^{9}BK by a residue with an aliphatic (Ala, Ile, Leu, Dleu, norleucine) or saturated cyclic hydrocarbon chain (cyclohexylalanine) produced antagonists of various potencies with a leucyl substitution being optimal (Regoli and Barabe, 1980). The effect of prolonging the N-terminal sequence of [Leu^8]desArg^{9}BK with a Lys residue confers a higher affinity in the rabbit and human BKB_1 receptors which parallels the relationship for the agonists (MacNeil *et al.,* 1995; Menke *et al.,* 1994). To date, no nonpeptide BKB_1 receptor antagonists have been reported. A metabolically stable BKB_1 receptor antagonist, Ac-Lys-[MeAla^6, Leu^8]desArg^{9}BK, has been developed that is resistant to inactivation by ACE, aminopeptidase M and neutral endopeptidase (Drapeau *et al.,* 1993). Recently, metabolically stable BKB_2 and mixed BKB_1 and BKB_2 antagonists have been produced. B9858 [Lys-Lys-[Hyp^3, Igl^5, D-Igl^7, Oic^8]desArg^{9}BK is one such peptide and has some selectivity for BKB_1 receptors (Gera *et al.,* 1996; Gobeil *et al.,* 1997). The desArg^9 fragment of Hoe 140 exhibits increased affinity towards the BKB_1 receptor, however this metabolite of Hoe 140 has not been found *in vivo* (Rhaleb *et al.,* 1992). Another recently produced antagonist with mixed BKB_1 and BKB_2 receptor antagonist affinity is B9430 (D-Arg-[Hyp^3, Igl^5, D-Igl^7, Oic^8]BK) (Burkard *et al.,* 1996). B9430 has been shown to abolish the hypotensive responses to BK and desArg^{9}BK in the dog without affecting responses to other agonists (Stewart *et al.,* 1996).

The prototype BKB_1 receptor antagonist, [Leu^8]desArg^{9}BK has been shown to exhibit partial agonist behaviour in isolated rat colon (Teather and Cuthbert, 1997) and isolated mouse stomach (Allogho *et al.,* 1995). This may have practical implications, since one

of the therapeutic effects of BKB_1 receptor antagonists are their analgesic properties, based on models in these species (see section 1.4.2). The antagonist R715 (AcLys[Db-Nal^{7}Ile^8]desArg^{9}BK) was recently developed for the pharmacological characterisation of the mouse BKB_1 receptor (Allogho *et al.*, 1995; Gobeil *et al.*, 1997). However, despite the high selectivity of this compound for the receptor, the affinity is fairly low. In terms of affinity and selectivity, no existing peptide antagonist is ideal to fully characterise the analgesic potential of BKB_1 receptor blockade in rodents (Rupniak *et al.*, 1997).

1.2.2 Desensitisation

(Marceau *et al.*, 1998)

Both BKB_1 and BKB_2 receptors exhibit desensitisation (ref). However, the absence of cross-desensitisation is another characteristic for defining the distinct kinin receptors. This applies to the adaptation mechanisms present in, or closely associated with, the receptor molecule as opposed to downstream desensitising mechanisms (e.g depletion of intracellular ion stores). In rat mesangial cells which express both BKB_1 and BKB_2 receptors the response to BK can be down-regulated without repressing the response to desArg^{9}BK as determined by a functional assay measuring the intracellular calcium concentration (Bascands *et al.*, 1993). The reverse effect was also seen in these cells. In addition, in bovine endothelial cells and rabbit mesenteric artery smooth muscle cells the down-regulation of the BK response did not effect the response elicited by desArg^{9}BK, however, the converse effect was not demonstrated (Mathis *et al.*, 1996; Smith *et al.*, 1995).

The BK receptors differ in the kinetics of their ligand-induced internalisation. Exposure of human BKB_2 receptors to BK results in a rapid receptor mediated internalisation in combination with increased association of BKB_2 receptors and Gα subunits with

calveolae (Austin *et al.*, 1997; de Weerd and Leeb-Lundberg, 1997). In contrast, the human BKB_1 receptor does not show ligand-induced receptor internalisation (Austin *et al.*, 1997). Chimeric studies of human BKB_2 and BKB_1 receptors show that the cytoplasmic carboxy terminus of the human BKB_2 receptor contains sequences necessary and sufficient to permit rapid ligand-induced internalisation of the human BK receptors (Faussner *et al.*, 1998). Another study confirmed the significant role of the carboxy terminal tail and identified specific tyrosine residues in the intracellular domains important for ligand-induced internalisation of the BKB_2 receptor (Prado *et al.*, 1997). Determination of the *in vivo* phosphorylation sites of the rat BKB_2 receptor indicate mutually exclusive palmitoylation of Cys^{356} or phosphorylation at Try^{352} within the carboxy terminus which may be significant for internalisation of the receptor (Soskic *et al.*, 1999). These differences in receptor down-regulation might signify individual roles for BK receptor subtypes in pathophysiology.

1.2.3 Signal transduction pathways

Both BKB_1 and BKB_2 receptors belong to the superfamily of G-protein coupled receptors as detailed in the following section. The signal transduction mechanisms of the BK receptors have been widely studied in numerous tissues. Receptor stimulation and coupling to G-proteins causes activation of phospholipase C (PLC) which generates diacylglycerol and inositol phosphates (IP). Diacylglycerol activates protein kinase C (PKC) and IP_3 mobilises internal calcium stores. The human BKB_1 receptor is coupled to the G proteins, $G\alpha q/11$ and $G\alpha i_{1/2}$ with $G\alpha q/11$ contributing to the receptor mediated activation of PLC (Austin *et al.*, 1997). Increased phosphatidylinositol turnover following stimulation with BKB_1 receptor agonists has been shown both with the naturally expressed receptor (Issandou and Darbon, 1991; Levesque *et al.*, 1993; Butt *et*

al., 1995; Smith *et al.,* 1995; Mathis *et al.,* 1996) and also for the stably expressed human BKB_1 receptor (Austin *et al.,* 1997; Bastian *et al.,* 1997). Further demonstration that the BKB_1 receptor couples to PLC are shown by measurements of intracellular calcium following stimulation with BKB_1 receptor agonists (Austin *et al.,* 1997; Bastian *et al.,* 1997; Bascands *et al.,* 1993; Marsh and Hill; 1994; Mathis *et al.,* 1996; Smith *et al.,* 1995; Menke *et al.,* 1994; Bkaily *et al.,* 1997; MacNeil *et al.,* 1997; Tsukagoshi *et al.,* 1999). Although both receptors couple to the same signalling pathways, distinct differences in these pathways have been used to characterise the kinin receptors. Single cell fluorescence imaging of the calcium sensitive ratiometric fluorescent dye, Fura-2, loaded into rabbit vascular smooth muscle cells reveals that both desArg^{9}BK and BK trigger rapid increases in cytosolic free Ca^{2+}, however the desArg^{9}BK response was biphasic and sustained whereas the BK response was transient (Mathis *et al.,* 1996). The continuation of the sustained phase may be dependent on the influx of extracellular Ca^{2+}, although the mechanism for this remains unclear (Mathis *et al.,* 1996; Marsh and Hill, 1994; Bascands *et al.,* 1993). Homologous desensitisation of the BKB_2 receptor may be responsible for these differences in the signalling pathways (Mathis *et al.,* 1996; Smith *et al.,* 1995).

Activation of the kinin receptors has been reported to induce an increase in basal mitogenesis (Goldstein and Wall, 1984; Bascands *et al.,* 1993; Issandou and Darbon, 1991; Levesque *et al.,* 1995a). In contrast, another study has shown that BK and desArg^{9}BK can inhibit mitogenesis in serum deprived quiescent cells (Patel and Schrey, 1992). Recent studies have shown that BKB_1 and BKB_2 receptors were capable of suppressing platelet-derived growth factor (PDGF) induced DNA synthesis (Dixon and Dennis, 1997; McAllister *et al.,* 1993). These data suggest that neither receptor is

specifically coupled to stimulation or inhibition of mitogenesis. The specific cellular pathways by which kinins regulate mitogenesis are unclear, although similar to BK, desArg^{9}BK has been shown to increase intracellular Ca^{2+} and activate protein kinase C leading to an increase in mitogenesis (Issandou and Darbon, 1991). In contrast, the anti-mitogenic effects of BK were reversed in the presence of a BKB_1 receptor antagonist whereas a BKB_2 receptor antagonist was without effect (Patel and Schrey, 1992). A more recent study shows that alternative signal transduction pathways may be involved in mitogenesis since the anti-proliferative effects of kinins in cultured arterial smooth muscle cells are not mediated by the activation of phosphoinositidase C, cyclic adenosine 3',5'-monophosphate (cAMP), prostaglandins or nitric oxide (Dixon and Dennis, 1997).

Activation of the Ca^{2+}-dependent enzymes, phospholipase A_2 and endothelial nitric oxide following activation of the BKB_1 receptors may account for increases in the production of eicosanoids (Levesque *et al.,* 1995a) and nitric oxide (Drummond and Cocks, 1995; Pruneau *et al.,* 1993). Kinin receptor stimulation may also involve activation of mitogen-activated protein (MAP) kinase pathways (Jaffa *et al.,* 1997; Zwick *et al.,* 1997; Naraba *et al.,* 1998, Blaukat *et al.,* 1999; Velarde *et al.,* 1999). Naraba *et al.,* (1998) have recently shown that both BKB_1 and BKB_2 receptors activate the transcription factor activator protein (AP) -1 through the MAP kinase, extracellular signal related kinase (ERK), pathway. Stimulation of BKB_2 receptors has been shown to activate the transcription factor NF-κB (Pan *et al.,* 1996). Further analysis by Pan and co-workers (1999) showed the requirement of both RhoA and phosphatidyl inositol (PI) -3 kinase in this signalling pathway.

1.2.4 Distinct BKB_1 and BKB_2 receptor genes

Molecular cloning of the kinin receptors shows that the protein coding regions are encoded by distinct genes. The BKB_2 receptor gene has been cloned and characterised from human (Hess *et al.,* 1992; Eggerickx *et al.,* 1992; McIntyre *et al.,* 1993), mouse (McIntyre *et al.,* 1993; Hess *et al.,* 1994) and rat (McEachern *et al.,* 1991; McIntyre *et al.,* 1994; Wang *et al.,* 1994) sources. The genomic structure of the human and rat BKB_2 receptor genes have been determined with the human BKB_2 receptor being localised to chromosome 14q32 (Ma *et al.,* 1994a; Kammerer *et al.,* 1995; Pesquero *et al.,* 1994). Analysis of the amino terminus of the rat and human BKB_2 receptors using domain specific antibodies, amino acid sequence analysis and *in vitro* transcription/translation reveals that of the several potential in-frame start codons, the most 5' of these is utilised for translation (AbdAlla *et al.,* 1996). However, the original cloning paper predicted that the rat BKB_2 receptor start site was most likely to correspond to the third ATG codon (McEachern *et al.,* 1991), this finding now appears unlikely, since the rat and human 5' sequences have three similar methionine codons.

Webb and co-workers exploited the *Xenopus* oocyte expression system to demonstrate that messenger ribonucleic acid (mRNA) of distinct sizes derived from the human lung fibroblast cell line, WI38, resulted in the expression of proteins with the pharmacological properties of the BKB_1 receptors and BKB_2 receptors (Phillips *et al.,* 1992; Webb *et al.,* 1994). An expression cloning strategy in *Xenopus* oocytes utilising the photoprotein aequorin as an indicator of the second messenger, Ca^{2+}, was used to clone the human BKB_1 receptor complementary deoxyribonucleic acid (cDNA) (Menke *et al.,* 1994). IMR-90 cells, another fibroblast cell line, was used as the source of mRNA for cloning the receptor. Treatment of the cells with the cytokine, interleukin-1

beta (IL-1β), was used to increase the amount of mRNA transcript encoding the BKB_1 receptor (see Section 1.3). At the same time our laboratory cloned the human receptor from WI38 cells (Jones *et al.,* 1999). From these studies human BKB_1 receptor clones were isolated that contained an open reading frame (ORF) encoding a 353 amino acid protein. This work is not a part of this thesis.

Both BK receptors exhibit the seven transmembrane (TM) structure characteristic of the G-protein coupled receptors. Although the BKB_1 receptor is more closely related to the BKB_2 receptor than to any other G-protein coupled receptor the amino acid sequence identity between the receptors is only 36%. In comparison, the BKB_1 receptor exhibits 30% homology with the angiotensin type II receptor. To date, the BKB_1 receptor has been isolated and characterised from the human (Menke *et al.,* 1994), rabbit (MacNeil *et al.,* 1995), mouse (Hess *et al.,* 1996; Pesquero *et al.,* 1996) and rat (described in this study and reported Jones *et al.,* 1999; Ni *et al.,* 1998b). The genomic structure of the human and rat BKB_1 receptor genes has been determined (Bachvarov *et al.,* 1996; Yang and Polgar, 1996; Ni *et al.,* 1998; this study and reported Jones *et al.,* 1999). Comparative analysis of the reported protein and genomic structures of the BKB_1 receptor will be discussed further in Chapter 3. The chromosomal location of the human BKB_1 receptor gene has been mapped to 14q32 between markers D14S265 and D14S267 (Chai *et al.,* 1996; Bachvarov *et al.,* 1998a). The production of antipeptide antibodies has been described by two groups, in both studies the most effective peptides corresponded to the carboxy terminal domain of the BKB_1 receptor (Hess *et al.,* 1996; Schanstra *et al.,* 1998). Western blot analysis reveals a band of molecular mass of approximately 40 kD corresponding to the predicted molecular mass for the human BKB_1 receptor (Hess *et al.,* 1996; Schanstra *et al.,* 1998). These antibodies cannot be

used for detection of the rat and mouse BKB_1 receptors since the rodent carboxy tails of the BKB_1 receptors are shorter than the human counterpart (see Chapter 3).

Although the BKB_1 and BKB_2 receptors are only 36% identical, chimeric receptor studies have shown that they are structurally compatible (Leeb *et al.*, 1997; Faussner *et al.*, 1998). Leeb and colleagues (1997) constructed chimeric proteins in which TM domain VI in the human kinin receptors were exchanged. Substitution of BKB_1 receptor TM-VI into the BKB_2 receptor reduces the affinity for the BKB_2 receptor agonists. The wild type affinity of the BKB_2 receptor was restored when two residues, Tyr^{259} and Ala^{263} were replaced with the corresponding BKB_2 receptor amino acids Phe^{259} and Thr^{263}. The converse experiment where the BKB_2 receptor TM-VI was substituted into the BKB_1 receptor prevented high affinity binding of the BKB_1 receptor agonist $desArg^{10}$kallidin. These results show that TM-VI is directly involved in distinguishing between the subtype selective agonists. Recent studies by the same group have focused on the role of TM-III exchange between the two kinin receptors (Fathy *et al.*, 1997). Their results show that TM-III contributes to the ability of the receptor to discriminate between subtype selective ligands. In particular, the positive charge of the lysine residue in position 118 of the BKB_2 receptor may repel the C-terminal arginine of the BKB_1 receptor selective ligands.

1.2.5 Knockouts

Targeted disruption of a gene by homologous recombination has become a valuable tool in discerning the physiological role of individual genes. However, there are limitations with interpreting data from knockout mice due to altered expression of related genes or physiologic compensation for the loss of a gene product. Knockout animals were

recently reported for both BKB_2 (Borkowski *et al.*, 1995) and BKB_1 receptors (Bader *et al.* ,1997). Elimination of the response to BK in BKB_2 receptor knockout mice are consistent with a single gene encoding the BKB_2 receptor. The absence of the BKB_2 receptor did not affect fertility or produce changes in morphology or litter size ruling out an essential role for the BKB_2 receptor in reproduction (Borkowski *et al.*, 1995). However, one report shows an increase in blood pressure in these animals (Madeddu *et al.*, 1997a). Furthermore, the BKB_2 receptor knockout mice display a greater hypertensive response to chronic sodium uptake compared to control animals suggesting a role of kinins in preventing salt-sensitive hypertension (Alfie *et al.*, 1996). Recent analysis of these mutant mice demonstrated a role for BKB_2 receptors in the regulation of sodium secretion in the urine (Alfie *et al.*, 1999).

The BKB_1 receptor knockout mice have been described quite recently (Bader *et al.*, 1997). Preliminary data from these mice show that the mean arterial pressure did not differ from control mice, ruling out the participation of the BKB_1 receptor in the control of blood pressure under normal conditions. Further analysis of the BK receptors knockout models will be useful to evaluate the roles of the receptors in the inflammatory response, the cardiovascular system, renal function and reproduction.

1.2.6 Different regulatory profiles

The kinin receptors can also be classified according to their different expression patterns. The BKB_2 receptor mediates the classic inflammatory actions of kinins in normal tissues (Bhoola *et al.*, 1992; Burch *et al.*, 1990; Hall, 1992) and is widely distributed throughout mammalian tissues (Hall, 1992; Ma *et al.*, 1994a). However, there is evidence for regulation of the BKB_2 receptor expression. Studies have shown

that the receptor can be up-regulated at the transcriptional level by IL-1, cAMP, PDGF and interferon gamma (IFN-γ) (Schmidlin *et al.*, 1998; Bathon *et al.*, 1992; Dixon, 1994; Dixon *et al.*, 1996; Lung *et al.*, 1998). Conversely, TNF-α has been shown to down-regulate BKB_2 receptor expression (Sawutz *et al.*, 1992). A recent study by Madeddu (1997b) and colleagues demonstrate a sexual dimorphism in BKB_2 receptor expression. A role for oestrogen in regulating BKB_2 receptor expression was further shown by analysis of reduced receptor mRNA levels.in ovariectomized rats and restoration to normal levels following the addition of oestrogen.

Accumulating evidence indicates that the BKB_1 receptor is normally absent or expressed in very low amounts in normal tissues but is rapidly up-regulated following certain types of tissue injury. The large number of *in vitro* and *in vivo* systems involving the BKB_1 receptor up-regulation are reviewed in the following two sections.

1.3 BKB_1 receptor up-regulation *in vitro* and *in vivo*

BKB_1 receptor up-regulation has been characterised in several *in vitro* and *in vivo* systems representing a variety of cell types and different species. More recent studies using molecular techniques have confirmed this receptor up-regulation at the mRNA level.

1.3.1 Tissue BKB_1 receptor up-regulation *in vitro*

Regoli and colleagues first described a "new type of receptor for BK" through a contractile response to BK and desArg^{9}BK that was observed in isolated rabbit aorta

strips (Regoli *et al.,* 1977). A year later the *de novo* synthesis of the BKB_1 receptor was reported in the rabbit isolated mesenteric vein (Regoli *et al.,* 1978). Since then a large number of *in vitro* preparations have demonstrated the phenomenon of BKB_1 receptor up-regulation, some of these data are summarised in Table 1.1. The spontaneous development of responses to desArg^{9}BK has been well characterised for the rabbit cardiovascular system. The rabbit aorta exhibits a contractile response to desArg^{9}BK, whereas other rabbit vascular preparations such as mesenteric artery, and carotid artery exhibit vasorelaxation when exposed to this compound (Churchill and Ward, 1986; Deblois and Marceau, 1987; Pruneau and Belichard, 1993).

Studies in the isolated vessels for other species including bovine, human and rat have provided sensitive and reliable preparations for studying both BKB_1 and BKB_2 receptors (Table 1.1). Other non-vascular smooth muscle preparations including the urinary bladder of the rabbit (Butt *et al.,* 1995), rat (Marceau *et al.,* 1980; Roslan *et al.,* 1995) and mouse (Busser *et al.,* 1996), the longitudinal muscle of the rat ileum (Meini *et al.,* 1996), the human colon (Couture *et al.,* 1981) and the rat colonic epithelium (Teather and Cuthbert, 1997) show post-isolation up-regulation of the BKB_1 receptor. In most of the tissues examined the BKB_2 receptors are co-expressed in the preparation and the responses to these receptors remain stable. The rat longitudinal muscle is exceptional in this respect expressing only BKB_1 receptors (Meini *et al.,* 1996).

Up-regulation of the response to BKB_1 receptor agonists is abolished by pre-treatment with protein synthesis inhibitors, such as cylcoheximide, and by the ribonucleic acid (RNA) synthesis inhibitor, actinomycin D (Regoli *et al.,* 1978; Deblois and Marceau, 1987; Bouthillier *et al.,* 1987; De Kimpe *et al.,* 1994; Sardi *et al.,* 1997; Teather and

Cuthbert, 1997). However, in the rabbit isolated aorta application of the protein synthesis inhibitor as a 'pulse' enhances the vascular responses to desArg^{9}BK (Deblois *et al.,* 1991). One explanation for this is the so-called "super-induction" of the corresponding BKB_1 receptor mRNA under these conditions involving up-regulation and/or stabilisation of mRNA (see Chapter 4). Another study shows the preventative effect of brefeldin A and tunicamycin on the up-regulation of the BKB_1 receptor mediated responses in the isolated rabbit aorta (Audet *et al.,* 1994). Brefeldin A inhibits the surface expression of newly synthesised protein targeted to the endoplasmic reticulum-Golgi pathway of maturation and secretion or expression at the cell surface (Helms and Rothman, 1992). Tunicamycin inhibits the first step in the pathway of the synthesis of the oligosaccharide portion of asparagine-linked glycoproteins (Elbein, 1981). These data are consistent with the *de novo* formation of the BKB_1 receptor as the molecular basis for the spontaneous up-regulation phenomenon.

The rabbit isolated aorta preparation has also been used to examine the effects of cytokines and other agents in inducing the responsiveness to desArg^{9}BK. As shown in Table 1.1 these include the bacterial products, lipopolysaccharide LPS and muramyl-dipeptide (MDP), phorbol myristate acetate (PMA), epidermal growth factor (EGF), endothelial cell growth factor, cycloheximide (a pulse exposure), oncostatin M and the cytokines, IL-1β and interleukin 2 (IL-2). EGF, unlike the other agents enhances the level of response to desArg^{9}BK immediately suggesting a direct effect on the smooth muscle cells involving sensitisation of pre-existing receptor that is independent of protein synthesis (Deblois *et al.,* 1989). In addition, various glucorticoids have been shown to surpress spontaneous, LPS and cytokine mediated responsiveness to desArg^{9}BK (Deblois *et al.,* 1988; Deblois *et al.,* 1991). In another model, the bovine

isolated mesenteric artery, IFN-γ increased the contractile response to desArg^{9}BK. Deblois and colleagues (1991) proposed a model to explain these diverse effects, with a central role for IL-1 in mediating the sensitisation to kinins. IL-1, a key mediator in host responses to infection and inflammation (Dinarello, 1984), was observed to be a potent stimulator of responses to desArg^{9}BK (Deblois *et al.,* 1988). Moreover, both LPS and MDP can promote the synthesis and release of IL-1 from macrophage-like cells (Dinarello and Kreueger, 1986; Warren *et al.,* 1986). In addition, IL-1 is produced by cultured vascular smooth muscle cells in response to LPS (Libby *et al.,* 1986). Kinins have also been shown to stimulate cytokine release from macrophages suggesting a complex autocrine interaction between these mediators (Tiffany and Burch, 1989; Tsukagoshi *et al.,* 1999). However, IL-1 receptor antagonist (IL-1Ra), the naturally occurring competitive antagonist of interleukin-1 alpha (IL-1α) and IL-1β, failed to inhibit both the spontaneous sensitisation to desArg^{9}BK in isolated aortic tissue and the LPS induced hypotensive responses to desArg^{9}BK in live rabbits (Petitclerc *et al.,* 1992, Whalley *et al.,* 1993). Although these studies rule out an obligatory role for IL-1, they suggest that the BKB_1 receptor may become an important mediator in pathological conditions associated with IL-1.

A recent study has examined the role of the MAP kinases in both the spontaneous and cytokine mediated contractile response to desArg^{9}BK in rabbit aortic rings (Larrivee *et al.,* 1998). This study showed that there is an increase in MAP kinase activity following tissue isolation. In particular, the activity of p38 MAP kinase is approximately 10 to 20 fold higher in control aortic rings compared to untreated cultured cells. The roles of the different kinase pathways in the regulation of expression of the BKB_1 receptors were examined by the use of selective inhibitory compounds. SB203580, an inhibitor of p38

MAP kinase activity inhibited the spontaneous up-regulation of responsiveness to desArg^{9}BK by approximately 75% and the development of the response to IL-1 and EGF by approximately 50%. PD98059, a MAP kinase/ ERK kinase-1 (MEK-1) inhibitor which prevents activation of the downstream ERK MAP kinase pathway, inhibited the spontaneous and IL-1β mediated up-regulation of responsiveness to desArg^{9}BK by approximately 40%, however the drug did not inhibit EGF induced responsiveness. The role of the c-Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway was not examined with inhibitory compounds although its activity was found to increase as a result of the isolation procedure and following the addition of IL-1β (Larrivee *et al.,* 1998). This study indicates that the MAP kinase pathways may mediate the effect of cytokines and tissue injury on BKB_1 receptor expression.

Table 1.1 *In vitro* studies of BKB_1 receptor up-regulation.

Tissue preparation	Mediator(s) of upregulation	Measurent of response to desArg^{9}BK	Reference
Vascular			
Rabbit aorta	Time (3-6 hours), IL-1β (3-6 hours), onconostatin M (3-6 hours), MDP (6 hours), cycloheximide (pulse exposure), IL-2 (3-6 hours), LPS (6 hours), PMA (3-6 hours), EGF	Contraction	Regoli *et al.*, 1977 Bouthillier *et al.*, 1987 Deblois *et al.*, 1988, 1989, 1991 Audet *et al.*, 1994 Levesque *et al.*, 1995 Larrivee *et al.*, 1998
Rabbit carotid artery	Time (5 hours)	Relaxation	Pruneau and Belichard, 1993
Human coronary artery	Time (3-9 hours)	Relaxation	Drummond and Cocks 1995b
Bovine coronary artery	Time (3-6 hours)	Relaxation	Drummonds and Cocks 1995a
Rabbit mesenteric artery	Time (5.5 hours)	Relaxation	Deblois and Marceau, 1987
Bovine mesenteric artery	IFN-γ (20 hours)	Contraction	De Kimpe *et al.*, 1994
Rabbit mesenteric vein	Time (6 hours)	Contraction	Regoli *et al.*, 1978
Human umbilical vein	Time (4-6 hours)	Contraction	Gobeil *et al.*, 1996 Sardi *et al.*, 1997
Rat portal vein	Time (4.5 hours)	Contraction	Campos and Calixto, 1994
Non-vascular			
Rat stomach	Time (3-4 hours)	Contraction	Allogho *et al.*, 1995
Rat colon epithelium	Time (3 hours)	Electrogenic chloride secretion	Teather and Cuthbert 1997
Rat ileum	Time (5 hours)	Contraction	Meini *et al.*, 1996
Human ileum	Time (overnight)	Contraction	Zuzack *et al.*, 1996
Rat bladder	Time (4, 20 hours)	Contraction	Marceau *et al.*, 1980; Roslan *et al.*, 1995
Rabbit bladder	Time (3 hours)	Contraction	Butt *et al.*, 1995
Mouse bladder	Time and LPS(3-5 hours)	Contraction	Busser *et al.*, 1998
Mouse SCG	Il-1β or IL-8 (18 hours)	Depolarisation	Seabrook *et al.*, 1995, 1997.

This table is not a complete list of all the *in vitro* tissues examined but rather are representative examples from different species and tissues.

Table 1.2 *In vivo* studies of BKB_1 receptor up-regulation.

Species	Pretreatment	Measurement of up-regulation	Reference
	Inflammation		
Rat	s.c. injection of BCG (75 days), or i.pl. injection of BK (7 days), or i.v. injection of LPS (24 hours)	Paw oedema	Campos *et al.*, 1995, 1996,1997; Campos and Calixto, 1995
Rat	Antigen induced chronic arthritis	Knee plasma extravasation	Cruwys *et al.*, 1994
Rabbit	Antigen induced arthritis	Contraction of aorta	Farmer *et al.*, 1991
Rat	Allergen induced bronchial hyperresponsiveness	Bronchial responsiveness, Northern blot analysis	Huang *et al.*, 1999
Human	Gastritis	Immunolabelling of stomach	Bhoola *et al.*, 1997
	Hyperalgesia and pain		
Rat	NGF induced hyperalgesia (1 day), or ultraviolet irradiation induced thermal hyperalgesia (24 hours), or Freuds adjuvant induced hyperalgesia (3 days), or i.a injection of IL-1β, IL-2 or IL-8, substance P or capsaicin.or i.pl injection of IL-1β (48 hours)	Thermal and/or mechanical hyperalgesia	Rueff *et al.*, 1996; Perkins and Kelly, 1993; Perkins *et al.*, 1993a; Davis and Perkins, 1994a,b; Davis and Perkins, 1996
Rat	i.cv. injection of LPS (24 hours)	Febrile response	Coelho *et al.*, 1997
	Cardiovascular		
Rat	i.v. injection of LPS (24 hours)	Hypotension	Tokumasu *et al.*, 1995; Nicolau *et al.*, 1996
Rabbit	i.v. injection of LPS (5 and 20 hours), or i.v. injection of IL-1β, or i.v. injection of cycloheximide, MDP or PMA (5 hours)	Hypotension	Regoli *et al.*, 1981, Marceau *et al.*, 1984; Bouthillier *et al.*, 1987, Deblois *et al.*, 1989, 1991, Nwator and Whalley, 1989, Drapeau *et al.*, 1991, Audet *et al.*, 1997
Pig	i.v. infusion of E.coli	Hypotension	Siebeck *et al.*, 1989, 1996, 1997
Pig	Intravesical infusion of LPS (8 hrs) and sick pigs	Autoradiographic visualisation of [^{3}H]desArg^{10}kallidn of arterial vessels	Schremmer-Danninger *et al.*, 1996
	Nephrology		
Human	renal carcinoma	*In situ* hybridisation of kidney	Wang *et al.*, 1996
Rat	i.pl. injection of LPS (18 hrs)	RT-PCR, $[Ca^{2+}]_i$ of nephron fragments	Marin-Castano *et al.*, 1998

This table does not include all of the *in vivo* studies but rather are representative examples of the inducible role of BKB_1 receptors in different models of pathophysiology. s.c.= subcutaneous, i.pl.= intraplantar, i.v.= intravenous, i.a.= intraarticular, i.cv.= intracerebroventricular.

1.3.2 BKB_1 receptor up-regulation *in vivo*

The up-regulation of BKB_1 receptors *in vivo* is now supported by a number of models as shown in Table 1.2, the best characterised being the rabbit cardiovascular model. Rabbits injected with a sub-lethal dose of bacterial LPS exhibit a hypotensive response to desArg^{9}BK (Regoli *et al.,* 1981). In contrast, control animals do not respond to desArg^{9}BK. The studies carried out on this model reiterate a number of important observations. Firstly, LPS pre-treatment does not alter the basal blood pressure or hypotensive response to BK. Secondly, LPS administered directly before anaesthesia does not induce the responsiveness to desArg^{9}BK indicating a time dependent increase necessary for protein synthesis. Thirdly, *ex vivo* tissues (for example strips of large arteries and veins) removed from these rabbits exhibit very early contractile responses suggesting that the response was acquired *in vivo* before sacrifice. In addition, desArg^{9}BK is a vasodilator of coronary arteries only in hearts removed from LPS-treated rabbits (Regoli *et al.,* 1981). More recently, the mechanisms by which BKB_1 receptor agonists elicit hypotension in LPS-treated rabbits have been studied with the metabolically protected BKB_1 receptor agonist, Sar[D-Phe^8]desArg^{9}BK (Drapeau *et al.,* 1991; Audet *et al.,* 1997). Injection of LPS has also been shown to elicit a hypotensive response in other species, including pig (Siebeck *et al.,* 1989) and rat (Tokumasu *et al.,* 1995; Nicolau *et al.,* 1996).

IL-1, MDP, PMA and pulse exposure to cycloheximide can all substitute for LPS to induce a state of responsiveness to desArg^{9}BK (Bouthillier *et al.,* 1987; Deblois *et al.,* 1991). In contrast to the observations made *in vitro*, dexamethasone does not modify the inductive effect produced *in vivo* by LPS, however, the reason for this remains unclear (Deblois *et al.,* 1988, 1989; Nwator and Whalley, 1989). In addition,

experimental neutropenia does not alter the responsiveness to desArg^{9}BK suggesting that neutrophils are not involved in the sensitisation process (Bouthillier *et al.,* 1987). A number of toxic treatments failed to induce a hypotensive response to desArg^{9}BK demonstrating that the response is highly selective and unrelated to the severity of the treatment (Bouthillier *et al.,* 1987; Marceau *et al.,* 1984).

Molecular evidence for an increase in BKB_1 receptors has been shown by receptor autoradiography using [^{3}H]desArg^{10}kallidin (Schremmer-Danninger *et al.,* 1996). This study showed an increase in binding in the muscle layer of thoracic aorta and pulmonary arteries of both LPS-treated and naturally sick pigs. The *de novo* synthesis of BKB_1 receptors is further substantiated by a parallel increase in BKB_1 receptor mRNA levels. Northern blot analysis of BKB_1 receptor mRNA levels from aortic smooth muscle isolated from rabbits with and without LPS treatment shows an increase in BKB_1 receptor mRNA from LPS-treated rabbits (Marceau *et al.,* 1997a). Increases in BKB_1 receptor mRNA levels following LPS treatment have been shown more recently by using reverse transcription polymerase chain reaction (RT-PCR) in the mouse and rat (Pesquero *et al.,* 1996; Ni *et al.,* 1998b; Marin-Castano *et al.,* 1998) and by Northern bot analysis in the rat (Jones *et al.,* 1999, this study). These studies revealed an increase in BKB_1 receptor transcripts in a number of isolated organs following LPS treatment (discussed in more detail in Chapter 3).

Other models of inflammation and tissue injury have been developed which demonstrate an increase in the response to BKB_1 receptor agonists compared to control conditions (Table 1.2). A role for BKB_1 receptor in mechanisms of inflammatory hyperalgesia is well established in rodents (Correa and Calixto, 1993; Davis and Perkins, 1994a,b;

Perkins *et al.,* 1993a; Shibata *et al.,* 1989). Studies by Davis and Perkins (1994a) have shown that the cytokines IL-1β, IL-2 and interleukin-8 (IL-8) can induce mechanical hyperalgesia when injected into the rat knee joint whereas interleukin-6 (IL-6) and TNF-α were without effect. The cytokine-mediated hyperalgesia was antagonised in the presence of [Leu^8]desArg^{9}BK. Furthermore, this study has shown that IL-1 and products of the cyclo-oxygenase (COX) pathway play a central role in the induction of the BKB_1 receptor mediated hyperalgesia. Nerve growth factor (NGF)-induced hyperalgesia can also be antagonised in the presence of [Leu^8]desArg^{9}BK (Rueff *et al.,* 1996).

The *in vivo* and the *in vitro* studies demonstrate up-regulation of the BKB_1 receptor in response to mediators via an increase in gene expression and subsequent protein synthesis. A number of inflammatory mediators which induce BKB_1 receptor up-regulation may also amplify and induce the synthesis of each other, thereby adding a further level of complexity in these systems. In addition desArg^{9}BK itself is a potent stimulator of IL-1 release from macrophages (Tiffany and Burch, 1989). These mediators may also have additional effects on the BKB_1 receptor agonist responses that act on a downstream event from the receptor, such as prostaglandin synthesis (Galizzi *et al.,* 1994).

Up-regulation of the BKB_1 receptor from a low or undetectable level of expression is well established. In contrast, several reports from *in vivo* and *in vitro* pharmacological studies suggest that BKB_1 receptors may be involved in the renal and cardiovascular system in normal dogs (Staszewska-Wooley *et al.,* 1991; Lortie *et al.,* 1992; Nakhostine *et al.,* 1993; Belichard *et al.,* 1996). Evidence from immediate contractile responses in

isolated mouse stomach and rat duodenum also suggest the existence of constitutive BKB_1 receptors (Feres *et al.,* 1992; Allogho *et al.,* 1995).

1.3.3 BKB_1 receptor up-regulation in cultured cell lines

Cellular models retaining the characteristics of the BKB_1 receptor up-regulation have recently been reported. These models have been utilised to study the receptor mRNA and protein up-regulation and receptor-mediated activation of signalling pathways in response to agonists. Radioligand binding studies have shown an increase in BKB_1 receptors over several hours following the addition of LPS, IL-1β or EGF to cultured rabbit aorta smooth muscle cells and mesenteric artery smooth muscle cells without affecting the ligand affinity (Galizzi *et al.,* 1994; Schneck *et al.,* 1994; Levesque *et al.,* 1995b). Furthermore, a correlative increase in phosphoinositide turnover in response to a BKB_1 receptor agonist has been demonstrated following the addition of IL-1β or EGF to these cells (Levesque *et al.,* 1993; Schneck *et al.,* 1994). A high level of BKB_1 receptors in these cells under basal conditions is shown, however this may be due to the cell culture environment. Indeed, recent studies by Ni and co-workers (1998a,b) examined the effects of mediators on BKB_1 receptor gene expression in serum-free medium to eliminate the inducing effects of serum. Expression of BKB_1 receptors has also been demonstrated in the human fibroblast cell lines, WI38 and IMR-90 by radioligand binding of [^{3}H]desArg^{10}kallidin (Menke *et al.,* 1994; Phagoo *et al.,* 1997, 1999; Yang *et al.,* 1998a, Schanstra *et al.,* 1998, Bastian *et al.,* 1998). Treatment of IMR-90 cells with IL-1β or IL-8 was found to increase the BKB_1 receptor density (Menke *et al.,* 1994, Bastian *et al.,* 1998; Zhou *et al.,* 1998). Similarly, IL-1β and the kinins, BK, desArg^{9}BK and desArg^{10}kallidin increased radioligand binding in WI38

cells (Phagoo *et al.,* 1997, 1999). IL-8 induced BKB_1 receptor expression was abolished by pre-treatment with metabolic inhibitors consistent with the results from the *in vitro* studies described in Section 1.3.1 (Bastian *et al.,* 1998). The concentration of human BKB_1 receptor in IMR-90 cells has also been evaluated by Western blot analysis (Schanstra *et al.,* 1998). This study showed an increase in a 40 kD band corresponding to the predicted size of the BKB_1 receptor following the addition of IL-1β or the BKB_1 receptor agonist, $desArg^{10}$kallidin.

Molecular analysis reveals that an increase in BKB_1 receptor mRNA levels precedes the increase in protein expression. EGF treatment of rabbit aortic cultured smooth muscle cells increases BKB_1 receptor mRNA levels 1.7 fold (Larrivee *et al.,* 1998). Other studies have shown IL-1β, IL-8, BK, $desArg^9$BK and $desArg^{10}$kallidin increase BKB_1 receptor mRNA levels in human lung fibroblasts (Bachvarov *et al.,* 1996; Zhou *et al.,* 1998; Schanstra *et al.,* 1998; Bastian *et al.,* 1998; Phagoo *et al.,* 1997, 1999). The involvement of IL-1β in the IL-8 and BK induced increase in BKB_1 receptor mRNA was demonstrated by inhibition of the up-regulation in the presence of IL-1Ra (Bastian *et al.,* 1998; Phagoo *et al.,* 1999). Recently, increased BKB_1 receptor mRNA levels have been reported in a number of rodent cell lines. RT-PCR analysis shows an increase in BKB_1 receptor mRNA levels following the addition of LPS, IL-1β, TNF-α and cycloheximide in rat aortic vascular smooth muscle cells (VSMCs) and following the addition of LPS in the rat smooth muscle cell line, A10 (Ni *et al.,* 1998a, b). In addition, real time quantitative RT-PCR reveals a 100-1000 fold increase in BKB_1 receptor mRNA levels following the addition of IL-1β in MH-S murine alveolar macrophage cells (Tsukagoshi *et al.,* 1999).

These findings confirm the cytokine and LPS driven up-regulation of BKB_1 receptor mRNA and protein. The mechanism and kinetics of activation of BKB_1 receptor inducing agents is an area which requires further investigation.

1.4 Pathophysiological relevance of BKB_1 receptor up-regulation

The previous sections show that the BKB_1 receptors can be induced by a variety of conditions and may exert an important role in certain pathological states. The following sections describe the evidence for a role of BKB_1 receptors in both experimental and naturally occurring pathophysiological states. Of particular interest are the potential therapeutic effects of BKB_1 receptors antagonists in conditions of prolonged inflammation and hyperalgesia. Indeed, the inducible nature of the BKB_1 receptor at the site of injury has made this an attractive target for drug development. To this end, stable non-peptide BKB_1 receptor antagonists would be useful to fully delineate the potential physiological roles of these receptors *in vivo*.

1.4.1 Inflammation

BK plays a major role in the inflammatory process. This was first demonstrated in 1960 when the administration of synthetic BK to animal tissues reproduced the classical signs of inflammation: redness, swelling, heat and pain (Elliot *et al.*, 1960). The availability of the metabolically stable BKB_2 receptor antagonist Hoe 140, has revealed that antagonism of the BKB_2 receptor can reduce the oedema seen after a wide variety of

inflammatory insults. Hoe 140 inhibited the carrageenin-induced inflammatory oedema in the rat paw (Wirth *et al.,* 1992) and formalin-induced oedema in the mouse paw (Correa and Calixto, 1993). These studies suggest that BK contributes to the formation and maintenance of inflammation. Indeed, the levels of both BK and desArg^{9}BK are increased in inflammatory conditions (Steranka *et al.,* 1987; Hargreaves *et al.,* 1988; Damas *et al.,* 1990; Burch and DeHaas, 1990; Decarie *et al.,* 1996).

Intraplantar administration of desArg^{9}BK in normal rats exhibits very little or no oedema producing activity (Campos and Calixto, 1995; Campos *et al.,* 1996; Campos *et al.,* 1997). In contrast, a dominant role for the BKB_1 receptor following a pathological insult is supported by several studies. DesArg^{9}BK was shown to elicit oedema formation in rats following a 24 hour pre-treatment with LPS or following long term systemic treatment with *Myobacterium bovis* bacillus Calmette-Guerin (BCG) (Campos *et al.,* 1996; Campos *et al.,* 1997). An increase in oedema formation was observed following multiple injections of BK in the rat paw (Campos and Calixto, 1995; Campos *et al.,* 1995). The BKB_1 receptor mediated oedema formation following the addition of LPS, BCG or BK are sensitive to dexamethasone and indomethacin (Campos *et al.,* 1995; Campos and Calixto, 1995; Campos *et al.,* 1996; Campos *et al.,* 1997). These data suggest the involvement of cytokine mediated mechanisms with a requirement of COX products in BKB_1 receptor activation. A constitutive BKB_1 receptor mediated inflammatory response has been observed in a murine model of pleurisy that was not changed following LPS treatment (Vianna and Calixto, 1998). Despite this, evidence from a number of inflammatory studies in various immunopathological models show an up-regulation of the BKB_1 receptor-mediated responses (Table 1.2). A study by Cruwys and colleagues (1994) examined the relative actions of BKB_1 and BKB_2 receptor

agonists and antagonists on plasma extravasation during the course of antigen-induced chronic arthritis in the affected joint. The majority of responses to BK in normal and acutely inflamed joints are mediated by BKB_2 receptors, whereas a functional role for BKB_1 receptors is evident in the chronic phases of the disease. Furthermore, aortas isolated from rabbits treated with Freunds complete adjuvant to induce arthritis responded to desArg^{9}BK immediately whereas in controls this response was time-dependent (Farmer *et al.,* 1991). These findings parallel *in vivo* studies with LPS described in section 1.3.2. More recently molecular approaches have confirmed the up-regulation of BKB_1 receptors in inflammation. Immunolabelling of kinin receptors, using BKB_1 receptor antibodies, in humans with gastritis has shown an induction of the BKB_1 receptor, with a decrease in the immunolocalisation of the BKB_2 receptor (Bhoola *et al.,* 1997). Contribution of the kinin receptors in a rat model of allergen-induced bronchial hyperresponsiveness has been examined by pharmacological and molecular approaches (Huang *et al.,* 1999). This study showed that the BKB_1 receptor antagonist, desArg^{10}[Hoe140], dose-dependently inhibited the allergen-induced bronchial hyperresponsiveness to acetylcholine. Complementary Northern blot analysis showed an increase in BKB_1 receptor mRNA levels after allergen exposure in sensitised lungs, while BKB_2 receptor mRNA levels remained unchanged (Huang *et al.,* 1999). *In vitro* and *in vivo* studies have demonstrated a role for inflammatory mediators, in particular IL-1, in the induction of BKB_1 receptors (described in section 1.3.), therefore the components required for expression and activation of the BKB_1 receptor are present within these models. Although it has been postulated that the up-regulation of the BKB_1 receptors is secondary to cytokine release, the mechanism of induction has not been completely defined.

Evidence also supports a role for BKB_1 receptor activation in the cellular component of the inflammatory response. Neutrophil polymorphs are the first of the blood leukocytes to enter the area of the inflammatory reaction attracted by a variety of substances, termed chemotaxins. BK was shown to cause increased vascular leakage by inducing the release of neutrophil elastase following activation of both BKB_1 and BKB_2 receptors (Carl *et al.*, 1996). BKB_1 receptors are also involved in the polymorphonuclear leukocyte accumulation induced by IL-1β *in vivo* in the mouse (Ahluwalia and Perretti, 1996). Ahluwalia and Perretti (1996) examined the leukocyte accumulation in IL-1β-treated air pouches and showed that induction of BKB_1 receptors in response to IL-1β act as mediators to release neuropeptides, substance P and calcitonin gene related peptide (CGRP), from nerve endings, which in turn stimulate the chemotactic response. The desArg^{9}BK mediated response in a mouse model of pleurisy also involves the activation of sensory neurones with the concomitant release of neuropeptides (Vianna and Calixto, 1998). Whether the BKB_1 receptor is present on the sensory nerve itself or on a non-neuronal cell, which could activate the nerve endings, is controversial and will be discussed in more detail in the following section.

1.4.2 Hyperalgesia and pain

BK is a potent pain producing substance, as demonstrated following application to a blister base in humans (Whalley *et al.*, 1987). Thus, BK has powerful excitatory and sensitising actions on sensory neurones (Dray *et al.*, 1988; Rang *et al.*, 1991). Studies using specific antagonists suggest that BK acts via the BKB_2 receptor to cause pain (Steranka *et al.*, 1987, 1988; Haley *et al.*, 1989; Dray *et al.*, 1992; Heapy *et al.*, 1993). Furthermore, BKB_2 receptors have been localised on sensory nerve terminals and small cells in the dorsal root ganglia (DRG) by autoradiography with [^{3}H]BK (Steranka *et al.*,

1988). The mechanism by which BK activates sensory neurones remains unclear although sensitivity to indomethacin suggests that the effects are mediated via prostanoid production (Weinreich, 1986; Weinreich and Wonderlin, 1987).

In most cases the BKB_1 receptor agonist, desArg^{9}BK was ineffective in these studies. One exception is in the acute nociceptive response to formalin, where both phases of the pain response were inhibited by [Leu^8]desArg^{9}BK (Shibata *et al.,* 1989). However, an increasing amount of evidence shows a role for the BKB_1 receptor in mechanisms of prolonged inflammatory hyperalgesia (Perkins *et al.,* 1993a; Perkins and Kelly, 1993; Dray and Perkins, 1993; Davis and Perkins, 1994a,b; Sufka and Roach, 1996; Rupniak *et al.,* 1997). Perkins and co-workers (1993) were the first to demonstrate the role of BKB_1 receptors in two models of persistent hyperalgesia: persistent inflammatory hyperalgesia was produced by exposing the rat hind paw to ultraviolet irradiation or by injecting Freund's adjuvant into the rat knee joint. In these studies the BKB_1 receptor antagonist [Leu^8]desArg^{9}BK reversed the hyperalgesia whereas the BKB_2 receptor antagonist, HOE 140, was ineffective or only weakly active. In addition, application of desArg^{9}BK increased the hyperalgesia in both models. These findings led to the proposal that the BKB_2 receptors may have a more significant role in the earlier stages of inflammatory pain, whereas the BKB_1 receptor becomes more important in the development and maintenance of chronic inflammatory hyperalgesia. Consistent with this proposal are experiments using a place preference behavioural paradigm experiment with normal and adjuvant inflamed rats (Sufka and Roach, 1996). These data show that [Leu^8]desArg^{9}BK but not HOE 140 produced negatively reinforcing effects (i.e. analgesia) but not positively reinforcing effects (i.e. abuse potential). Recently, it was demonstrated that in IL-1β pre-treated rat knee joints, desArg^{9}BK causes analgesia

when administered at low doses (50 pmol) (Davis and Perkins, 1997). This effect was blocked by naloxone showing that the effect was mediated through the release of peripheral opioids. Recent experiments have examined the role of BKB_1 receptors in the hyperalgesia induced by different chemical mediators. The cytokines, IL-1β, IL-2, IL-8, NGF, substance P, capsaicin and repeated application of BK all produced inflammatory hyperalgesia which was attenuated by the presence of [Leu^8]desArg^{9}BK in models of mechanical or thermal hyperalgesia (Davis and Perkins, 1994b; Perkins and Kelly, 1994; Rueff *et al.,* 1996; Davis and Perkins, 1996; Tonussi and Ferreira, 1997). Expression of the BKB_1 receptors was not involved in the TNF-α induced mechanical hyperalgesia (Perkins and Kelly, 1994). This is in accord with the *in vitro* and *in vivo* studies described in section 1.3.1 and 1.3.2 where TNF-α did not increase BKB_1 receptor expression (Deblois *et al.,* 1988, 1991). Administration of IL-1Ra blocked the hyperalgesia induced by IL-1β, IL-2, IL-8, substance P or capsaicin, suggesting the involvement of IL-1 in the hyperalgesia induced by these cytokines (Davis and Perkins, 1994b; 1996). Furthermore, the development of hyperalgesia to IL-1β was abolished following co-administration with indomethacin showing the requirement of COX products (Davis and Perkins, 1994b). Thus, cytokines and prostaglandins are implicated in BKB_1 receptor mediated inflammation and hyperalgesia. Furthermore, *in vitro* studies have shown that IL-1β potentiates BKB_1 receptor mediated prostanoid release from fibroblasts and vascular smooth muscle cells (Lerner and Modeer, 1991; Galizzi *et al.,* 1994). These data has led to the proposal that endogenous up-regulation of BKB_1 receptors by the cytokines causes the production of prostaglandins which in turn could result in hyperalgesia (Davis and Perkins 1994b).

Although these studies show that in inflammatory conditions there is an induction of BKB_1 receptor mediated-hyperalgesia they do not address the cellular location of the BKB_1 receptors that mediate the hyperalgesia. BKB_1 receptors are not involved in acute nociceptor activation (Dray *et al.,* 1992). A number of complementary experiments carried out in a study by Davis and colleagues (1996) did not locate the BKB_1 receptor on the sensory neurones suggesting that the receptor may be expressed by other cells that release mediators which sensitise or directly activate the sensory nociceptors. However, $desArg^9BK$ mediated inflammatory hyperalgesia is absent following sympathectomy (Khasar *et al.,* 1995). Khasar *et al.,* (1995) suggest that the presence of BKB_1 receptors on sympathetic post-ganglionic neurones which are functionally activated by cytokines. Other studies show that BKB_1 receptors can be induced in sympathetic neurones following treatment with IL-1β and the kininase II inhibitor, captopril (Seabrook *et al.,* 1995), although this was not observed following treatment with LPS (Babbedge *et al.,* 1995). Further molecular studies have shown the presence of BKB_1 receptor mRNA in both superior cervical ganglia (SCG) and DRG neurones, although this did not correlate with a functional response in the DRG's (Seabrook *et al.,* 1997). Inhibition of gold-labelled BK binding by a number of peptides showed that cultured rat DRG's could acquire BKB_1 receptor binding sites (Von Banchet *et al.,* 1996). In addition, immunolabelling of human brain sections using BKB_1 receptor antibodies, localised BKB_1 receptors on neurones of the thalamus, spinal cord and hypothalamus (Raidoo and Bhoola, 1997). Therefore, a subpopulation of neuronal cells could express BKB_1 receptors.

A central involvement for kinin receptors in the endotoxin induced febrile response has been examined by intracerebroventricular injection of specific kinin receptor antagonists

and measurement of the change in rat core temperature (Coehlo *et al.*, 1997). Central BKB_2 receptors were activated during the initial stages of the response followed by down-regulation or desensitisation and induction of BKB_1 receptors in the late phase of the response. Walker and colleagues (1996) also demonstrated involvement of central BKB_2 receptors in endotoxin induced fever but in contrast found no effect of the BKB_1 receptor antagonist. Differences in the dosing regime, routes of administration of LPS, or time of administration of the BKB_1 receptor antagonist between the two studies may explain these discrepancies.

1.4.3 Others (Cardiovascular system and nephrology)

1.4.3.1 Cardiovascular system

A role for up-regulated BKB_1 receptors *in vivo* in the circulation has been supported by a cardiovascular model in the rabbit (Table 1.2). In this model animals injected with a sub-lethal dose of bacterial LPS exhibit a dose-related hypotensive response to desArg^{9}BK 5 hours later (Regoli *et al.*, 1981). This hypotensive response following LPS treatment has also been demonstrated in the rat (Tokumasu *et al.*, 1995; Nicolau *et al.*, 1996) and in the pig (Siebeck *et al.*, 1989, 1996, 1997). The mechanism of hypotension has been investigated using the metabolically protected agonist Sar[D-Phe^8]desArg^{9}BK (Drapeau *et al.*, 1991; Audet *et al.*, 1997). The immediate decrease in mean arterial blood pressure is thought to result from peripheral vasodilation, with a reduction in cardiac output contributing to the maintenance of hypotension. The prolonged hypotension following the administration of Sar[D-Phe^8]desArg^{9}BK is due in part to prostaglandin release and to the slow rate of elimination of the agonist. The

presence of constitutive BKB_1 receptors in the dog, as described in section 1.4.2 mediates the vasodepressor effects to desArg^{9}BK (Nakhostine *et al.*, 1993).

Siebeck and co-workers (1996, 1997) have addressed the role of kinins in septic shock induced by injecting large doses of LPS into the pig. The use of BKB_2 receptor antagonists have been effective in increasing the survival of rats and pigs in response to endotoxic shock (Whalley *et al.*, 1992; Siebeck *et al.*, 1996). Intriguingly, this effect was reversed when a BKB_1 receptor antagonist was given simultaneously indicating a protective mechanism for BKB_1 receptors under these conditions (Siebeck *et al.*, 1996). A protective role for the BKB_1 receptor has also been implicated in models of ischaemia in the heart (Bouchard *et al.*, 1998; Chahine *et al.*, 1993; Foucart *et al.*, 1997). As reported in section 1.3.3, stimulation of the BKB_1 receptors can result in mitogenesis. This effect may be significant in angiogenesis and angioplasty where up-regulation of the BKB_1 receptors has been demonstrated (Hu and Fan, 1993; Pruneau *et al.*, 1994). Indeed, the BKB_1 receptor antagonist, [Leu^8]desArg^{9}BK has been shown to block the angiogenic response in rats (Hu and Fan, 1993).

1.4.3.2 Nephrology

Activation of the renal kallikrein-kinin system results in diuresis (increased secretion of urine from the kidneys), natriuresis (secretion of sodium in the urine), and increased renal blood flow (Majima and Katori, 1995). These effects play a protective role in the development of hypertension. This finding was confirmed by analysis in BKB_2 receptor knockout mice which exhibit an increased salt sensitive hypertensive response compared to normal mice (Alfie *et al.*, 1996). Recently, the role of BKB_2 receptors in natriuresis was shown using BKB_2 receptor knockout mice (Alfie *et al.*, 1999). An

enhanced effect of arginine vasopressin (AVP) in knockout mice compared to controls suggests that endogenous kinins acting through BKB_2 receptors oppose the antidiuretic effect of AVP *in vivo*.

The natriuretic effect of BK in normal dogs appears to be mediated by BKB_1 receptors (Lortie *et al.*, 1992). However, this role for BKB_1 receptors may be isolated to this species since they are exceptional in the expression of constitutive renal BKB_1 receptors (see Section 1.3.2). Examination of BKB_1 receptors in the human kidney by *in situ* hybridisation has shown an increase in BKB_1 receptor mRNA in human malignant kidney cells (Wang *et al.*, 1996). More recently up-regulation of BKB_1 receptors in the rat kidney following the addition of endotoxin has been demonstrated using RT-PCR analysis (Marin-Castano *et al.*, 1998). These studies suggest that under conditions of renal diseases, the effects of kinins may be mediated through BKB_1 receptors. Furthermore, an altered frequency of a BKB_1 receptor gene polymorphism was found in patients with a history of end stage renal failure (Bachvarov *et al.*, 1998a).

ACE inhibitors also show beneficial therapeutic effects in patients with high blood pressure, cardiac hypertrophy, congestive heart failure and diabetic nephropathy (Marceau *et al.*, 1997b). Pharmacological ACE inhibition by intravenous injection of enalapril or captopril in rabbits has been shown to increase the responsiveness to $desArg^9BK$ (Nwator and Whalley, 1989), although other studies were unable to reproduce this effect (Deblois *et al.*, 1991; Marceau *et al.*, 1997b). Nevertheless, some of the beneficial and side effects of ACE inhibitors are thought to be derived from the effect of these drugs in preventing kinin inactivation (Linz *et al.*, 1995; Marceau, 1997b).

1.5 Regulation of the BKB_1 receptor gene

Prior to the studies in this thesis, the mechanisms of up-regulation of the BKB_1 receptor gene were unknown. In the last few years however, several studies have characterised the transcriptional and post-transcriptional effects of the human BKB_1 receptor gene following the addition of inducing agents. The rat BKB_1 receptor 5' regulatory region and promoter has recently been reported and will be discussed in Chapter 5 (Ni *et al.*, 1998b; and this thesis). The conflicting results of analysis of the human BKB_1 receptor gene regulation will be described in the following sections.

1.5.1 Transcriptional regulation of the human BKB_1 receptor gene

The human BKB_1 receptor genomic structure comprises three exons spanning approximately 10 kilobases (kb) (Bachvarov *et al.*, 1996; Yang and Polgar, 1996). The entire ORF and 3' untranslated region (UTR) are located on exon 3. The 5' UTR is located on all three exons separated by introns of 6-7 kb and 0.9 kb. Sequence analysis of the 5' flanking region reveals the presence of a consensus TATA box along with numerous putative transcription factor binding sites.

The 5' flanking region and intronic sequences have been examined for regulatory activity by synthesis of reporter genes constructs. Some of these constructs are shown in Figure 1.3. Transient transfection of the constructs has been carried out in the human cell lines, IMR-90, simian virus 40 (SV40) transformed IMR-90, HEK-293 and HepG2 (Yang and Polgar, 1996; Chai *et al.*, 1996; Ni *et al.*, 1998a; Yang *et al.*, 1998a; Schanstra *et al.*, 1998; Marceau *et al.*, 1998), the rat cell cultures, VSMCs, rat-1 (Ni *et al.*, 1998a; Yang *et al.*, 1998a), bovine arterial endothelial cells (BAECs) (Ni *et al.*,

1998a) and the monkey cell line, COS (Yang *et al.,* 1998a). IMR-90 cells and rat VSMCs show an increase in BKB_1 receptor mRNA levels following the addition of inducing agents (Bachvarov *et al.,* 1996; Zhou *et al.,* 1998; Schanstra *et al.,* 1998; Ni *et al.,* 1998a). Zhou *et al.,* (1998) report that this increase corresponds to an 8 fold induction of BKB_1 receptor mRNA levels. In contrast IL-1β failed to induce BKB_1 receptor expression in HepG2 cells (Ni *et al.,* 1998a). BKB_1 receptor mRNA levels in HEK-293 cells, rat-1 cells, BAECs and COS cells have not been determined.

Regulatory activity of the intronic sequences show that a 917 bp fragment (fragment 1 in Figure 1.3), from intron 1 has no activity (Yang and Polgar, 1996), whereas fragments of 872 bp and 1900 bp (fragments 2 and 3, Figure 1.3) spanning intron 2 will drive reporter gene expression (Yang and Polgar, 1996; Chai *et al.,* 1996; Ni *et al.,* 1998a). These constructs did not confer inducibility of the reporter gene following the addition of IL-1β (Ni *et al.,* 1998a). In addition, fragment 2 exhibited one tenth of the activity of a construct spanning the 5' flanking region (fragment 4, Figure 1.3) (Yang and Polgar, 1996). Similarly, fragment 3 exhibited one seventh of the activity of a 2600 kb fragment spanning the 5' flanking region (fragment 5, Figure 1.3) (Ni *et al.,* 1998a). However in the non-inducible cell line, HepG2, fragment 3 exhibited approximately 8 fold more activity than fragment 5 (Ni *et al.,* 1998a). These results suggest that a sequence within intron 2 may function as an alternative promoter, possibly functional in basal conditions.

The most abundant transcription is driven by the 5' region flanking exon 1. Analysis of fragments spanning this region also exhibit cell type-specificity, having greater regulatory activity in BKB_1 receptor expressing cell lines (Yang *et al.,* 1998a). Detailed

analysis of the 5' flanking region by Yang and co-workers (1998a) has revealed the presence of positive and negative regulatory elements and a cell type-specific enhancer. The enhancer region is located –548 bp to –448 bp relative to the transcription initiation site (TIS) with NF-1-like and AP-1 sites crucial for activation. Assessment of the inducibility of a 1800 bp fragment (fragment 6, Figure 1.3) in SV40 transformed IMR-90 cells showed no significant increase in the presence of LPS, TNF-α or PMA, however, a two fold activity was observed on the addition of BK. Furthermore, a 4200 bp fragment spanning the 5' flanking region was reportedly not importantly modulated by IL-1 or dexamethasone in IMR-90 cells (Marceau *et al.*, 1998). The basal levels of expression of BKB_1 receptors in these cell lines may be one explanation for the lack of significant induction following the addition of inducing agents. Accordingly, Marceau and colleagues (1998) reported a doubling of reporter gene expression in HEK-293 cells when co-transfected with the human IL-1 receptor type I gene. In contrast, a recent study shows that a 1300 bp 5' flanking fragment (fragment 7, Figure 1.3) up-regulates the reporter gene by approximately 5 fold following the addition of IL-1β and desArg^{10}kallidin in IMR-90 cells (Schanstra *et al.*, 1998). The reason for these discrepancies is unclear. Schanstra and co-workers (1998) identified a nuclear factor-kappaB (NF-κB)-like site located approximately –1200 bp relative to the TIS that confers IL-1β and desArg^{10}kallidin inducibility of the reporter gene. NF-κB is a transcription factor involved in the regulation of a large number of genes involved in the immune and inflammatory response (Baldwin, 1996). Activation of NF-κB is discussed in more detail in section 1.6. Another study also reports the involvement of NF-κB in the inducible response of the human BKB_1 receptor gene to LPS in rat VSMCs (Ni *et al.*, 1998a). However, this study identifies a separate NF-κB-like element at position

−73 to −50 bp responsible for LPS-induction. Differences in the cell lines or the inducing agents may be responsible for these discrepancies. However, an inhibitor of NF-κB, pyrrolidinedithiocarbamate (PDTC), failed to block the IL-1β mediated up-regulation of BKB_1 receptor mRNA in IMR-90 cells (Zhou *et al.,* 1998). Thus these data are difficult to reconcile with each other and suggest that the inducible activity of the inducing agents may be acting through more than one transcriptional pathway depending on the cell type.

An allelic polymorphism in the human BKB_1 receptor gene has been located in the 5' flanking region and corresponds to a G to C transition at position −699 relative to the TIS (Bachvarov *et al.,* 1998a,b). Promoter analysis shows that the C containing allele exhibits a 40% increase in the expression of a reporter gene compared to an identical construct containing the G allele following transient transfection in IMR-90 cells. The frequency of this polymorphism (G–699 to C) was 33.3% in normal patients and exhibited a significant decrease in patients with a history of end stage renal failure (20.6%) and with inflammatory bowel disease, (5.7%) (Bachvarov *et al.,* 1998a,b). This may underlie a genetic predisposition to other inflammatory conditions involving the BKB_1 receptor.

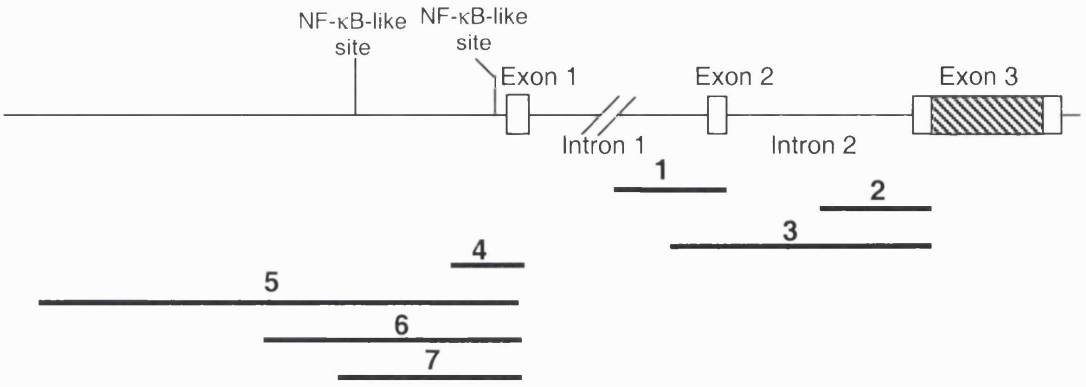


Figure 1.3 Schematic diagram of the human BKB_1 receptor gene (Yang and Polgar, 1996; Bachvarov *et al.*, 1996).

Exons are indicated by solid boxes, the coding region is shown by a hatched box inside exon 3. The broken line in intron 1 represents the large size of this intron. The location of the NF-κB-like sites in the 5' flanking sequence are indicated. Some of the fragments tested for promoter activity in reporter gene constructs are indicated by numbered lines. (**1**) a 917 bp fragment spanning the 3' end of intron 1 and the 5' end of exon 2 (Yang and Polgar, 1996); (**2**) an 827 bp fragment 5' of the coding sequence spanning intron 2 (Yang and Polgar, 1996); (**3**) a 1900 bp fragment 5' of the coding sequence, spanning all of intron 2, exon 2 and the 3'end of intron 1 (Chai *et al.*, 1996, Ni *et al.*, 1998a); (**4**) a 451 bp fragment (−361 to +89 bp) (Yang and Polgar, 1996); (**5**) a 2600 bp fragment (−2582 to +34 bp) which drives inducible reporter gene expression (Ni *et al.*, 1998a); (**6**) a 1800 bp fragment (−1748 to +89 bp) which does not increase expression following the addition of inducing agents (Yang *et al.*, 1998a); (**7**) a 1300 bp fragment (−1191 to +43 bp) which drives inducible reporter gene expression (Schanstra *et al.*, 1998). Nucleotide numbers are relative to the TIS reported by Yang and Polgar (1996).

1.5.2 Post-transcriptional regulation of the human BKB_1 receptor gene

Post-transcriptional mechanisms may also contribute to the IL-1β mediated up-regulation of BKB_1 receptor mRNA levels in IMR-90 cells (Zhou *et al.*, 1998). Addition of IL-1β to these cells doubled the BKB_1 receptor mRNA half life.

Transcriptional and post-transcriptional mechanisms of regulation of the BKB_1 receptor gene will be discussed further in Chapters, 4 and 5.

1.5.3 Signal transduction pathways involved in BKB_1 receptor up-regulation

Evidence presented in the sections above show that a number of inducing agents, in particular, LPS and IL-1, can up-regulate the BKB_1 receptor gene. Analysis of the promoter and 5' flanking sequences of the human BKB_1 receptor has provided information on the elements ultimately responsible for the increase in expression with different inducing agents. However, the intracellular mechanisms through which these changes occur are largely undetermined. The failure of protein synthesis inhibitors to block the IL-1β and LPS-induced increase in BKB_1 receptor mRNA levels have shown that new protein synthesis is not involved in these activating cascades (Zhou *et al.*, 1998; Ni *et al.*, 1998a). A role for the MAP kinases in the spontaneous, IL-1 and EGF-mediated contractile response to desArg^{9}BK in rabbit aortic rings has been demonstrated using specific MAP kinase inhibitors (Larrivee *et al.*, 1998). This was supported by analysis of rabbit aortic cultured smooth muscle cells which showed a correlation between the MAP kinase activation by the cytokines and the increase in BKB_1 receptor mRNA (Larrivee *et al.*, 1998). Another study showed that phorbol ester-mediated activation of PKC up-regulated BKB_1 receptor gene expression in a MAP kinase and NF-κB dependent manner in IMR-90 cells (Zhou *et al.*, 1998). In contrast, the IL-1β-induced BKB_1 receptor mRNA up-regulation in IMR-90 cells was not blocked using a MAP kinase inhibitor, a PKC inhibitor or a protein kinase A (PKA) inhibitor (Zhou *et al.*, 1998). This study showed a role for protein tyrosine kinases in the IL-1β-mediated receptor mRNA up-regulation in these cells. These data suggest that the intracellular

signalling pathways may differ according to the cell type and the stimulus involved. The signal transduction pathways activated in response to IL-1 and LPS will be briefly reviewed in the following section, focusing on the signal transduction pathways regulating the transcription factor NF-κB and the MAP kinases cascades.

1.6 Signal transduction pathways utilised by IL-1 and LPS

1.6.1 Signal transduction pathways utilised by IL-1

The IL-1 gene family is composed of intereukin-1alpha IL-1α, IL-1β, and IL-1Ra, each of which is synthesised as a precursor protein (Dinarello, 1994). IL-1Ra can block the binding and consequently the biological activities of IL-1α and IL-1β (Eisenberg *et al.*, 1990; Carter *et al.*, 1990). The major receptor that mediates the effects of IL-1 is the type I IL-1 receptor (IL-1RI) (Sims *et al.*, 1988). A second receptor termed IL-1RII has been identified which does not signal and acts as a decoy receptor (Stylianou *et al.*, 1992; Colotta *et al.*, 1993). The cytoplasmic domain of IL-1RI shares significant homology with the cytoplasmic region of the *Drosophila melanogaster* protein Toll (Gay and Keith, 1991). Subsequently, a number of proteins with homologous cytoplasmic signalling domains to IL-1RI and Toll have been described in diverse species including the human Toll like receptors (TLRs) (reviewed by O'Neill and Greene, 1998). More recent studies have shown that IL-1RI must complex with another IL-1 receptor family member, IL-1 accessory protein (IL-1AcP), to transduce a signal (Greenfeeder *et al.*, 1995).

IL-1 stimulation does not increase intracellular calcium levels or directly activate phosphatidylinositol hydrolysis. However, almost every other signal transduction system has been reported to be activated in response to IL-1 (Bankers-Fulbright *et al.*, 1996). In the last two years there has been an acceleration in the understanding of the IL-1 signal transduction pathways, particularly in the activation of NF-κB. A large number of IL-1 inducible genes are regulated by NF-κB. Indeed, as discussed in section 1.5, a role for NF-κB in the up-regulation of the human BKB_1 receptor gene has been postulated.

1.6.1.1 IL-1-activated signal transduction pathways to NF-κB

NF-κB consists of homodimers and heterodimers of proteins that belong to the Rel family, to date five proteins have been identified in mammalian cells: p65, c-Rel, RelB, p50/p105 and p52/p100 (reviewed in Baldwin, 1996). The retention of NF-κB in the cytoplasm is due to its interaction with inhibitory proteins called IκBs, of which the most important are thought to be IκBα, IκBβ and IκBε (Baeuerle and Baltimore, 1996; Whiteside *et al.*, 1997). The key event in the activation of NF-κB is the phosphorylation and degradation of the inhibitory protein, IκB. This results in the release of NF-κB, which translocates to the nucleus and activates target gene expression. The molecular events linking the IL-1R signalling complex to the induction of NF-κB have recently been characterised. Upon binding of IL-1 to its receptor and association with the IL-1AcP the complex recruits and activates the adaptor protein, MyD88 (Wesche *et al.*, 1997; Burns *et al.*, 1998). MyD88 in turn recruits two distinct putative serine-threonine kinases, called IL-1 receptor-activated kinase (IRAK)-1 and IRAK-2, to the receptor complex (Muzio *et al.*, 1997). IRAK-1 and IRAK-2 subsequently interact with the

adapter molecule, TNF receptor-associated factor 6 (TRAF6) (Cao *et al.*, 1996; Muzio *et al.*, 1998), which links them to the protein kinase, NF-κB-inducing kinase (NIK) (Song *et al.*, 1997). TRAF6 resembles another member of the TRAF family, TRAF2, which is required for NF-κB activation through the TNF receptors (Song *et al.*, 1997). Like TRAF6, TRAF2 also interacts with NIK, and thus this may be a point at which signals from unrelated receptors converge (Malinin *et al.*, 1997). NIK belongs to the MAP kinase kinase kinase (MAP3K) family and associates with the IκB kinase (IKK), IKK1 and IKK2 (or IKKα and IKKβ) which directly phosphorylates IκB (DiDonato *et al.*, 1997; Stancovski et al. 1997; Woronicz *et al.*, 1997; Delhase *et al.*, 1999). IKK1 and IKK2 were identified as components of a high molecular weight complex, termed the IKK signalsome, containing a number of proteins involved in the activation of NF-κB (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). More recently Ninomiya-Tsuji and co-workers (1999) showed that the MAP kinase kinase kinase (MAP3K), TAK1, interacts with TRAF6 and acts upstream of NIK in the IL-1 activated signalling cascade. Phosphorylation of IκB initiates the ubiquitin-proteasome-mediated degradation pathway which liberates and activates NF-κB (Baeuerle and Baltimore, 1996).

Evidence now exists for a second IL-1 signalling pathway leading to phosphorylation of the NF-κB p65/RelA subunit in parallel to the cascade leading to IκB degradation (Naumann *et al.*, 1994; Sizemore *et al.*, 1999; Bergmann *et al.*, 1998; Nasuhara *et al.*, 1999). Recent studies show that this pathway is dependent on recruitment of PI 3-kinase to the receptor and its subsequent activation (Sizemore *et al.*, 1999; Marmiroli *et al.*, 1998). Inhibition of IL-1 signalling using PI 3-kinase inhibitors further supports a role for this signal transducer in IL-1 mediated responses (Reddy *et al.*, 1997;

Donaldson *et al.,* 1996; Sizemore *et al.,* 1999). However, the downstream targets of PI 3-kinase in this pathway remain to be elucidated. Possible candidates are the PKC isoforms, in particular PKCζ, which have been shown to regulate the phosphorylation of NF-κB (Bergmann *et al.,* 1998; Anrather *et al.,* 1999; Bonizzi *et al.,* 1999). Other targets of PI 3-kinase include MAPK activation (Bondeva *et al.,* 1998) and Akt also known as protein kinase B (PKB) (Sizemore *et al.,* 1999; Kane *et al.,* 1999). In addition, NF-κB activation has been demonstrated following serine phosphorylation by the PKAc subunit through a cAMP independent mechanism (Zhong *et al.,* 1997, 1998). The pathways of IL-1-mediated NF-κB activation are shown in Figure 1.4. Activation of MAP kinase cascades have also been shown to activate NF-κB after release from IκB, this will be described in the following section.

1.6.1.2 IL-1 activated protein kinase cascades

Three MAP kinase cascades have been identified and named according to the final enzyme in each series, these are the ERKs, the JNK/SAPKs and p38 MAP kinase. The MAP kinases are proline directed kinases, requiring dual specific phosphorylation of both tyrosine and threonine residues for maximal activation (Su and Karin, 1996). Considerable evidence shows that IL-1 activates the three subtypes of MAP kinases (Kyriakis *et al.,* 1994,1996; O'Neill *et al.,* 1998; Paul *et al.,* 1997; Saklatvala *et al.,* 1999). Various transcription factors have been identified as targets for the MAP kinases, these include c-Jun, c-Fos, activating transcription factor (ATF)-1 and ATF-2, Elk-1, cAMP response element binding protein (CREB) (reviewed in Treisman, 1996; Su and Karin, 1996; Schulze-Osthoff *et al.,* 1997). More recently, activation of NF-κB has been shown following IL-1, TNF and phorbol ester stimulation of the p38 pathway

(Beyaert *et al.,* 1996; Schulze-Osthoff *et al.,* 1997; Wesselborg *et al.,* 1997; Bergmann *et al.,* 1998). Studies in IL-1 signalling have attempted to identify upstream and downstream targets of the MAP kinase cascades. Interestingly, two components of the IκB degradation pathway, TRAF6 and TAK1, can activate the JNK pathway (Martin *et al.,* 1997; Ninomiya-Tsuji *et al.,* 1999). These data suggest bifurcation of the IL-1 induced JNK and NF-κB activation pathways occur at these proteins. Low-molecular-weight G proteins are important upstream regulators of MAP kinases and furthermore can be activated in response to IL-1 (Zhang *et al.,* 1995; Popoff *et al.,* 1996; Singh *et al.,* 1999). The precise targets following IL-1 mediated activation of the MAP kinase pathways remain to be determined. Other kinases activated by IL-1 include the TNF/IL-1-induced protein kinase (TIP kinase) which is exclusively regulated by IL-1 and TNF (Guesdon *et al.,* 1997), the sphingomyelin-ceramide pathway (Kolesnick and Golde, 1994) and casein kinase II (Bird *et al.,* 1997).

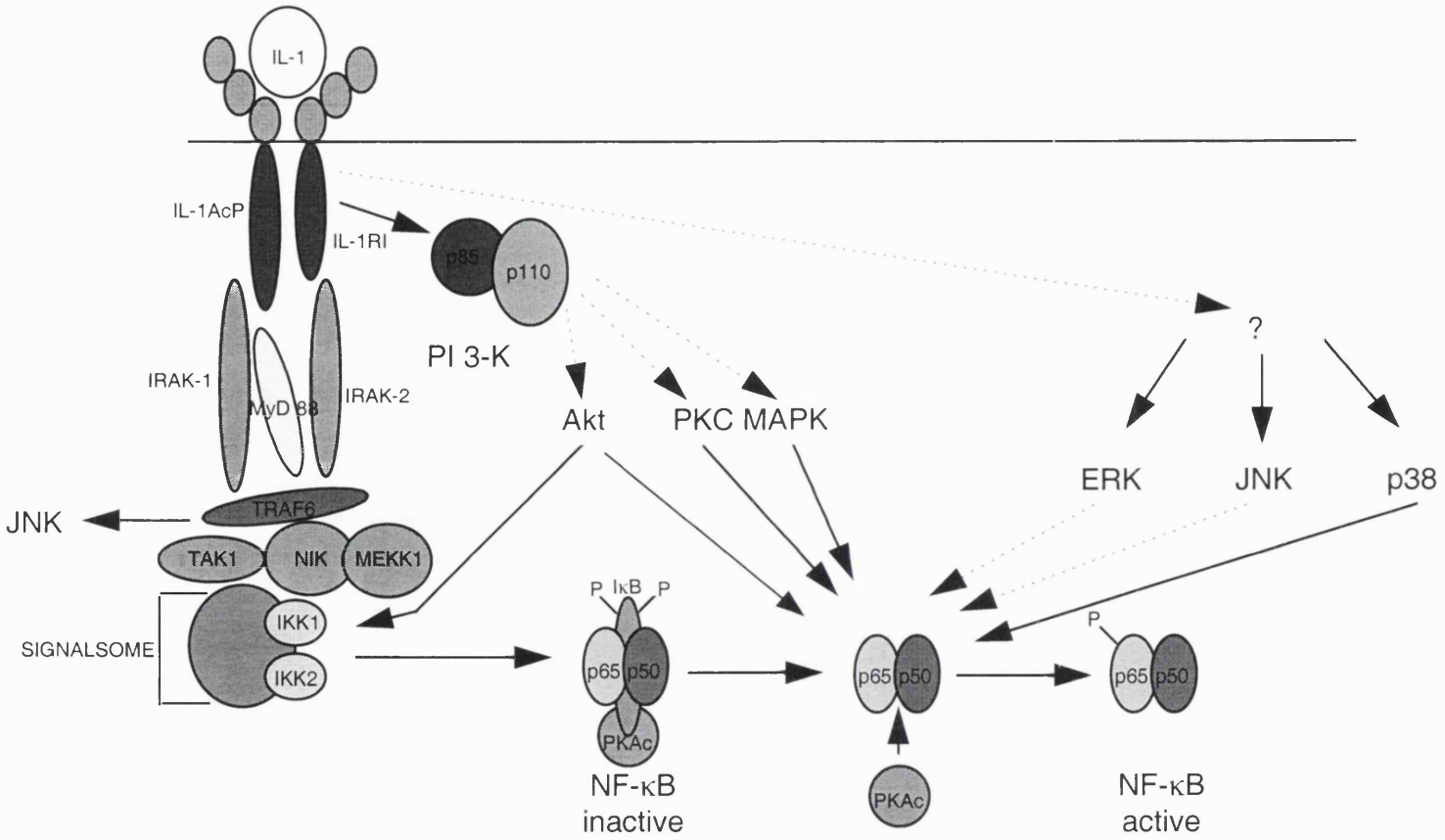


Figure 1.4 Schematic diagram of the IL-1-mediated activation of NF-κB.

All terms are described in the text. Pathways that are reasonably well characterised are shown as solid arrows, while speculative ones are shown as dashed. **P**'s represent phosphorylation, although the sites of p65 phosphorylation by the protein kinases have not been characterised.

1.6.2 Signal transduction pathways utilised by LPS

LPS or endotoxin, is the major component of the outer surface of Gram-negative bacteria. As demonstrated in tables 1.1. and 1.2, LPS has been used as a potent inducer of BKB_1 receptor expression. Stimulation of monocytes by LPS activates many second messengers and signal transduction pathways some of which may be the result of autocrine effects, such as LPS induced IL-1 release (Sweet and Hume, 1996).

Activation of LPS responsive cells, such as monocytes and macrophages, occurs after LPS interacts with circulating LPS binding protein and CD14, a glycosylphophatidylinositol-linked cell surface glycoprotein necessary for sensitive responses to LPS (Ulevitch and Tobias, 1995). CD14 is devoid of a cytoplasmic domain and does not elicit intracellular signalling events directly. More recently a number of studies show a role for members of the TLR family in the transduction of LPS signals across the plasma membrane (Yang *et al.,* 1998b,1999; Kirschning *et al.,* 1998; Chow *et al.,* 1999). Toll was originally identified in *Drosophila* and is essential for the establishment of dorsoventral polarity in the embryo and in the induction of an anti-fungal response (Morisatio and Anderson, 1995; Lemaitre *et al.,* 1996). In humans five homologues of Toll, designated TLR1-5 have been identified (Medzhitov *et al.,* 1997; Rock *et al.,* 1998). These proteins are members of the IL-1 receptor family and furthermore use overlapping signalling components as described in the following section.

1.6.2.1 LPS transduction pathways to NF-κB

Accumulating evidence suggest that activation of NF-κB by LPS uses the same pathway of IκB degradation as that described above for IL-1. LPS activates both IKK-1 and

IKK-2 and NIK and MEKK1 (O'Connell *et al.,* 1998; Swantek *et al.,* 1999). Other studies have linked LPS activation of TLRs, in particular TLR2, to the proximal signalling events leading to activation of NF-κB (Medzhitov *et al.,* 1998; Kirschning *et al.,* 1998; Muzio *et al.,* 1998; Yang *et al.,* 1999, Zhang *et al.,* 1999). Dominant negative versions of MyD88, IRAK, TRAF6 or NIK were used to show the involvement of these proteins in LPS-mediated NF-κB activation (Yang *et al.,* 1999; Zhang *et al.,* 1999). In addition, IL-1Ra had no effect in this system demonstrating that the NF-κB activation was not due to autocrine release of IL-1 (Zhang *et al.,* 1999). Similar to IL-1, LPS-activated NF-κB can also be regulated following IκB degradation (Yoza *et al.,* 1996). A role for protein-tyrosine kinase activation has been shown in this process (Yoza *et al.,* 1996).

1.6.2.2 LPS activated protein kinase cascades

Activation of the three major MAP kinase cascades, ERKs, JNK/SAPKs and p38 has been demonstrated in response to LPS (Han *et al.,* 1994; Hall *et al.,* 1999; Carter *et al.,* 1999). Hall and co-workers (1999) showed the transient phosphorylation of the transcription factor JunD correlating to JNK/SAPK activation in response to LPS treatment. However, activation of specific upstream and downstream regulators in response to LPS remains largely uncharacterised.

1.7 Aims of the study

The search for a possible role for inducible BKB_1 receptors in pathophysiology has resulted in increased analysis of this receptor subtype. A large number of these studies have been developed in rodents, however, prior to the studies in this thesis the rat BKB_1 receptor gene had not been isolated. Moreover, species-specifc differences between the receptors in their affinity for BKB_1 receptor agonists were apparent. In addition, the lack of molecular tools, such as species-specific probes, has hampered progress in understanding the mechanisms of regulation of the BKB_1 receptor gene.

Therefore, the aims of this thesis were as follows:

1. **To determine the cDNA sequence and genomic structure of the rat BKB_1 receptor gene.**
2. **To analyse the expression patterns of the BKB_1 receptor mRNA.**
3. **To investigate the mechanisms of regulation of the rat BKB_1 receptor gene.**

1.8 Scientific approach

Aim 1

For the isolation of a rat clone containing the BKB_1 receptor gene a rat genomic library will be screened under low stringency hybridisation conditions. The isolated clone will then be used to screen a cDNA library to isolate a rat BKB_1 receptor cDNA clone. Alignment of the genomic and cDNA sequences will be used to elucidate the structural organisation of the receptor gene and sequences of the exon-intron boundaries. The cDNA ends of the transcripts will be mapped by primer extension analysis and rapid amplification of cDNA ends (RACE).

Aim 2

Northern blot analysis will be used to examine the BKB_1 receptor mRNA expression pattern in isolated tissues from control and LPS-treated rats. Analysis of inducible BKB_1 receptor mRNA levels in rat cultured cell lines will be used to identify a cellular model in which to study the mechanisms of BKB_1 receptor regulation. The expression profiles of BKB_1 receptor mRNA levels following the addition of a number of inducing agents will be examined by Northern blot studies. This should provide information about the potency of these agents and the time course of inducible BKB_1 receptor gene expression.

Aim 3

To address the mechanisms of BKB_1 receptor gene regulation, the receptor mRNA levels will be examined in the presence of transcriptional and translational inhibitors. To examine the role of signal transduction components the BKB_1 receptor mRNA levels will be measured in the presence of specific inhibitory compounds. Similarly, the role of NF-κB activation pathways will be addressed by analysis of the BKB_1 receptor mRNA levels in the presence of a dominant negative IκB protein. To delineate important regulatory elements in the 5' flanking sequence of the gene, reporter gene constructs will be synthesised and analysed following transient transfection into cultured cell lines. In addition, the interaction of transcription factors to with regulatory elements will be examined using electromobility shift assays (EMSA).

Chapter 2

Methods and Materials

2.1 DNA probe labelling

DNA fragments were labelled using a random priming method with the Amersham Megaprime labelling system (Amersham Pharmacia Biotech). 25 ng of DNA and 5 µl of random primer (supplied with kit) in a total volume of 26 µl were denatured by boiling for 5 min and quenching on ice for a further 5 min. 4 µl of each deoxynucleotide triphosphate (dNTP) (dTTP, dGTP and dCTP) (supplied with kit), 5 µl of 10 × reaction buffer (supplied with kit), 5 µl of α-^{32}P dATP (110 TBq/mmol, Amersham Pharmacia Biotech) and 2 µl of Klenow fragment (2 units) were added to give a final volume of 50 µl. The reaction was then incubated for 15 min at 37°C. Unincorporated nucleotide was removed by passing through a G-50 sephadex Nick column (Amersham Pharmacia Biotech). After disposal of excess liquid, the column was equilibriated with 3 ml of 1 × Tris/Ethylenediaminetetra-acetic acid (EDTA) (TE) buffer (0.1 M Tris, pH 8, 0.01 M EDTA). The reaction was then added to the column, 300 µl of 1 × TE was then added and allowed to enter the gel bed. The labelled probe was eluted by the addition of a further 500 µl of 1 × TE to the column. 1 µl of the purified probe was spotted onto 3 mm Whatman paper (Whatman) and the specific activity was measured using a Bioscan QC2000. Before hybridisation the probe was denatured by boiling for 5 min and quenched on ice.

Oligonucleotides were labelled by a 3' tailing method as follows: 10 pmoles of primer were incubated in 1 × T4 polynucleotide kinase buffer, (70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) (New England Biolabs (NEB)), 3 µl of γ-^{32}P ATP (110 TBq /mmol, Amersham Pharmacia Biotech) and 1 µl of T4 polynucleotide kinase (10 units, NEB) in a total volume of 20 µl for 30 mins at 37°C. For oligonucleotides longer than 20 bases the unincorporated nucleotide was removed by passing the reaction through a G-50 sephadex Nick as described above. Counts were measured as described above.

2.2 Genomic library screening

A Sprague Dawley rat DNA library in the SuperCos 1 vector (Stratagene) was screened by hybridisation with a 750 bp random prime labelled Xba I/Eco R1 fragment from a human BKB_1 receptor cDNA clone (Jones *et al.*, 1999). Approximately 1×10^6 colony forming units (cfu) were screened on Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) with the α–^{32}P dATP labelled human fragment. Colonies were transferred to nylon membranes by duplicate lifts from each of the plates. The colonies were lysed by placing them on Whatman 3 mm paper soaked in 10% sodium dodecyl sulphate (SDS) for 5 min. The DNA was denatured by placing the membranes on Whatman 3 mm paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and then neutralised by transferring the membranes to Whatman 3 mm soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) 0.001 M EDTA) for a further 5 mins. Filters were then washed in 2 × standard sodium saline-citrate (SSC) buffer (0.3 M NaCl, 0.03 M sodium citrate), to remove cell debris and fixed by baking for 2 hours at 80°C. The filters were prehybridised at 42°C for 2-3 hours in

hybridisation buffer containing 50% formamide, 5 × SSC, 5 × Denhardts (0.1% bovine serum albumin (BSA), 0.1% Ficoll and 0.1% polyvinylpyrrolidine, (Sigma)), 0.1% SDS and 100 μg/ml herring sperm DNA (Sigma). After prehybridisation, labelled probe equivalent to 1×10^6 dpm was added and hybridisation was performed at 42°C for 12-15 hours. The membranes were washed twice with 2 × SSC, 0.1% SDS at room temperature, once with 1 × SSC, 0.1% SDS at 50°C and finally once with 0.1 × SSC, 0.1% SDS at 50°C. The membranes were then exposed to X-ray film (Kodak) at −70°C for 15-18 hours. 40 colonies were picked that corresponded to hybridisation signals and pooled into 8 groups, each with 5 colonies. Secondary and tertiary screens, plating approximately 200 and 20 cfu per plate, respectively, were carried out to isolate single clones.

2.3 cDNA library synthesis and screening

A λ ZAP express library (Stratagene) was constructed using poly(A)$^+$ RNA extracted from rat bladders and incubated in Kreb's balanced salt solution (100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2mM KH_2PO_4, 11.7 mM glucose, 2.5 mM $CaCl_2$) to allow maximum expression of the BKB_1 receptor mRNA (Marceau *et al.*, 1980). 5 μg of poly(A)$^+$ RNA was made up to a total volume of 37.5 μl with 0.1% diethyl pyrocarbonate (DEPC) treated water and used for first strand synthesis. The poly(A)$^+$ RNA was added to a microcentrifuge tube containing 5 μl of 10 × first strand buffer, 3 μl of first strand methyl nucleotide mixture, 2 μl of linker primer (1.4 μg/μl) and 1 μl of RNase block ribonuclease inhibitor (40U/μl) (all reagents supplied with kit). The reaction was allowed to anneal for ten minutes at room temperature. 1.5 μl of

moloney murine leukemia virus reverse transcriptase (MMLV-RT) 50 U/μl was then added to a final reaction volume of 50 μl. 5 μl of the reaction volume was transferred to a tube containing 0.5 μl of α-^{32}P dATP (110 TBq /mmol, Amersham Pharmacia Biotech) for analysis of the quality and quantity of the first strand synthesis. The samples were then incubated at 37°C for 1 hour and transferred to ice. For second strand synthesis the following components were added on ice, 20 μl of 10 × second strand buffer, 6 μl of second strand nucleotide mixture, 106 μl of sterile water, 2 μl of α-^{32}P dATP (110 TBq/mmol, Amersham Pharmacia Biotech), 3.5 μl of RNase H (0.9 U/μl) and 11 μl of DNA polymerase I (9.1 U/μl) (all reagents supplied with kit). The reaction was incubated at 16°C for 2.5 hours and then placed on ice. 23 μl of blunting dNTP mix and 2 μl of cloned Pfu DNA polymerase (reagents supplied with kit) were added to the reaction and incubated for 30 mins at 72°C to blunt the cDNA termini. The sample was extracted once with an equal volume of phenol/chloroform (1:1) and once with chloroform. The cDNA pellet was then ethanol precipitated, and washed in 70% ethanol, lyophilised until dry and finally resuspended in 9 μl of Eco RI adaptors (supplied with kit). A further 1 μl of 10 × ligase buffer, 1 μl of 10 mM rATP and 1 μl of T4 DNA ligase (4 U/μl) (all reagents supplied with kit) were added and the reaction was incubated at 8°C for 2 days to allow ligation of the Eco RI adaptors. The ligase was heat inactivated by incubating at 70°C for 30 mins. The adaptor ends were kinased by the addition of 1 μl of 10 × ligase buffer, 2 μl of 10 mM rATP, 6 μl of sterile water and 1 μl of T4 polynucleotide kinase (10 U/μl) (all reagents supplied with kit) at 37°C for 30 mins. Xho I digestion of the reaction mix was performed by the addition of 28 μl of Xho I buffer and 3 μl of Xho I (40 U/μl) at 37 °C for 1.5 hours. After the addition of 10 × STE (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 10 mM EDTA) buffer the sample

was run through a Sephacryl S-500 (Amersham Pharmacia Biotech) column at 400 × g. Three separate fractions were collected and ethanol precipitated. The cDNA pellets were then resuspended in 5 µl of sterile water and 2 µl of 6 × gel loading dye, (0.05% w/v bromophenol blue, 40% w/v sucrose, 0.01M EDTA, pH 8, and 0.5% w/v SDS, Sigma) and size fractionated by gel electrophoresis. cDNA between 1 and 1.7 kb was gel purified using Jetsorb (Genomed) and ethanol precipitated. The pellet was resuspended in 1 µl of water and ligated into the Uni-ZAP XR vector arms by addition of the following reagents, (all supplied with the kit) 1 µl of Uni-ZAP XR vector (1 µg), 0.5 µl of 10 × ligase buffer, 0.5 µl of 10 mM rATP (pH 7.5) and 0.5 µl of T4 DNA ligase (4 U/µl) in a final volume of 5 µl. The reaction was incubated for 15 hours at 12°C. All of the reaction was packaged using Stratagenes' Gigapack III Gold packaging extract. The DNA was added to the packaging extract (supplied with kit). 15 µl of sonic extract (supplied with kit) was added and the packaging reaction was incubated at room temperature for 2 hours. 500 µl of SM buffer (0.1 M NaCl, 0.015M $MgSO_4$, 0.05M Tris, 0.01% gelatin) and 20 µl of chloroform were added to the packaged DNA and the phage were stored at 4°C for titering. Approximately 4×10^5 plaque forming units (pfu) were screened on Hybond N^+ membranes with an α-^{32}P dATP labelled 900 bp Pst I fragment spanning the ORF of the rat BKB_1 receptor gene (Probe A). Nylon filters were prepared and hybridised as described above for genomic library screening. The membranes were washed twice with 2 × SSC, 0.1% SDS at room temperature, once with 1 × SSC, 0.1% SDS at 50°C and finally once with 0.1 × SSC, 0.1% SDS at 58°C. The membranes were then exposed to X-ray film at −70°C for 4 hours and colonies picked that gave hybridisation signals on both replica membranes. A secondary screen was carried out to isolate a single cDNA clone. A full length cDNA sequence was

obtained by RT-PCR using primers, sense 5'-AGGTCACCATCAAAAACACAGG-3' and antisense, 5'CTTCTTGGCTCCCTGCTGTT-3' and using 1 µl of cDNA used for the synthesis of the cDNA library. RT-PCR was carried out using the conditions described in section 2.15.

2.4 Plasmid DNA purification

Small scale purification of plasmid DNA was carried out using Promega Wizard Plus miniprep DNA purification systems. 3 mls of overnight cultures in Luria Bertani (LB) medium (Life Technologies) plus the selective antibiotic (either ampicillin (Sigma) at 50 µg/ml or kanamycin (Sigma) at 50 µg/ml) were pelleted by centrifugation at 13,000 rpm for 1 min. The cell pellet was resuspended in 200 µl of resuspension buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). 200 µl of cell lysis buffer (0.2 M NaOH and 1% SDS) was added and mixed by inversion until clear. 200 µl of neutralisation buffer (1.32 M potassium acetate) was added, mixed followed by centrifugation of the lysate at 13,000 rpm for 5 mins. Plasmid DNA was then processed using a vacuum manifold (Promega Vac-Man). For each miniprep a 2 ml syringe barrel was attached to the Luer-Lok extension of a minicolumn. The minicolumn/syringe barrel was inserted into the vacuum manifold. 1 ml of resuspended resin was applied to each minicolumn/syringe assembly, the cleared lysate from each miniprep was then added to the barrel and a vacuum applied to pull the resin/lysate mix into the minicolumn. 2 ml of column wash solution (80 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA and 55% ethanol) was added and the vacuum reapplied. The resin was dried by keeping the vacuum applied for an additional 30 seconds after the solution had passed through the column. The minicolumn was transferred to a

microcentrifuge tube and centrifuged for 2 mins at 13,000 rpm to remove any residual wash solution. The plasmid DNA was eluted by applying 50 µl of water, pre-heated to 50°, to the minicolumn and after waiting 1 minute, spinning for 20 sec at 13,000 rpm.

For large scale preparations of DNA, Qiagen plasmid purification maxi kits were used. 200 ml cultures were grown overnight in LB plus the selective antibiotic (ampicillin or kanamycin, concentrations as described for miniprep DNA purification). The bacterial cells were harvested by centrifugation at 4,000 rpm for 15 min at 4 °C. The pellet was resuspended in 10 ml of resuspension buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA and 100 µg/ml RNase A). Cells were lysed by the addition of 10 ml of lysis buffer (200 mM NaOH and 1% SDS) and incubating at room temperature for 5 mins. 10 ml of neutralisation buffer (3 M potassium acetate, pH 5.5) were added and the lysate placed on ice for 20 min, followed by centrifugation at 13,000 rpm in a Sorvall SS-34 rotor. The supernatant containing the plasmid DNA was transferred to a pre-equilibriated Qiagen tip-500 (tips were equilibriated by the addition of 10 ml of equilibriation buffer, (750 mM NaCl, 50 mM 3-[N-morpholino]propanesulphonic acid (MOPS), pH 7.0, 15% isopropanol and 0.15% Triton X-100) and allowed to enter the resin by gravity flow. The tip was washed by the addition of 2 × 30 ml of medium salt wash buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0, and 15% isopropanol) to remove contaminants. Plasmid DNA was eluted by the addition of 15 ml of high salt elution buffer (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, and 15% isopropanol). The eluted plasmid DNA was desalted and precipitated by adding 0.7 volumes of isopropanol at room temperature. The plasmid DNA pellet was washed in 70% ethanol, air dried and resuspended in 1 ml of 1 × TE.

The DNA concentrations were determined by reading the absorbance at 260 nm using a spectrophotometer.

2.5 Southern blot analysis

The DNA samples were combined with 1 × Sigma gel loading buffer and run alongside DNA molecular weight markers (Life Technologies) on 1% agarose gels in 1 × Tris-acetate 0.04 M /EDTA 0.001 M (TAE) electrophoresis buffer. The DNA gel was denatured for 20 minutes in 250 mM HCl and neutralised for a further 20 minutes in 0.4 M NaOH. The gel was blotted onto Hybond N^+ nylon membrane by capillary transfer in 0.4 M NaOH for 15-18 hours. Following transfer the membrane was briefly rinsed in 2 × SSC. Prehybridisation and hybridisation were carried out as described in section 2.2 for genomic library screening. Membranes were exposed to the Molecular Dynamics phosphorimager screen overnight and then scanned with a Molecular Dynamics STORM 840.

2.6 Mapping the cosmid clone, RB1

The rat BKB_1 receptor clone, RB1, in SuperCos 1 was mapped by endonuclease digestion and Southern blot analysis. 4 μg of RB1 DNA were digested with Not I. The digested DNA was divided into 2 samples. One of the DNA samples was then partially digested with Eco RI. The Not I digested DNA was made up to 100 μl in 1 × restriction enzyme buffer and divided into 5 tubes, with tube 1 containing 30 μl of mixture, tubes 2-4 containing 20 μl of mixture and tube 5 containing 10 μl of mixture. Tubes 1 to 5 were then placed on ice. 10 units of Eco RI were added to tube 1, the reaction was

mixed and then 10 µl of this mixture was transferred to tube 2. This serial dilution was repeated for all five tubes on ice. The samples were then digested at 37°C for 15 min and the reactions were stopped by the addition of 1 × Sigma gel loading buffer. All the samples were separated by electrophoresis on a 0.7% agarose gel and Southern blotted as described in section 2.5. End-labelled T7 or T3 oligonucleotides (Promega) were used for hybridisation. Blots were hybridised with Amersham rapid hybridisation buffer for 1 hour at 42°C. Membranes were washed once with 6 × SSC, 0.1% SDS for 10 mins at room temperature, followed by a further wash at 42°C for 15 mins. Hybridisation with probes spanning the BKB_1 receptor gene were used to determine the location and orientation of the BKB_1 receptor coding sequence within RB1. A 5.2 kb Eco RI fragment and an overlapping 3' 2.2 kb Bam HI fragment spanning the BKB_1 receptor gene were subcloned into pBluescript II KS (Stratagene). A 1.6 kb Bam HI fragment located directly upstream of the 2.2 kb Bam HI fragment and within the 5.2 kb fragment was subcloned into pBluescript II KS. These fragments were sequenced as described in section 2.7.

2.7 DNA sequencing

The nucleotide sequences of the cloned receptor fragments were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T7 sequencing kit (Amersham Pharmacia Biotech). 2 µg of DNA were diluted in 32 µl of water, the DNA was denatured by adding 8 µl of 2 M NaOH and incubating at room temperature for 10 min. The DNA was then ethanol precipitated and the pellet washed in 70% ethanol, dried, and respuspended in 10 µl of water. 10 pmole of sequencing primer and 2 µl of annealing buffer (supplied with kit) were added in a final volume of 14 µl and incubated

5 min at 65 °C, followed by for 5 min at 37 °C and finally for 5 min at room temperature to allow the primer to anneal to the DNA. Extension reactions were carried out by the addition of 3 µl of labelling mix (supplied with kit) and 0.5 µl of α-^{33}P dATP (100 TBq /mmol, Amersham Pharmacia Biotech) and incubating at room temperature for 5 min. Termination reactions were carried out by the addition of 4.5 µl of labelled reaction to each of 4 tubes containing a different dideoxyribonucleoside triphosphates, either GTP, ATP, TTP or CTP (supplied with kit). Reactions were incubated for a further 5 min at 37°C and then stopped by the addition of 5 µl of Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) (supplied with kit). Samples were denatured by heating to 70°C for 2 min before loading onto a 6% polyacrylamide gel (Flowgen) in 1 × Tris-borate 0.09 M/EDTA 0.002 M (TBE) electrophoresis buffer. The sequences obtained were analysed using the DNAstar software package. The sequence of the 6 kb Eco RI to Bam HI fragment containing the BKB_1 receptor gene is shown in Appendix 1.

2.8 Total RNA isolation

Total RNA was prepared using Sigma Tri-Reagent by the method of Chomzynski and Saachi (1987). Female Wistar rats (approximately 200 g) were given an intravenous injection of LPS (3 mg/kg). The animals were sacrificed 4 hours after the injection of LPS and tissues were removed and frozen in liquid nitrogen. The tissues and cells were stored at −70°C until the RNA was extracted. Tissues were homogenised in 1 ml of Tri Reagent (Sigma) per 100 mg of tissue using a Polytron. For cell cultures, cells were lysed directly on the culture dish following removal of the media and washing with phosphate buffered saline (PBS), 1 ml of Tri Reagent was used per 20 cm^2 of the culture

dish. Following cell lysis samples were transferred to corex tubes and 0.2 ml chloroform was added per ml of Tri Reagent and the samples were shaken vigorously. The samples were centrifuged at 12,000 × g for 15 min at 4°C and the upper aqueous phase removed to a fresh tube. The RNA was then precipitated with 0.5 volumes of isopropanol. After washing the pellet in 75% ethanol and air drying the RNA was dissolved in 0.5 ml-1 ml of DEPC-treated water (0.1%) containing 30 units of RNasin ribonuclease inhibitor (Promega). The concentration of RNA was determined by reading the absorbance at 260 nm and purity was assessed by the A_{260}/A_{280} ratio.

2.9 Poly(A)$^+$ RNA isolation

Poly(A)$^+$ RNA was purified from total RNA by binding to oligo dT cellulose using Ambion Poly(A) Pure (Ambion). 5 M NaCl was added to 0.2-2 mg of total RNA to a final concentration of 0.45 M. High salt binding buffer (supplied with kit) was added to a final volume of approximately 4 ml and the sample was denatured at 65°C for 5 min and quenched on ice for 1 min. 100 mg of oligo dT was added to the RNA and the sample was rocked at room temperature for 1 hour. The resin was pelleted by centrifugation at 2,000 × g for 3 min and then resuspended in a further 10 ml of high salt binding buffer. The resin was pelleted as before. This was repeated a total of three times with high salt binding buffer. Three further washes in low salt wash buffer (supplied with kit) were also carried out to remove additional ribosomal RNA. After the third wash the resin was resuspended in 0.5 ml of wash buffer and transferred to a spin column. The poly(A)$^+$ RNA was eluted with 2 × 200 µl of elution buffer (10 mM Tris pH 7.5 and 1 mM EDTA) prewarmed to 65°C and precipitated with 2 µl glycogen (5 mg/ml), 0.1 volumes of 5 M ammonium acetate, and 2.5 volumes of ethanol. The pellet

was washed in 75% ethanol and dissolved in 50 µl of nuclease free water (0.1% DEPC, 0.1 mM EDTA). The concentration was determined by reading the absorbance at 260nm using a spectrophotometer.

2.10 Northern blot analysis

Poly(A)$^+$ RNA (4 µg/lane) was made up to a total volume of 10 µl with DEPC-treated water (0.1%). 5 µl of Sigma RNA gel loading buffer (62.5% v/v formamide, 1.14 M formaldehyde, 200 µg/ml bromophenol blue, 200 µg/ml xylene cyanole, 50 µg/ml ethidium bromide, and 1.25 × MOPS-EDTA-sodium acetate) was added to each sample, this was denatured at 65 °C for 10 min and quenched on ice. The samples were loaded, alongside RNA millennium markers (Ambion Inc), on a 1% denaturing agarose gel, made up in 17 % formaldehyde and 1 × formaldehyde gel-running buffer buffer pH 7.0 (0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA (pH 8.0)) and electrophoresed in 1 × formaldehyde gel-running buffer. The gel was then rinsed in DEPC-treated water (0.1%) and blotted onto Ambion Brightstar charged nylon membrane (Ambion Inc) by capillary transfer for 15-18 hours in 20 × SSC transfer buffer. The membrane was baked at 80°C for 2 hours. The filters were prehybridised at 42°C for 2-3 hours in hybridisation buffer containing 50% formamide, 5 × SSC, 5 × Denhardts Reagent, 0.5% SDS, 10% dextran sulphate and 100 µg/ml herring sperm DNA. Labelled probe equivalent to 1 × 10^6 dpm was added and hybridisation was performed at 42°C for 12-15 hours. The membranes were washed once with 2 × SSC, 0.5% SDS at room temperature, once with 1 × SSC, 0.5% SDS at 50°C and finally once with 0.1 × SSC, 0.5% SDS at 65°C. The membranes were exposed to a phosphorimager screen

overnight then scanned with a Molecular Dynamics STORM 840. Probe A was used for analysis of the BKB_1 receptor mRNA levels. Quantitation of the BKB_1 receptor mRNA levels was determined by hybridisation with a random prime labelled 103 bp rat cyclophilin fragment excised from pTRI-cyclophilin (Ambion Inc.).

2.11 Primer extension anaiysis

For primer extension analysis, two oligonucleotides, primer I (5'-CCGGATAGAAAAGCAAAGAGC-3') located in exon 1 and primer II (5'-GCTGGAGCTCCAACAAGACCTCGGACGCCA-3') located in exon 2 were end-labelled and purified on a G-50 sephadex Nick column as described in section 2.1. The equivalent of 4×10^6 cpm of primer I was annealed to 50 μg of total RNA isolated from rat bladders (treated as described in section 2.2) and primer II was annealed to 5 μg of poly(A)$^+$ from control and LPS-treated rat uterus tissue in 30 μl of hybridisation buffer (40 mM sodium citrate, 1 mM EDTA, 0.4 M NaCl and 80% formamide). Hybridisation was carried out at 30°C for 12-15 hours. The RNA-primer mixtures were precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of 100% ethanol. The pellet was washed with 75% ethanol/25% 0.1 M sodium acetate and air dried. Reverse transcription reactions were performed on the RNA-primer hybrids in 25 μl of reverse transcriptase buffer containing; 0.56 mM dNTPs, 1 × Superscript buffer (Life Technologies), 20 units of RNase inhibitor (Promega), 200 units of Superscript II (Life Technologies). Reverse transcription proceeded for 1 hour at 42°C and then for 15 min at 50°C to overcome any secondary structure in the 5' end of the RNA. Finally the reaction was heated to 65°C for 10 mins. The RNA was then digested by incubation with 10 units of RNase One (Promega) in the presence of 20 μM EDTA at 37°C for 40

min. The digested mixture was extracted once with phenol/chloroform and then ethanol precipitated, dried and resuspended in 4 µl of 1 × TE and 4 µl of RNA gel loading buffer. Samples were denatured by boiling for 3 min and then quenched on ice. The samples was run on a 6% polyacrylamide gel (Flowgen) in 1 × TBE electrophoresis buffer with a parallel sequencing reaction. The sequencing reaction for the extension products generated with primer I used the same primer and the genomic DNA as a template. The sequencing reaction for the extension products generated with primer II used the universal primer (5'-GTTTTCCCAGTCACGACGTTGTA-3') primed on pUC18 (Stratagene). The gels were dried under vacuum and exposed to X-ray film for up to 1 week.

2.12 Rapid amplification of cDNA ends (RACE)

RACE was carried out using a modified procedure of Frohman *et al.*, (1988).

2.12.1 5' RACE

First strand cDNA was synthesised using Superscript II reverse transcriptase on poly(A)$^+$ from control and 4 hour LPS-treated rat uterus tissue. Reverse transcription was carried out in a total volume of 20 µl, containing: 100 ng of poly(A)$^+$ RNA, 4 µl of 5 × first strand buffer, 2 µl of 0.1 M DTT, 4 µl of 2.5 mM dNTPs, 200 ng of random hexamers (Promega) and 1 µl of Superscript II (200 units) at 42°C for 45 min followed by 50°C for 15 min. Reverse transcription was terminated by incubation at 75°C for 15 min. The reaction was ethanol precipitated and resuspended in 5 µl of water, the cDNA/RNA hybrid was then denatured by boiling for 2 min and quenched on ice. The cDNA was tailed using terminal transferase (Boehringer Mannheim). The following

reaction mix was prepared on ice and incubated at 37°C for 30 min: 2 µl of 5 × TdT buffer (1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 µg/ml BSA), 1 µl of 15 mM $CoCl_2$, 1 µl of 1 mM dATP, 5 µl of cDNA mix and 1 µl of terminal transferase (all reagents supplied with kit). The enzyme was inactivated by heating at 65°C for 2 min. The tailed cDNA was ethanol precipitated and the pellet resuspended in 10 µl of water. The tailed cDNA was subsequently amplified using a T_{17} adapter primer (5'-GACTCGAGTCGACATCGA$(dT)_{17}$-3') and a BKB_1 receptor-specific primer (5'-GAGCCTGTAGCGGTCCTG-3') located at the 5' end of exon 2. The PCR amplification was performed in a total volume of 50 µl containing 1 µM of each primer, 0.2 mM dNTPs, 5 µl of Taq polymerase buffer (Perkin Elmer) and 1 µl of Taq polymerase (Perkin Elmer). PCR amplification was carried out using 30 cycles of: 95°C for 40 s, 42°C for 2 min and 72°C for 3 min. Finally the reaction was incubated at 72°C for 10 min. 1 µl of the first round PCR products was then used for a second round of amplification using the adapter primer (5'-GACTCGAGTCGACATCG-3') and a BKB_1 receptor-specific primer (5'-CTATGGTTAAGCGCTGCTGCCT-3') internal to the BKB_1 receptor-specific product amplified. PCR amplification was carried out using 35 cycles of: 95°C for 30 s, 55°C for 1 min and 72°C for 3 min. The reaction was then incubated for a further 10 min at 72°C. 10 µl of the reaction product was analysed on a 1% agarose gel and Southern blotted. This was hybridised with a third end labelled BKB_1 receptor primer (5'-GCTGGAGCTCCAACAAGACCTCGGACGCCA-3') lying internal to the BKB_1 receptor primers used for amplification. The 5' RACE products were cloned directly into the pCR 2.1 vector (Invitrogen) and sequenced.

2.12.2 3' RACE

3' RACE cDNA was synthesised using poly(A)$^+$ RNA isolated from control and 4 hour IL-1β-treated JTC-19 cells. 100 ng of poly(A)$^+$ RNA were primed for cDNA synthesis using the T_{17} adapter primer and the conditions used for 5'RACE. The cDNA was subsequently amplified using the adapter primer and a BKB_1 receptor-specific primer (5'-GGACAGAAGGAGGCCAGCAGGAC-3') located at the 3' end of exon 2. A second round of amplification was carried out using the adapter primer and a BKB_1 receptor-specific primer (5'-GAGTGATCCAGGACTGCTC-3') located internal to the BKB_1 receptor-specific product amplified. The PCR reactions were performed as described for 5' RACE. 10 μl of reaction products were analysed as described in section 2.12.1 and hybridised with a third end labelled BKB_1 receptor-specific primer (5'-CTTTATAAATGATGCAACCGAGAA-3') lying internal to the BKB_1 receptor products amplified. The 3' RACE products were cloned directly into the pCR2.1 vector and sequenced.

2.13 Cell culture

Rat embryonic lung fibroblasts, JTC-19, rat smooth muscle cells, A10, rat liver hepatoma cells, H4, and human foetal lung fibroblasts, IMR-90, were obtained from European collection of animal cell cultures (ECACC). JTC-19 cells were cultured in 3% CO_2 at 37°C in Dulbecco's minimal essential medium (DMEM)/Nutrient Mix F12 (1:1) without L-glutamine with pyridoxine (Life Technologies) supplemented with 10% foetal bovine serum (FBS) (Life Technologies). H4, A10, and IMR-90 cells were cultured in 5% CO_2 at 37°C in DMEM supplemented with 10% FBS. When confluent, the cells were seeded at 1:4. The media was removed and the cells were washed in 1 ×

PBS. Cells were trypsinised by the addition of 1 ml of 1 × Trypsin/EDTA (0.25% trypsin, 1 mM EDTA) (Life Technologies) per 34 cm^2 of culture dish. Where mediators were added to cells, the cells were used between passages 3 and 10 (where each passage was between 4-7 days).

2.14 Measurement of [^{3}H]desArg^{10}kallidin binding

BKB_1 receptor number was determined by binding of [^{3}H]desArg^{10}kallidin to intact JTC-19 cells in culture. 1×10^5 cells per well of a 24-well plate were grown for 15-18 hours. Cells were treated with mediators for times shown in the figure legends. Cells were then washed with 1 ml of binding buffer (10 mM TES, 300 mM sucrose, 0.1% BSA, 10 μM thiorpan (Sigma), 1 μM enalopril (Sigma) and 1 μM MERGEPTA, pH 7.4 (Calbiochem)). 0.5 ml of binding buffer containing 2 nM [^{3}H]desArg^{10}kallidin (740 Gbq/mmol (NEN)) was then added to each well. Non-specific binding was determined in the presence of 3 μM unlabelled desArg^{10}kallidin. After binding for 1 hour at room temperature, the cells were washed 3 times with 0.5 ml of wash buffer (50 mM Tris-Cl pH 7.4 and 300 mM sucrose). The cells were solubilised with 0.2% SDS for 30 mins, the lysate was transferred to scintillation vials and the wells were washed with a further 0.5 ml of wash buffer, which was transferred to the scintillation vials. 4 ml of Ready Flow (Beckman) was added to each of the scintillation vials and the amount of radioactivity determined in a Wallac scintillation counter. All determinations were performed in triplicate. Student's unpaired *t* tests were performed to determine significant differences between groups. *P* values < 0.05 were considered significant.

2.15 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out to analyse the inducible expression and alternative splicing of BKB_1 receptor mRNA. Total RNA (1 μg) or poly $(A)^+$ RNA (500 ng) from control and treated rat tissues and control and treated rat cell lines were treated with RNase free DNase (Promega) for 1 hour at 37°C. Reverse transcription was carried out using Superscript II reverse transcriptase in a 20 μl reaction with random hexamers as described in 5'RACE (section 2.12.1). The PCR reactions were performed using 1 μl of first-strand cDNA, in a 50 μl reaction containing 1 μM of each primer, 0.2 mM dNTPs, 5 μl of Taq polymerase buffer and 1 μl of Taq polymerase. PCR amplifications were carried out using 25 cycles of 95°C for 30s, 58°C for 30s and 72°C for 30s. Finally the reaction was incubated at 72°C for 10 mins. Primers used for analysis of inducible expression in rat cell lines were; sense 5'-TCTTTGGCCTCTTGGGGAACCT-3' and anitsense 5'-CAAGCCTCGTGGGGGAAAAG-3'. Amplified products were separated on a 1% agarose gel and Southern blotted. BKB_1 receptor specific products were analysed by hybridisation with an end-labelled primer 5'-TGGTGGTGGCAGCAACGACAGA-3'. Intron spanning primers used for analysis of BKB_1 receptor splice variants were; sense 5'-GAGCTGCCCCAGGACAGA-3' and antisense 5'-TCGCAGGAGGTAATGTTGG-3'. Amplified products were separated on a 4-20% polyacrylamide gel (Novex) in 1 × TBE gel electrophoresis buffer, stained with Vistra Green nucleic acid gel stain (Amersham Pharmacia Biotech) and visualised with a Molecular Dynamics STORM 840.

2.16 Construction of reporter plasmids

A 2.9 kb fragment spanning the nucleotides −2838 to +88 bp of the rat BKB_1 including all of exon 1 was cloned into PGL3-Basic (Promega). The resulting construct was named pG2926luc. The construct pG731luc spanning the nucleotides −643 to +88 bp was constructed by Bam HI digestion of pG2926luc to remove a 2.2 kb 5' fragment. Two further constructs (pG485 and pG79luc) were generated by PCR using oligonucleotide linker primers based on the reporter constructs described by Ni *et al.*, (1998b). The construct pG485luc spanning the nucleotides −477 to +8 bp was generated using the sense oligonucleotide, 5'-GAGAGGTACCCCTAGTAATTGCCCTTCA-3', with an additional Kpn I restriction site added to the 5' end and the antisense oligonucleotide, 5'-GAGAAAGCTTGCAGCTCCCTGGACACA-3', with an additional Hind III restriction site added to the 3' end. The construct pG79luc spanning the nucleotides −71 to +8 bp was generated using the sense oligonucleotide 5'-GAGAGGTACCTTTTTGGGTAATCCCCTG-3' with an additional Kpn I restriction site added to the 5' end and the same antisense oligonucleotide as above. PCR products were amplified using 10 pg of cosmid RB1 template using the conditions described in section 2.15 for RT-PCR. PCR products were digested with the restriction enzymes Kpn I and Hind III and cloned into Kpn I/Hind III cut PGL3-Basic. All of the constructs were sequenced to verify the absence of errors introduced by PCR and to check their orientation. The human BKB_1 receptor-directed reporter gene constructs with and without the upstream NF-κB-like site (Schanstra *et al.*, 1998) were a generous gift from Dr. Jean-Loup Bascands.

2.17 DNA transfections

DNA was transfected into cultured cells using lipid transfection technology with either FuGENE 6 transfection reagent (Boehringer Mannheim) or LipofectAMINE reagent (Life Technologies).

2.17.1 Transient transfections

The day before transfection cells were seeded at a concentration of 6×10^4 per well of a 24-well plate. Test plasmids were co-transfected with a plasmid (at a ratio 1:500) containing the *Renilla* luciferase under the control of the SV40 promoter, pRL-SV40 (Promega) as a control to monitor the transfection efficiency. Using the FuGENE 6 transfection protocol, 1.2 µl of FuGENE 6 was diluted in 40 µl of serum-free media and added to 0.8 µg of DNA for 15 mins. The cell media was replaced with 500 µl of normal growth media to which the transfection complexes were added and incubated for 5 hours after which the transfection mixture was removed and replaced with normal media. Using LipofectAMINE, 0.4 µg of DNA were diluted in 25 µl of serum-free media and precomplexed with 2 µl of lipofectAMINE for 15 min. Media was removed from the cells and replaced with 200 µl of serum-free media to which the DNA-lipofectAMINE mixture was added. After 5 hours the transfection mixture was replaced with normal growth medium. 48 hours following transfection, cells were treated with or without mediators for 3 hrs before harvesting the cells for luciferase activity. For analysis of BKB_1 receptor expression, the transfection was scaled up to 80 cm^2 culture flasks using 28.8 µl of FuGENE 6 diluted in 960 µl of serum-free media added to 19.2 µg of DNA. Media was removed from the cells and replaced with 12 ml of normal media. After 5 hours the transfection mixture was replaced with normal growth

medium. 48 hours following transfection, cells were treated with or without mediators and total and poly (A)+ were purified as described in sections 2.8 and 2.9.

The transfection efficiency of JTC-19 cells using FuGENE 6 reagent was determined by transfecting cells in either 24-well plates or 80 cm^2 culture flasks with a β-Gal-CMV plasmid. 48 hours following transfection the media was removed and cells were washed with PBS. The cells were fixed for 10 mins at room temperature in a solution of 50% methanol and 50% acetone. The cells were washed 3 times with 1 × PBS and overlaid with 1 × PBS containing 1 mg/ml X-gal (Sigma), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in. The cells were left for 12 hours. Transfection efficiency was determined by the percentage of blue stained cells indicating the presence of the β-galactosidase enzyme.

2.17.2 Stable transfections

The IκB mutant (IκB-mut) construct which contains a deletion of the base pairs encoding the first 36 amino acids (Brockman *et al.,* 1995) was a kind gift from Dr. Dean Ballard. The IκB-mut construct was co-transfected with pMC1-Neo (Stratagene) at a ratio of 1:10. The transfection was carried out using the FuGENE 6 transfection reagent and scaled up using 25 cm^2 culture flasks. 48 hours following transfection, cells were cultured in the presence of 350 μg/ml G418 to select for those cells that expressed the neomycin gene. Following selection cells were diluted so that when they were transferred to a 96 well plate, each well contained approximately 1 cell. Single clone populations were examined for IκB-mut expression by immunoprecipitation and immunoblotting as described in section 2.19.

2.18 Dual-Luciferase assay

Following treatment of the transfected cells with mediators, luciferase activities were measured using the dual-luciferase assay kit (Promega). Media was removed from cells and replaced with 100 µl of passive lysis buffer. Lysis occurred whilst shaking at room temperature for 15 min. 10 µl of the lysate was transferred to a 96 well plate for measurement of luciferase activity. 100 µl of luciferase assay reagent, LARII, was added successively to each well by injector on a luminoskan. A 2 second premeasurement delay was followed by a 10 second measurement of firefly luciferase activity for each sample. *Renilla* luciferase activities were measured in the same way by the addition of 100 µl of Stop and Glo reagent. Background levels were determined by measurement of control non-transfected cells. Values were adjusted for background and firefly luciferase activity normalised with *Renilla* luciferase activity. All the values are the mean ± standard error of the mean number (n) (SEM) of the experiments performed.

2.19 Immunoprecipitation and immunoblotting

JTC-19 cells and the stable cell line expressing IκB-mut were cultured in 9-cm dishes to confluency. Cell monolayers were washed in ice cold 1 × PBS and then lysed in 0.5 ml of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 2 mM ethyleneglycol Glycol-bis(β-aminoethyl Ether) (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM pepstatin, 10 µg/ml aproprotin, and 1% Nonidet P-40, for 20 mins on ice. Extracts were scraped from the dish and transferred to eppendorfs and cleared by centrifugation at 14,000 rpm for 15 mins at 4°C. The supernatant was removed and protein concentrations were determined by the Bradford method (Bio-Rad). 500 µg of protein were pre-cleared for 1 hour at 4°C with 30 µl of the agarose-

conjugate, protein A/G PLUS-agarose (Santa Cruz Biotechnology). The beads were then pelleted by centrifugation at 2,500 rpm for 5 mins at 4°C. The supernatant was mixed for 15 hours with either 4 μg of anti-FLAG goat polyclonal IgG (D-8) (Santa Cruz Biotechnology) or 4 μg of anti-IκB-α rabbit polyclonal IgG (C-21) (Santa Cruz Biotechnology). The samples were then immunoprecipitated by the addition of 30 μl of protein A/G PLUS-agarose for 2 hours at 4°C. Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 mins at 4°C, and washed three times with lysis buffer. After the final wash, the pellet was resuspended in 30 μl of Laemmli's sample buffer (0.16 M Tris-HCl ,pH 6.8, 5% SDS, 25% glycerol, 0.0025% bromophenol blue, Bio-Rad) with 5% 2-mercaptoethanol. Samples were boiled for 5 mins and resolved by electrophoresis on a Tris-glycine gel (Bio-Rad) in 1 × Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3, and 0.1% SDS) followed by electrotransfer to nitrocellulose membranes (Schleicher and Schuell) in 1 × transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol). The membrane was blocked by incubation for 1 hour in 1 × PBS, 0.1% Tween (Sigma), (PBS-T) containing 10% nonfat dry milk followed by a 1 hour incubation with the primary antibody, anti-FLAG mouse monoclonal antibody (1/500) (Stratagene) in a solution containing PBS-T containing 2.5% nonfat dry milk. Membranes were washed three times with PBS-T and incubated for 1 hour with a 1/5,000 dilution of a peroxidase-coupled anti-mouse antibody (Pierce) in PBS-T containing 2.5% nonfat dry milk. Membranes were washed three times in PBS-T and detection of the antibody-protein complex was carried out using chemiluminescence detection reagents (Pierce) and exposure to high performance chemiluminescence film (Amersham Pharmacia Biotech).

2.20 Nuclear Extract preparation

Control and treated JTC-19 cells were harvested by centrifugation at 2,000 rpm for 5 min in 1 × PBS. The cell pellet was resuspended in 10 ml of ice cold buffer A (10 mM N-[2-Hydroxyethyl]piperazine-N'[2-ethanesulfonic acid (HEPES) pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF) with 0.1% Nonidet P40 per 170 cm^2 culture flask and homogenised slowly with 25 strokes of a Dounce homogeniser. The homogenate was centrifuged for 5 min at 2,000 rpm and resuspended in a further 10 ml of ice cold buffer A and centrifuged as described above. For EMSAs, nuclei were resuspended at 10^8 nuclei/ml in ice cold buffer C (5 mM HEPES pH, 7.9, 26% v/v glycerol, 0.2 mM EDTA and 1.5 mM $MgCl_2$), NaCl was then added to a final concentration of 300 mM and the nuclei proteins extracted on ice for 30 min. After centrifugation at 14,000 rpm for 20 mins at 4°C, the supernatants were transferred to 2 ml cryogen tubes (Nunc) and stored in liquid nitrogen. Protein concentrations were determined by the Bradford method (Bio-Rad).

2.21 Nuclear Run-On

Nuclei from control and treated JTC-19 cells were prepared as described in nuclear extract preparation. The transcription run-on was performed according to a modified method of Greenberg and Ziff (1984).

2.22 DNase I hypersensitive site assay

DNase I hypersensitive sites were assayed in JTC-19 cells by a modified method according to Forrester *et al.,* (1987).

2.23 Electromobility shift assay (EMSA)

The EMSA was carried out using probes corresponding to the AP-1 site, CRE site and NF-κB-like site in the rat 5' flanking sequence and probes corresponding to a consensus NF-κB site and AP-1 site (Table 2.1). To obtain the double stranded oligonucleotides, single stranded oligonucleotides with Hind III linkers (sense and antisense) were synthesised by Genosys. These were dissolved in 1 × TE to a stock concentration of 100 mM. Stocks were diluted in water to a concentration of 10 μM and mixed in equal amounts. Samples were denatured by placing in a boiling water bath and then cooled slowly to room temperature. The double stranded oligonucleotides were labelled by filling in the overhangs using Klenow polymerase (Stratagene) and α-^{32}P dATP. 10 pmoles of double stranded oligonucleotide were labelled in a 20 μl reaction containing 2 μl of 5 × buffer, 0.5 mM dCTP, dGTP and dTTP, 2 μl of α-^{32}P dATP and 1 μl of Klenow (100 units) at 37°C for 30 minutes. 1 μl of 5 mM dNTPS were added and the reaction incubated for a further 10 min at 37°C. The labelled oligonucleotides were purified on a 6% polyacrylamide gel in 1 × TBE buffer. The oligonucleotides were eluted in 300 μl of 1 × TE for 15-18 hr at 37°C. Using a scintillation counter, 1 μl of the probe was used to determine the specific activity. Binding reactions were performed using 40,000 cpm of labelled oligonucleotide probe, 0.2-0.7 μg of nuclear protein, 1 μl of calf thymus DNA, and 10 μl of 2 × gel shift buffer (12 mM HEPES, pH 7.9, 60 mM

KCl, 1mM EDTA, 1mM PMSF, 1mM DTT and 15% glycerol) in a final volume of 20 µl at room temperature for 20 minutes. For competition assays, 700-1,000 fold molar excess of unlabelled specific oligonucleotides, or oligonucleotides spanning the rat BKB_1 receptor AP-1site, NF-κB-like site and NF-κB site (Table 1.2), or mutant oligonucleotides (Table 1.2) or oligonucleotides containing consensus AP-1 site, CRE site and NF-kB sites (Promega) were added to the binding reaction for 20 min on ice before the probe was added. The nuclear proteins bound to the CRE site were identified by preincubation of the nuclear extract for 20 min on ice with an antibody (0.2 µg) directed against the CREB protein (Santa Cruz Biotechnology). After the binding reaction the samples were electrophoresed for 1.5 hours on a 4% nondenaturing polyacrylamide gel (19:1) in 0.25 × TBE electrophoresis buffer. Gels were dried under vacuum and exposed to X-ray film at −70°C for up to three days.

Table 2.1 Sequences of the double-stranded oligonucleotides used in the EMSA.

Primer	Sequence
R AP-1 (–78 to –72 bp)	5'-AAGCTTG**AGACTCA**CTTTTGGA-3' 3'- AC**TCTGAGT**GAAAACCTTCGA-5'
Mut AP-1	5'-AAGCTTGctACTCACTTTTGGA-3' 3'- ACgaTGAGTGAAAACCTTCGA-5'
AP-1	5'-GATCCGCTTGATGAGTCAGCCGGAA-3' 3'- GCGAACTACTCAGTCGGCCTTCTAG-5'
R CRE (– 51 to –46 bp)	5'-AAGCTTGT**GACATC**ATGGGA-3' 3'- ACA**CTGTAG**TACCCTTCGA-5'
Mut CRE	5'-AAGCTTGcaACATCATGGGA-3' 3'- ACgtTGTAGTACCCTTCGA-5'
Ru NF-κB (–64 to –57 bp)	5'-GATCCAAGT**GGGAAAGCCC**ATG-3' 3'- TTCA**CCCTTTCGGG**TACCTAG-5'
Rd NF-κB (–1192 to –1183 bp)	5'-AAGCTTG**GGTAATCCC**CTGTGA-3' 3'- AC**CCATTAGGG**GACACTTCGAA-5'
Mut NF-κB	5'-AAGCTTGctTAATCCCCTGTGA-3' 3'- ACgaATTAGGGGACACTTCGAA-5'
NF-κB	5'-GATCAGTTGAGGGGACTTTCCCAGGC-3' 3'- TCAACTCCCCTGAAAGGGTCCGCTAG-5'

The NF-κB and AP-1 oligonucleotides were modified, from those available from Promega to contain 5' overhangs. Mut= mutated oligonucleotide, mutated nucleotides shown in lower case. R= site present in the rat 5' flanking sequence. Ru= rat upstream, Rd= rat downstream. The putative transcription factor binding sequences in the rat 5' flanking sequence are shown in bold.

2.24 Materials

The materials and reagents used in this study which have not been described in the text are shown below.

2.24.1 Mediators added to cell cultures

Rat recombinant IL-1β, rat recombinant TNF-α, and mouse recombinant IL-1Ra were purchased from R and D systems. Actinomycin D, cycloheximide, LPS (from E. coli serotype 0111:B4), dibutyryl cAMP (dbcAMP) forskolin and G418 were obtained from

Sigma. SB203580 and PD98059 and LY294002 were obtained from Calbiochem. DesArg^{9}BK was obtained from Bachem. DesArg^{10}kallidin was obtained from Peninsula.

2.24.2 Materials for buffers and hybridisation solutions

Boric acid, HEPES, EDTA, MOPS, SDS, PBS, TES, KCl, NaCl, NaOH, sodium acetate, sodium citrate, dextran sulphate, DEPC, formaldehyde, formamide, Tris-base, Tris-HCl, Triton X-100, $MgCl_2$, gelatin, β-mercaptoethanol, PMSF were obtained from Sigma. HCl, sucrose and glycine was obtained from BDH. Nonidet P40 was obtained from Boehringer Mannheim. Glycerol was obtained from Life Technologies.

2.24.3 Other materials

All restriction enzymes and restriction enzyme buffers were obtained from NEB. Ammonium acetate, chloroform, phenol, ethanol, isopropanol, methanol, acetone, were obtained from Sigma. Agarose was obtained from Life Technologies.

Chapter 3

Isolation and characterisation of the rat BKB_1 receptor gene

3.1 Introduction

Kinins exert their effects by the activation of specific G-protein coupled receptors. Two mammalian receptors, BKB_1 and BKB_2 have been characterised by a number of criteria described in Chapter 1. Most of the *in vivo* effects assigned to the kinins are thought to be mediated by BKB_2 receptors (Hall *et al.,* 1992; Bhoola *et al.,* 1992), with BKB_2 receptors being constitutively expressed in many mammalian tissues (Hall, 1992; Ma *et al.,* 1994a). In contrast, the BKB_1 receptor is either absent, or present in low levels, but unlike the BKB_2 receptor its expression can be dynamically regulated. Initial studies carried out in isolated vascular tissues showed that the responsiveness to BKB_1 receptor agonists occurs as a function of time in a process dependent on both RNA and protein synthesis (Regoli *et al.,* 1978; Deblois *et al.,* 1987; Bouthillier *et al.,* 1987). Pursuant to these studies, a number of agents, in particular IL-1 and LPS, were shown to induce the responsiveness to desArg^{9}BK both *in vitro* (Deblois *et al.,* 1988, 1989, 1991; Bouthillier *et al.,* 1987), and *in vivo* (Regoli *et al.,* 1981; Deblois *et al.,* 1989, 1991; Bouthillier *et al.,* 1987; Marceau *et al.,* 1984). To date, BKB_1 receptor up-regulation has been determined in both vascular and non-vascular tissues for a number of species including

humans and rats (Chapter 1, Table 1.1). A role for inducible BKB_1 receptors in pathophysiology has been demonstrated in a number of models of tissue injury and inflammation, many of which have been developed in rodents (Chapter 1, Table 1.2). However, very little is known about the role of the BKB_1 receptors in healthy tissues.

At the onset of these studies, the *in vivo* and *in vitro* characterisation of the BKB_1 receptors had been performed using pharmacological agents with no molecular confirmation of the up-regulation process. Moreover, although *de-novo* synthesis of the BKB_1 receptors was verified from pharmacological studies with metabolic inhibitors, knowledge of the regulation at the level of the BKB_1 receptor gene was unknown. Recent studies have reported the isolation of cDNA clones for the human and rabbit BKB_1 receptors (Menke *et al.,* 1994; MacNeil *et al.,* 1995). These studies showed that the BKB_1 receptor encodes a protein belonging to the superfamily of G-protein coupled receptors. Interestingly, although the human and rabbit BKB_1 receptors show protein sequence homology of 78%, they exhibit significant species-related pharmacological differences (MacNeil *et al.,* 1995). For example, the affinity of the human and rabbit receptors for the agonist desArg^{10}kallidin are similar, whereas the rabbit receptor has more than 10 fold higher affinity for the agonist desArg^{9}BK. The pharmacological characteristics of BKB_1 receptors in the mouse macrophage cell line, RAW264, have been studied using [H^{3}]desArg^{10}Pro^{2}kallidin binding in the presence of competing ligands (Burch and Kyle, 1992). However, molecular cloning was required to determine a detailed pharmacological profile for the rodent receptors (Hess *et al.,* 1996; Pesquero *et al.,* 1996; Ni *et al.,* 1998b; Jones *et al.,* 1999). In contrast to humans, kallidin and its derivative desArg^{10}kallidin are not formed in rodents (Furoto-Kato *et al.,* 1985, Hess *et al.,* 1996). Therefore, it is possible that the human BKB_1 receptors have evolved high

affinity towards kallidin and its derivatives and rodent receptors, in the absence of kallidin have evolved high affinity for bradykinin and its derivatives instead.

This chapter describes the isolation of both genomic and cDNA clones for the rat BKB_1 receptor and characterisation of the genomic structure. Using this DNA as a species-specific probe, the molecular nature of the BKB_1 receptor up-regulation is established. In addition, a rat embryonic fibroblast cell line, JTC-19, retaining the characteristics of BKB_1 receptor up-regulation is reported.

3.2 Results

3.2.1 Isolation of genomic and cDNA clones for the rat BKB_1 receptor

A plasmid clone containing the human BKB_1 receptor cDNA was isolated by expression cloning in *Xenopus* oocytes from a cDNA library synthesised using mRNA purified from the human fibroblast cell line, WI38. This work is briefly described in Jones *et al.,* (1999), but was not a part of this thesis. The isolation of the rat sequences described in Jones *et al.,* (1999) forms a major part of this thesis. A rat genomic library constructed in the cosmid vector, SuperCos 1, was screened to isolate a clone containing the rat BKB_1 receptor gene. A 750 bp Xba I to Eco RI fragment located in the ORF of the human BKB_1 receptor cDNA sequence was used as a probe for screening the library under low stringency hybridisation conditions (see Methods and Materials). From 1 x 10^6 clones screened, one was obtained that contained the rat BKB_1 receptor, hereafter called RB1. The cosmid clone was mapped using restriction enzymes and subsequent Southern blot analysis with the human BKB_1 receptor as a probe. A 2.2 kb Bam HI fragment containing the BKB_1 receptor gene was identified which was subcloned into pBluescript KSII. Restriction digestion with Eco RI excised a 0.9 kb fragment from the 3'end of the subclone, which did not hybridise to the human probe, leaving a 1.3 kb insert. This insert was sequenced in its entirety and a predicted ORF corresponding to the rat BKB_1 receptor protein was identified. The predicted ORF spanned 1014 bp encoding a 338 amino acid protein.

In order to obtain the cDNA sequence for the rat BKB_1 receptor, an 864 bp Pst I fragment (Probe A) located within the putative rat BKB_1 receptor ORF was used to

screen a rat bladder cDNA library. The cDNA library was synthesised with Poly(A)$^+$ RNA purified from rat bladders, treated as described in Methods and Materials, to obtain maximum expression of the BKB_1 receptor mRNA. From 1 x 10^5 clones screened, a single clone was isolated and sequenced. The clone contained a single ORF of 780 bp flanked by 103 bp of 5' untranslated sequence and 159 bp of 3' untranslated sequence. The cryptic poly(A) addition signal, CATAAA, was not an exact match to the consensus sequence (AATAAA) and was located 28 bp upstream from the start of the poly(A) tail. Sequencing of the entire cDNA revealed that there was a deletion in the region encoding the predicted TM domains VI and VII. Consequently, the following primers were synthesised, 5'-AGGTCACCATCAAAAACACAGG-3' and 5'-CTTCTTGGCTCCCTGCTGTT-3', corresponding to the most 5' and 3' cDNA sequence, respectively. Using these primers, RT-PCR was performed on the same source of poly(A)$^+$ RNA as used for construction of the cDNA library. A 1.3 kb product corresponding to the predicted size for an undeleted clone was amplified and cloned into pCR2. Sequence analysis of the PCR product revealed identical sequence to the deleted clone but with the inclusion of TM domains VI and VII corresponding to an ORF of 1014 bp.

Based on the hydrophobicity profile, the amino acid sequence of the rat BKB_1 receptor cDNA was predicted to have seven TM domains as determined for the human BKB_1 receptor (Menke *et al.,* 1994; Jones *et al.,* 1999), this is characteristic of the superfamily of G-protein coupled receptors. Figure 3.1 shows an alignment of the predicted 338 amino acid ORF from the rat receptor with that of the mouse (Hess *et al.,* 1996; Pesquero *et al.,* 1996), rabbit (MacNeil *et al.,* 1995) and human receptors (Menke *et al.,* 1994; Jones *et al.,* 1999). Some features of G-protein coupled receptors observed in the

human BKB_1 receptor are conserved in all four species (Menke *et al.,* 1994). These include three potential N-linked glycosylation sites and two conserved cysteine residues that are proposed to form a disulphide bridge. However, whereas the rat and mouse ORFs are similar, there are marked differences compared to the human and rabbit receptor sequences. Two striking differences are evident between the predicted protein sequence of the rat receptor with both the rabbit and human BKB_1 receptors (Figure 3.1). Firstly, the rat BKB_1 receptor protein is 26 amino acids shorter than the human and the rabbit sequences at the C-terminus. Secondly, the rat contains an extra 11 amino acids within the first intracellular loop. Like the rat, the predicted mouse BKB_1 receptor sequence also has a longer first intracellular loop, in this case with an extra 8 amino acids, and the same shortened tail as seen in the rat receptor. In addition, the rat sequence lacks a consensus site for palmitoylation which is found in the human and rabbit receptors. Palmitoylation has been shown for the BKB_2 receptor but has yet to be determined for the BKB_1 receptor (Soskic *et al.,* 1999). Determination of the receptor homologies between the species showed that the rat and human predicted proteins have 73.1% identity, the rat and rabbit proteins have 71.6% identity and the rat and mouse have 89.9% identity. The pharmacological profile for the rat receptor was characteristic of a BKB_1 receptor, exhibiting high affinity for the desArg metabolites, desArg^{9}BK and desArg^{10}kallidin (Jones *et al.,* 1999).

3.2.2 A distinct tissue expression pattern for the BKB_1 receptor mRNA

Detection of the receptor protein is preferable to detection of mRNA levels in regulation studies, as this represents the physiological end point of gene regulation. However, specific antibodies for the rat BKB_1 receptor were not available while the studies in this

thesis were performed. Therefore, Northern blot analysis was used to determine whether there was a correlative increase in the BKB_1 receptor mRNA levels in isolated organs from LPS-treated rats compared to control animals. Poly(A)$^+$ RNA was isolated from *ex-vivo* tissues from LPS-treated and control rats, and analysed by Northern blot studies as described in Methods and Materials. Hybridisation with probe A showed the presence of three mRNA transcripts of 1.6, 4 and 8 kb (Figure 3.2A). The large transcripts of 4 and 8 kb were only detected in Northern blots from tissues that exhibited high levels of induced expression. The 1.6 kb transcript corresponds to the expected size of the mRNA from the cDNA clone and is equivalent to the reported human BKB_1 receptor mRNA transcript of 1.4-1.7 kb (Bachvarov *et al.,* 1996; Chai *et al.,* 1996; Phagoo *et al.,* 1997). For quantitation of the 1.6 kb transcript, blots were stripped and re-probed with cyclophilin (Figure 3.2A). All future measurements of BKB_1 receptor mRNA levels by Northern blot analysis were detected and quantified according to this protocol, unless stated otherwise. Figure 3.2B shows that low levels of BKB_1 receptor mRNA are found in control tissues, with the exception of spleen where BKB_1 receptor mRNA levels were undetectable. Interestingly, control uterus exhibited almost 10 fold higher BKB_1 receptor mRNA levels than any of the other control tissues tested. Treatment of rats with LPS showed an up-regulation of the receptor mRNA in all of the tissues tested, in heart (8 fold), bladder (25 fold), lung (25 fold), kidney (7 fold), spleen, and uterus (14 fold). Of the isolated tissues from LPS-treated rats, the highest BKB_1 receptor mRNA levels were observed in the uterus. This tissue exhibited 5 fold more receptor mRNA levels compared to the lung, the tissue with the next highest receptor mRNA levels.

3.2.3 Expression of BKB_1 receptor mRNA in cultured cell lines

Due to limitations in the lifespan of primary cell cultures and and the low quantity of cells, primary cell cultures were not used for analysis in these studies. Therefore, to study the mechanisms of BKB_1 receptor up-regulation, a rat cell line retaining these inducible characteristics was identified. A number of functional responses, including mitogenesis (Bascands *et al.,* 1993; Issandou and Darbon, 1991) and cytokine release (Tiffany and Burch, 1989), have been attributed to BKB_1 receptors in rat cell lines. However, these responses were observed in the absence of inducing agents suggesting a high constitutive level of BKB_1 receptor expression under the cell culture conditions used. Menke *et al.,* (1994) observed that binding of [^{3}H]desArg^{10}kallidin in IMR-90 human foetal lung fibroblast cells was up-regulated following treatment with IL-1β. This study analysed the rat embryonic lung cell line, JTC-19 and the mouse fibroblast cell line, NIH-3T3 for inducible BKB_1 receptor mRNA levels. Since the effects of LPS, in some cell types, can be mediated through the autocrine effects of several mediators, including IL-1 (Sweet and Hume, 1996), IL-1β was used as a more specific inducing agent to analyse BKB_1 receptor gene expression. NIH-3T3 and JTC-19 cells were treated with IL-1β (10 ng/ml) for four hours prior to RNA isolation. Previous studies established a dose response curve for IL-1β and showed that 10 ng/ml was super-maximal for induction of BKB_1 receptor mRNA levels (data not shown). RT-PCR was performed and the amplified products were run through a 1% agarose gel and Southern blotted (see Methods and Materials). An oligonucleotide located within the PCR product was end labelled using γ-^{32}P ATP and used as a probe to confirm the specificity of the amplification products. The results of the analysis in Figure 3.3 show that both JTC-19 cells and NIH-3T3 cells express BKB_1 receptor mRNA which is up-regulated

following treatment with IL-1β. For quantitative analysis, JTC-19 cells were further analysed by Northern blot studies using poly(A)$^+$ RNA (Figure 3.4). Three mRNA transcripts of 1.6, 4 and 8 kb were detected following IL-1β-treatment as described for the Northern blot analysis from LPS-treated rat tissues (see Figure 3.2). This corresponded to a 16 fold induction of BKB_1 receptor mRNA levels (Figure 3.4B). In contrast, the same dose of IL-1β failed to induce BKB_1 receptor mRNA levels in rat H4 liver hepatoma cells (data not shown). In addition, H4 cells did not express BKB_1 receptor mRNA when treated with LPS as an alternative inducing agent. The presence of undegraded mRNA from H4 cells was verified by stripping and re-probing the blots with cyclophilin. More recent studies have shown that rat VSMCs, and the rat smooth muscle cell line, A10, express inducible BKB_1 receptor mRNA levels (Ni *et al.*, 1998a,b). BKB_1 receptor mRNA levels from control, IL-1β and LPS-treated A10 cells were examined in this study by Northern blot analysis (data not shown). The addition of IL-1β and LPS resulted in a 3 and 4 fold increase in BKB_1 receptor mRNA levels, respectively. Thus, these data show that the JTC-19 cell line is a good model system in which to study the up-regulation of the rat BKB_1 receptor gene. Consequently, these cells were used in subsequent studies of BKB_1 receptor gene expression as described in Chapters 4 and 5.

3.2.4 Characterisation of the genomic BKB_1 receptor clone, RB1

As a first step to study the regulatory mechanisms that contribute to the BKB_1 receptor regulation, the gene structure was determined. To elucidate the structural organisation of the BKB_1 receptor gene and the sequences of the exon-intron boundaries, the

genomic and cDNA sequences were aligned. The sequences were identical over the ORF and the 3' UTR demonstrating that the protein coding region is contained within one exon. This resembles a large number of other G-protein coupled receptors, such as muscarinic M_1 and M_4 receptors (Pepitoni *et al.*, 1997; Wood *et al.*, 1995), angiotensin II AT_1 and AT_2 receptors (Curnow *et al.*, 1992; Langford *et al.*, 1992; Takeuchi *et al.*, 1993; Nakajima *et al.*, 1993) and α_2 and β adrenergic receptors (Kobilka *et al.*, 1987a,b; Emorine *et al.*, 1987). Alignment of the 5' non-coding cDNA and genomic sequences showed evidence for a splice site located 51 bp upstream of the start codon. Restriction digests and Southern blot analysis of RB1 using an end-labelled oligonucleotide probe located within the 5' end of the cDNA identified a 1.6 kb Bam HI fragment containing the 52 bp of upstream non-coding sequence. Sub-cloning and sequencing of this fragment revealed that it was located directly upstream of the 2.2 kb Bam HI fragment containing the BKB_1 receptor coding exon. An intervening intron of 1126 bp spanning the Bam HI fragments was identified. The remaining 52 bp of cDNA sequence was found to be contiguous with the genomic sequence. Thus, these data indicate that the rat BKB_1 receptor gene is encoded on 2 exons with an intervening intron of 1126 bp (Figure 3.5C).

The restriction enzymes Not I and Eco RI were used to map RB1 and determine the location of the BKB_1 receptor gene within the insert. A Not I complete restriction digest and Eco RI partial restriction digests of the cosmid were separated by electrophoresis and analysed by Southern blotting as described in Methods and Materials. T7 and T3 oligonucleotides located within the polylinker were end-labelled with γ-^{32}P ATP and used as probes for Southern blot analysis. Figure 3.6A and 3.6B show restriction maps of the Eco RI and Not I sites in RB1. Hybridisation with probe A from the coding

region was used to determine the position of the coding exon. To determine the orientation of the coding sequence a 900 bp Eco RI to Bam HI fragment located downstream of the coding sequence was used as a probe. As illustrated in Figure 3.6A and 3.6B the cosmid contains approximately 13 kb of 5' sequence and 20 kb of 3' sequence relative to the coding exon. The 5.2 kb Eco RI fragement spanning the BKB_1 receptor gene was subcloned into pBluescript KSII and sequenced. Figure 3.5C represents a restriction map of the Eco RI to Bam HI fragment showing the location of the BKB_1 receptor gene. Sites used to generate fragments for subcloning and for the synthesis of probes are highlighted. The sequence of this fragment is shown in Appendix I.

3.2.5 Organisation of the rat BKB_1 receptor gene

The existence of multiple mRNA transcripts was noted in previous Northern experiments (see Figures 3.2 and 3.4). Such transcripts could be explained by the use of alternative splice variants, unspliced intermediate transcripts, alternative polyadenylation sites or different subtypes of receptor. To elucidate the exon-intron boundaries and the start and end of the transcripts, the cDNA ends were mapped using primer extension analysis and RACE. Primer extension analysis can be used to determine the location of the TIS(s). Furthermore, this technique can be used to quantitate the amount of a given RNA product. RACE was developed by Frohman *et al.*, (1988), this method is based on PCR and is specifically designed to amplify the 5' and 3' ends of mRNA molecules for rare sequences. Enriched sources of BKB_1 receptor transcripts from LPS-treated rat bladder and uterus and from IL-1β-treated JTC-19 cells were used in these investigations to increase the yield of BKB_1 receptor cDNA products.

3.2.5.1 Identification of multiple transcription initiation sites using primer extension analysis

Primer extension analysis was performed using two different antisense primers for the synthesis of extension products, primer I, located at the 3' end of exon 1 and primer II, located at the 5' end of the coding exon. Comparison of the extension products generated with these primers would identify possible differential splicing to alternative upstream exons. Primer I was hybridised with 50 μg of total RNA from rat bladder (treated as described in Methods and Materials). Extension products were synthesised according to Methods and Materials and separated by electrophoresis on a 6% polyacrylamide gel. The result in Figure 3.6A shows two bands corresponding to TISs at the first and second of three G residues. Nucleotide numbering of the rat sequence starts at +1 at the first of these two G residues. Primer II, was hybridised to 5 μg of poly(A)$^+$ RNA isolated from control and LPS-treated rats uterus tissue. As shown in Figure 3.7B transcripts were only detected in poly(A)$^+$ RNA derived from LPS-treated rat uterus tissue. The extension products from this poly(A)$^+$ RNA revealed the presence of two sets of transcripts. The large initiations shown by arrow 1 in Figure 3.6B correspond to TISs at the second and third of the three G residues consistent with the products generated using primer I. In contrast, the transcripts shown by arrow 2 in Figure 3.6B are 41 bps shorter. The smaller transcripts were only identified using primer II suggesting differential splicing of alternative 5' sequences to the coding exon.

3.2.5.2 Alternative splice acceptor sites generate multiple BKB_1 receptor transcripts

5' RACE was performed on cDNA synthesised from RNA isolated from control and LPS-treated rat uterus tissue and from control and IL-1β-treated (4 hours) JTC-19 cells as described in Methods and Materials. Products were analysed by separation on a 1% agarose gel, Southern blotting and hybridisation with a primer located within the PCR products. Southern analysis showed a smear of DNA between 300 to 500 bp from the treated samples. The lack of hybridisation to a specific band was probably due to non-specific amplification. PCR products were cloned into pCR 2.1 and DNA was isolated from 10 recombinants and sequenced. Sequence analysis of the clones revealed TISs located 30 and 31 bp upstream from the 5' end of the cDNA clone at the first and second of three G residues as determined using primer extension analysis (Figure 3.7). The additional 31 bp of sequence is contiguous with the genomic sequence and thus verifies the two exon structure for the rat BKB_1 receptor gene. Furthermore, sequence analysis revealed the presence of an alternative splice acceptor site. This alternative splice acceptor site is located 10 bp upstream of the start codon resulting in a 41 bp difference between the mRNA transcripts as shown in Figure 3.7. Moreover, this finding suggests that the smaller products in the primer extension analysis with primer II correspond to mRNA transcripts generated using the 3' splice acceptor site (Figure 3.6B arrow 2). The 3' splice acceptor site is equivalent to the location of the splice site in the human BKB_1 receptor gene (Bachvarov *et al.,* 1996; Yang and Polgar, 1996). The 41 bp size difference between the alternative mRNA transcripts would be indistinguishable by Northern blot analysis. The functional significance, if any, of the alternative mRNA

transcripts remains to be determined. Expression profiles for these mRNA transcripts are assessed in Chapter 4.

Thus, these data from RACE studies and primer extension analysis show that the rat BKB_1 receptor gene consists of two exons of 87 or 88 bp (depending on which start site is used) and 1224 bp, separated by an intron of 1126 bp. However, this gene structure does not explain the presence of the additional 4 and 8 kb transcripts identified by Northern blot studies.

3.2.5.3 Alternative 3' processing of BKB_1 receptor mRNA generates multiple transcripts

To analyse the 3' end of the BKB_1 receptor cDNA, 3'RACE was performed. RACE cDNA was synthesised from control and IL-1β-treated (4 hour) JTC-19 cells. Products were analysed by Southern blotting and hybridisation with a primer located within the PCR products. PCR products were cloned into pGEM-T and DNA was prepared from six recombinants and sequenced. Two of the clones appeared to be PCR artifacts. Three of the clones showed the same 3' end as identified previously (Figure 3.8). These corresponded to a coding exon of 1244 bp and contain the poly(A) addition site (CATAAA). The remaining clone contained an additional 372 bp of sequence which was contiguous with the genomic sequence. Sequence analysis revealed the presence of a poly(A) addition site (AGTAAA) within this region located 374 bp downstream of the first identified poly(A) site. Assuming the poly(A) tails are of equivalent length, utilisation of the downstream poly(A) addition site would generate mRNA transcripts 372 bp longer than those generated using the upstream first identified poly(A) addition

site. However, alternative mRNA transcripts exhibiting this size difference were not detected by Northern blot analysis. The functional significance of these alternative 3' UTRs thus remains to be determined.

The 1.6 kb transcript corresponds to the predicted BKB_1 receptor mRNA size, however, the origin of the 4 and 8 kb transcripts could not be explained. RACE studies and primer extension analysis showed no evidence for the large transcripts. These large, hybridising bands were present after washing the blots to high stringency (data not shown) suggesting that they are due to transcription of the BKB_1 receptor gene. Therefore, transcription must be occurring either further upstream, due to alternative TISs, or proceeding further downstream, due to read-through at the 3' end of the gene. To elucidate the nature of the 4 and 8 kb transcripts, probes were synthesised spanning the 5' and 3' flanking regions of the BKB_1 receptor gene and used in Northern blot studies. BKB_1 receptor mRNA levels were analysed from JTC-19 cells treated with IL-1β for 2 hours. Using a 594 bp probe located −1941 to −2535 bp, hybridisation products were not detected (data not shown). Figure 3.9B shows the Northern blot analysis using a 450 bp probe spanning the genomic DNA starting at the sequence located 6 bp downstream of the most 3' poly(A) addition site. Two mRNA transcripts of 4 and 8 kb were detected using this probe. To confirm the existence of all three transcripts the same blot was stripped and reprobed with probe A, the canonical rat BKB_1 receptor probe (Figure 3.9A). Thus, these findings suggest that the large transcripts are not generated by differential 5' processing but are produced by 3' read-through and inefficient termination of the transcript.

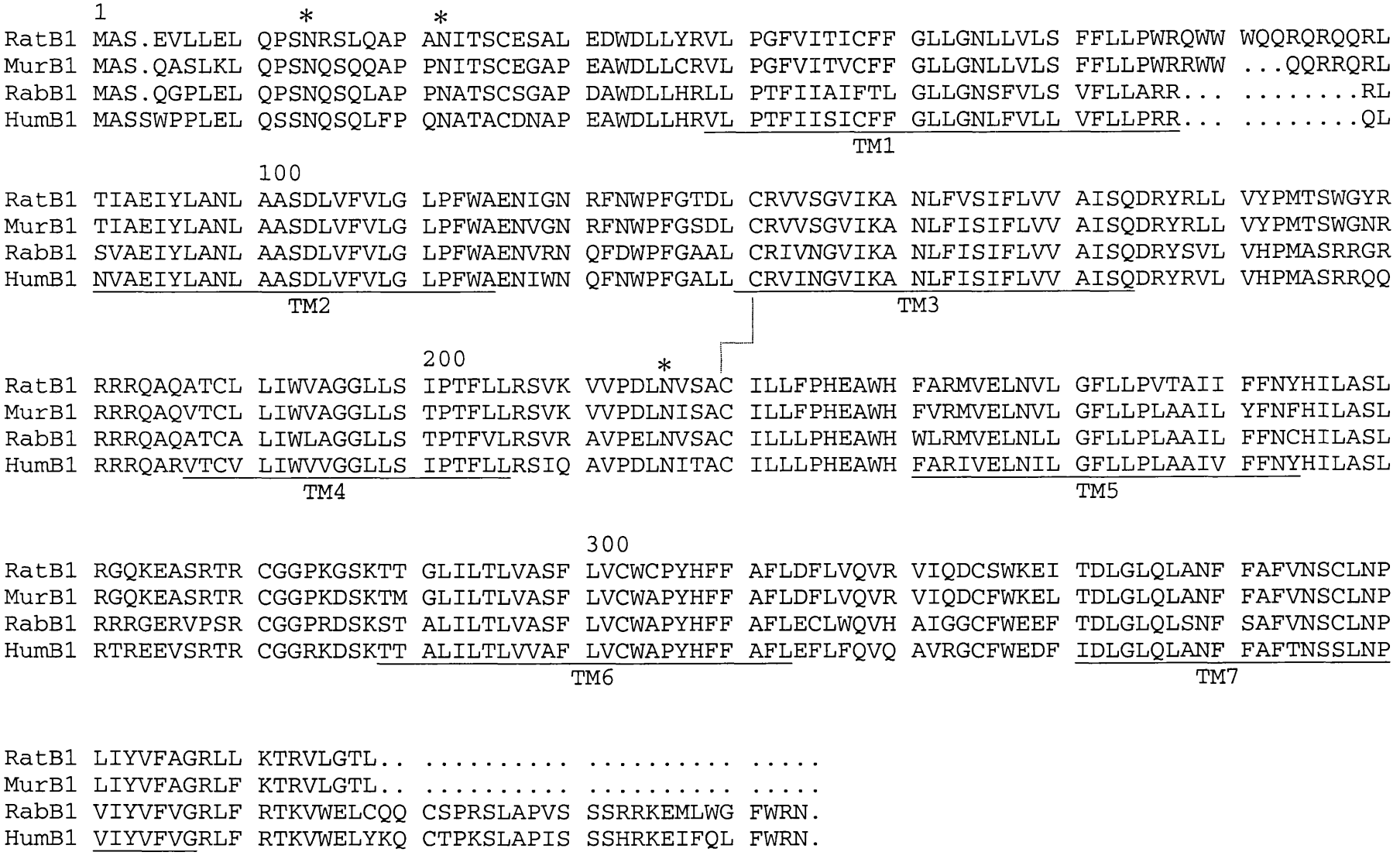
3.2.6 Comparison of the rat and human BKB_1 receptor gene structure

Whilst this work was carried out, the human BKB_1 receptor gene structure was reported (Bachvarov *et al.*, 1996, Yang and Polgar., 1996). In comparison with the rat, the human BKB_1 receptor gene contains 3 exons of 92, 119 and 1086 bp separated by introns of approximately 7000 bp and 900 bp (Figure 3.11A). Consequently, comparative Southern blot analysis of restriction digests from rat genomic DNA and cosmid RB1 DNA were carried out to eliminate the possibility of a deletion within the cosmid clone. Restriction digests were separated by electrophoresis on a 1% agarose gel and transferred to nylon membranes by Southern blotting. Hybridisation with probe A showed the presence of equivalent cosmid and genomic Bam HI and Eco RI fragments of 2.2 kb and 5.2 kb, respectively (Figure 3.10). A map corresponding to the rat BKB_1 receptor genomic structure is illustrated in Figure 3.11A.

To elucidate the nature of the differences in gene structure between the rat and human BKB_1 receptor genes, the non-coding exon sequences were aligned (Figure 3.11B). Alignment of the rat and human BKB_1 receptor non-coding exon sequences revealed 64.7% identity between exon 1 of the rat with exon 1 of the human. In contrast, alignment of exon 1 of the rat with exon 2 of the human shows 35.2% identity. These data suggests that exon 1 of the rat BKB_1 receptor gene is equivalent to exon 1 of the human BKB_1 receptor gene and one can further speculate that the human exon 2 arose by duplication and divergent evolution of the primordial precursor of exon 1.

Figure 3.1 BKB_1 receptor amino acid alignment.

Predicted ORFs from the rat (this study, Jones *et al.,* 1999; Ni *et al.,* 1998b), mouse (Pesquero *et al.,* 1996; Hess *et al.,* 1996), rabbit (MacNeil *et al.,* 1995) and human (Menke *et al.,* 1994; Jones *et al.,* 1999) were aligned using the GCG PILEUP program. Predicted transmembrane domains are underlined. Potential sites of N-linked glycosylation are shown by an asterisk. The conserved cysteine residues of the second and third extracellular loops ,which may form a disulphide bond, are joined by the dotted line.


1
*
*
RatB1 MAS.EVLLEL QPSNRSLQAP ANITSCESAL EDWDLLYRVL PGFVITICFF GLLGNLLVLS FFLLPWRQWW WQQRQRQQRL
MurB1 MAS.QASLKL QPSNQSQQAP PNITSCEGAP EAWDLLCRVL PGFVITVCFF GLLGNLLVLS FFLLPWRRWW ...QQRRQRL
RabB1 MAS.QGPLEL QPSNQSQLAP PNATSCSGAP DAWDLLHRLL PTFIIAIFTL GLLGNSFVLS VFLLARR... ....RL
HumB1 MASSWPPLEL QSSNQSQLFP QNATACDNAP EAWDLLHRVL PTFIISICFF GLLGNLFVLL VFLLPRR... ....QL
TM1
100
RatB1 TIAEIYLANL AASDLVFVLG LPFWAENIGN RFNWPFGTDL CRVVSGVIKA NLFVSIFLVV AISQDRYRLL VYPMTSWGYR
MurB1 TIAEIYLANL AASDLVFVLG LPFWAENVGN RFNWPFGSDL CRVVSGVIKA NLFISIFLVV AISQDRYRLL VYPMTSWGNR
RabB1 SVAEIYLANL AASDLVFVLG LPFWAENVRN QFDWPFGAAL CRIVNGVIKA NLFISIFLVV AISQDRYSVL VHPMASRRGR
HumB1 NVAEIYLANL AASDLVFVLG LPFWAENIWN QFNWPFGALL CRVINGVIKA NLFISIFLVV AISQDRYRVL VHPMASRRQQ
TM2
TM3
200
*
RatB1 RRRQAQATCL LIWVAGGLLS IPTFLLRSVK VVPDLNVSAC ILLFPHEAWH FARMVELNVL GFLLPVTAII FFNYHILASL
MurB1 RRRQAQVTCL LIWVAGGLLS TPTFLLRSVK VVPDLNISAC ILLFPHEAWH FVRMVELNVL GFLLPLAAIL YFNFHILASL
RabB1 RRRQAQATCA LIWLAGGLLS TPTFVLRSVR AVPELNVSAC ILLLPHEAWH WLRMVELNLL GFLLPLAAIL FFNCHILASL
HumB1 RRRQARVTCV LIWVVGGLLS IPTFLLRSIQ AVPDLNITAC ILLLPHEAWH FARIVELNIL GFLLPLAAIV FFNYHILASL
TM4
TM5
300
RatB1 RGQKEASRTR CGGPKGSKTT GLILTLVASF LVCWCPYHFF AFLDFLVQVR VIQDCSWKEI TDLGLQLANF FAFVNSCLNP
MurB1 RGQKEASRTR CGGPKDSKTM GLILTLVASF LVCWAPYHFF AFLDFLVQVR VIQDCFWKEL TDLGLQLANF FAFVNSCLNP
RabB1 RRRGERVPSR CGGPRDSKST ALILTLVASF LVCWAPYHFF AFLECLWQVH AIGGCFWEEF TDLGLQLSNF SAFVNSCLNP
HumB1 RTREEVSRTR CGGRKDSKTT ALILTLVVAF LVCWAPYHFF AFLEFLFQVQ AVRGCFWEDF IDLGLQLANF FAFTNSSLNP
TM6
TM7
RatB1 LIYVFAGRLL KTRVLGTL.. ....
MurB1 LIYVFAGRLF KTRVLGTL.. ....
RabB1 VIYVFVGRLF RTKVWELCQQ CSPRSLAPVS SSRRKEMLWG FWRN.
HumB1 VIYVFVGRLF RTKVWELYKQ CTPKSLAPIS SSHRKEIFQL FWRN.

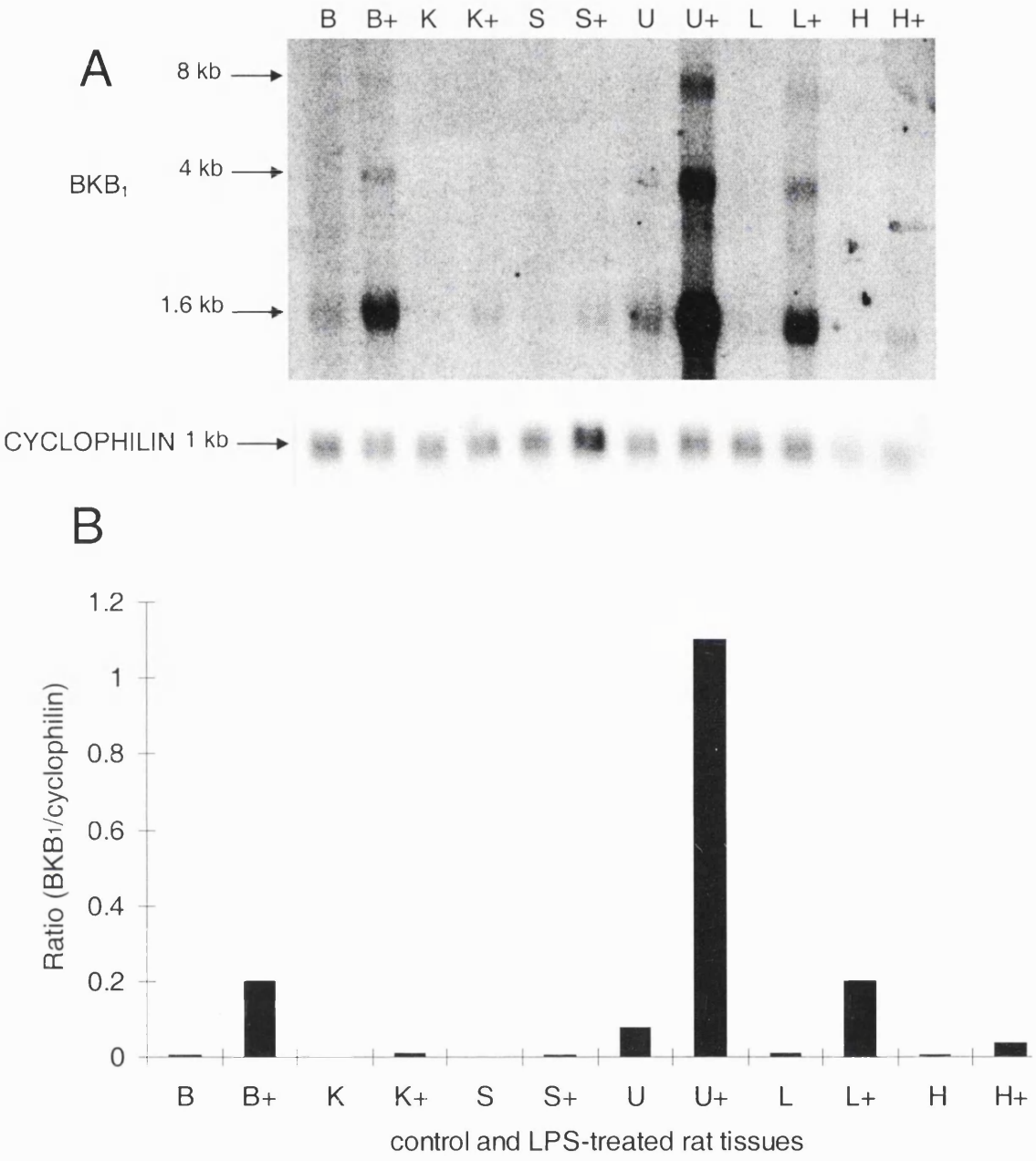


Figure 3.2 BKB_1 receptor mRNA levels in control and LPS-treated rat tissues.

A Northern blot analysis showing BKB_1 receptor mRNA levels from control and 4 hour LPS-treated (i.v. injection of 3 mg/kg) *ex-vivo* rat tissues. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT software and normalised to the respective cyclophilin band. The normalised signal was plotted against the tissues. B= Bladder, K= Kidney, S= Spleen , U= Uterus, L= Lung, H= Heart, the presence of a + sign indicates treatment of the rat with LPS.

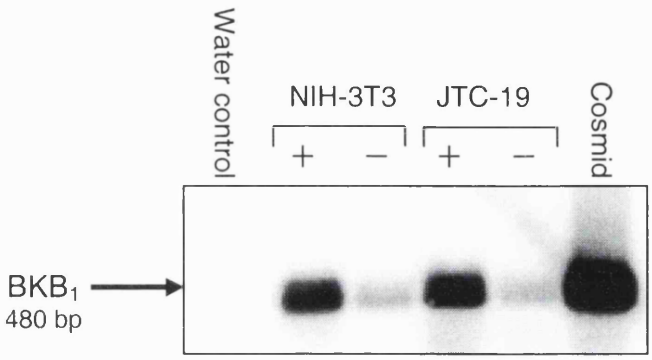


Figure 3.3 RT-PCR Southern blot analysis of BKB_1 receptor mRNA from rat and mouse cell lines.

Total RNA was extracted from control and 4 hour IL-1β-treated (10 ng/ml) JTC-19 cells and NIH-3T3 cells. 1 μg of total RNA was treated with DNase I and used in the reverse transcription reaction using reverse transcriptase as described in Methods and materials. PCR was conducted for 30 cycles using cDNA or 10 pg of RB1 template as a positive control, using primers spanning the ORF and the products separated by electrophoresis. Hybridisation was carried out using an ^{32}P end-labelled oligonucleotide located within the amplified product. The + sign indicates treatment with IL-1β.

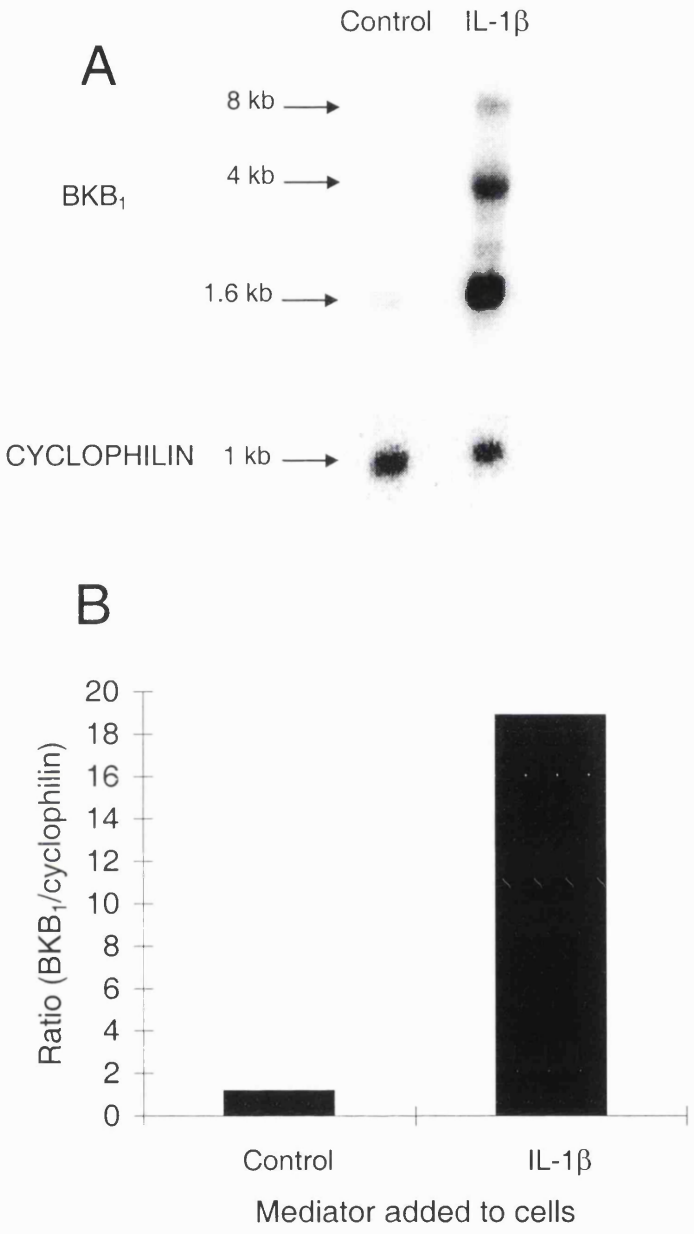


Figure 3.4 Effect of IL-1β on BKB_1 receptor mRNA levels in JTC-19 cells.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from control and 4 hour IL-1β-treated (10 ng/ml) JTC-19 cells. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel to allow quantitation. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band intensity. The normalised signal was plotted against the appropriate treatment. The Northern blots shown are representative of two separate experiments.

Figure 3.5 Restriction map of the BKB_1 receptor cosmid clone, RB1.

A and **B** show the cosmid insert RB1 with the relative positions of the restriction enzymes Not I in (**A**) and Eco RI in (**B**). The boxed region represents the cosmid insert. The shaded area within the boxed cosmid insert represents the coding exon. The lines adjoining the end of the cosmid insert represent the Supercos 1 sequence with the position of the T7 and T3 sequences shown. **C** shows an amplified Eco RI–Bam HI fragment of RB1 containing the BKB_1 receptor gene. The boxes represent the exons, with the hatched box representing the ORF for the receptor. Restriction enzymes used for the generation of fragments for subcloning and as probes are shown, E= Eco RI, X= Xba I, B= Bam HI and P= Pst I. The location of Probe A, the canonical rat BKB_1 receptor probe used in these studies is illustrated.

FIGURE 3.5

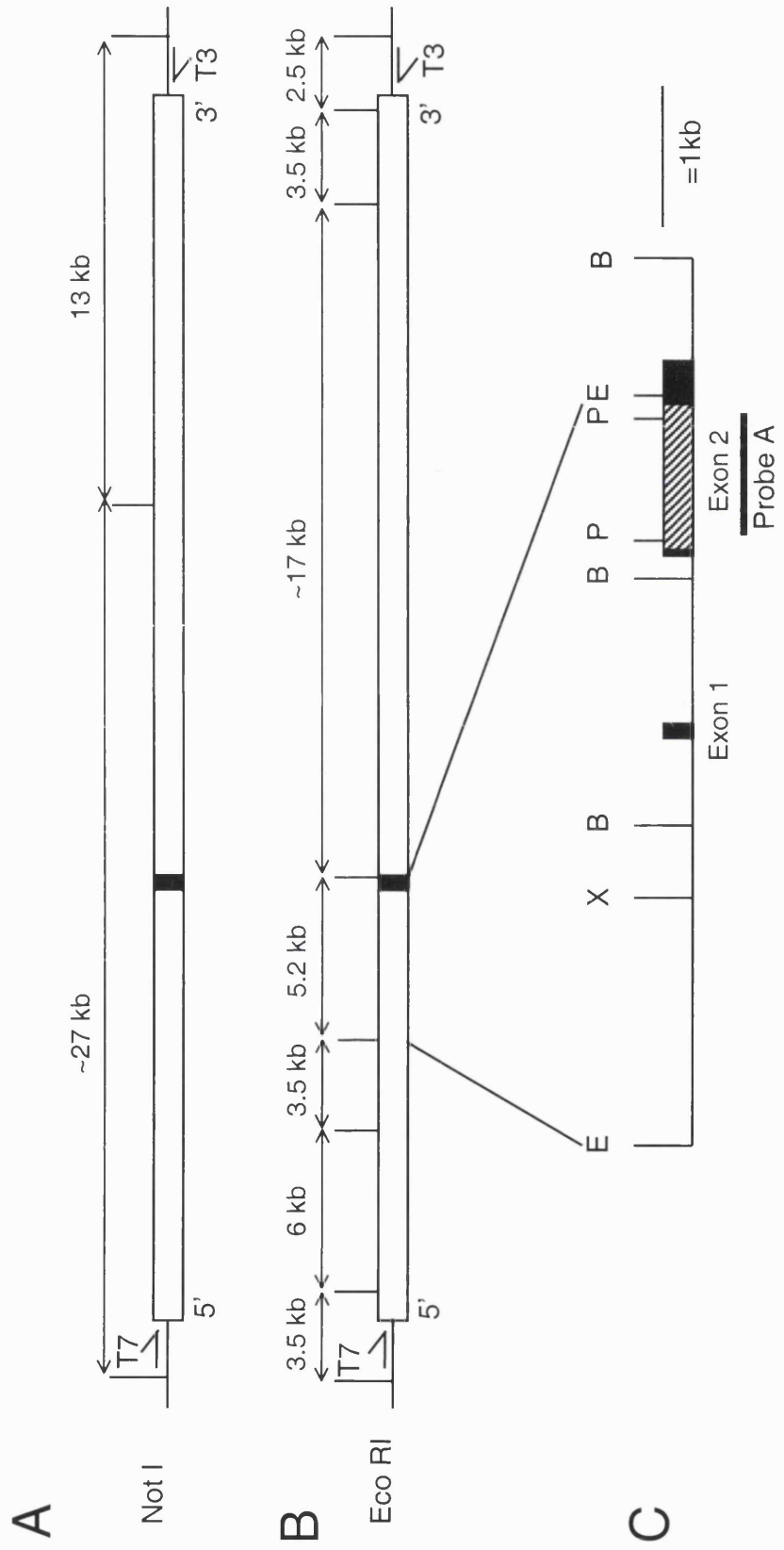
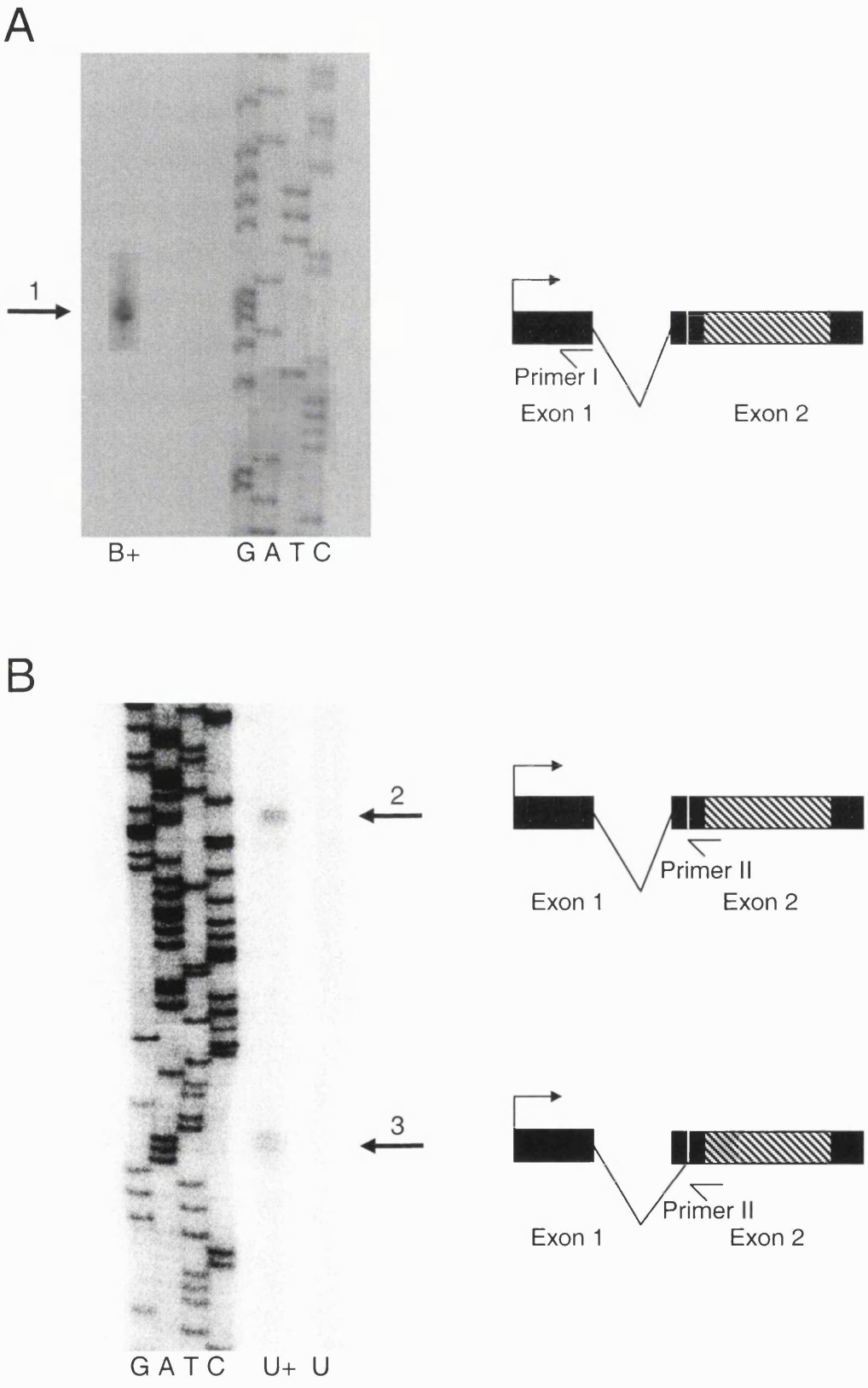

A
Not I
13 kb
~27 kb
3' T3
5'
T7
B
Eco RI
3.5 kb
2.5 kb
~17 kb
5.2 kb
3.5 kb
6 kb
3.5 kb
3' T3
5'
T7
C
=1kb
B
PE
B P
X
B
E
Exon 2
Probe A
Exon 1

Figure 3.6 Determination of the transcription initiation sites by primer extension analysis.

A 50 μg of total RNA isolated from rat bladders (treated as described in Methods and Materials) was hybridised with an oligonucleotide, primer I (5'-CCGGATAGAAAAGCAAAGAGC- 3'), located in exon 1. Extension products were synthesised with reverse transcriptase as described in Methods and Materials and size fractionated on a denaturing polyacrylamide gel alongside a Sanger dideoxynucleotide sequencing ladder (G,A,T,C) primed on a genomic plasmid subclone with the same primer. Arrow 1 indicates the initiations. Arrow 1 corresponds to two TISs located 87 and 88 bp from the 3' end of exon 1. The TIS is represented diagrammatically on the genomic BKB_1 gene receptor in (**A**) (right hand panel). Exons are indicated by solid boxes, the coding region is shown by a hatched box inside exon 2. The arrow shows the location of the TIS.

B 5 μg of poly(A)$^+$ RNA isolated from control (U) and LPS-treated (U+) rat uterus were hybridised with an oligonucleotide, primer II (5'-GCTGGAGCTCCAACAAGACCTCGGACGCCA-3'), located in exon 2 and extended with reverse transcriptase as described in Methods and Materials. Extension products were size fractionated on a denaturing polyacrylamide gel alongside a Sanger dideoxynucleotide sequencing ladder (G,A,T,C) primed on pUC18 with the universal primer (5'-GTTTTCCCAGTCACGACGTTGTA-3'). Arrows numbered 2 and 3 indicate the initiations. Arrow 2 corresponds to two TISs located 87 and 88 bp from the 3' end of exon 1. Arrow 3 corresponds to two TISs 41 bp smaller than the initiation sites shown by arrow 2, these products can be explained by an alternative splice acceptor site identified using 5' RACE. The TISs and possible splice patterns are represented diagrammatically on the genomic BKB_1 receptor structure shown in (**B**) (right hand panel). The exon structure is the same as described in (**A**), the arrow shows the location of the TIS, the location of primer II is shown. The primer extensions shown are representative of three separate experiments.

FIGURE 3.6


A
1
B+
G A T C
Primer I
Exon 1
Exon 2
B
2
3
Primer II
Exon 1
Exon 2
Primer II
Exon 1
Exon 2
G A T C
U+ U

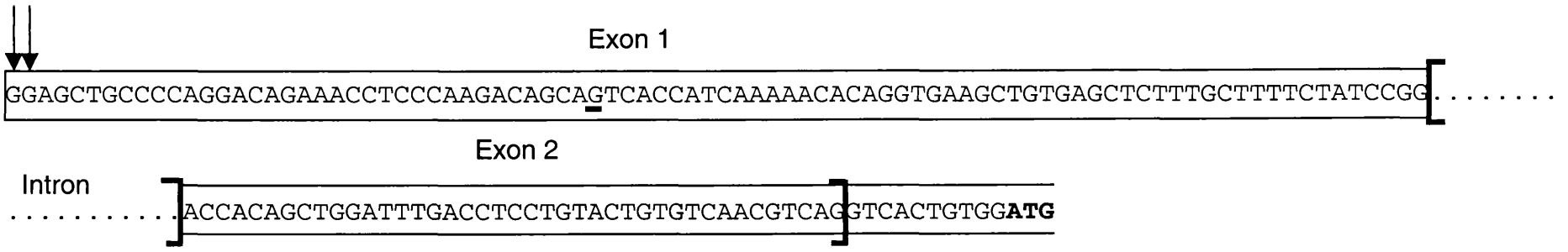


Figure 3.7 5' UTR sequence of the BKB_1 receptor cDNA ends isolated by 5' RACE.

5'RACE products were isolated from LPS-treated rat uterus and from IL-1β-treated JTC-19 cells (see Methods and Materials). Nucleotide sequence for the products are shown. The TISs are indicated by arrows. The first nucleotide of the cDNA clone isolated from the rat bladder cDNA library is underlined. The start codon (ATG) of the coding region is presented in boldface. The splice donor site is represented by the [. Alternative splice acceptor sites are represented by].

TGATGCAACCGAGAAGCCTCATGCCCATGTGGCCCAACAGGAAGGGACTCTTCCAATTATCCTGCTGGAATTCAAACAGCAGGGAGCCAAGAAGCC

TGGCTTCTTGACCAACCATCTCTGGTAT**CATAAA**GACCATCTGGTCAGCTGACCCACAGCCCACAAAGATACCCAGGGTAAAAGTTATTAGCAGGG

TAGTCGGGTAGTCGGGACCTTGAAGGGCAATTACTAGGGCTCTGGAGATTAGGCTGATCCTGAAGTTTTTCGGGACAGAGGGAGAAGCATAGCCAC

AGCGGTTCTTTTAAGAGGGGATTTTGGAGGGCTGCAATGCATGGACAGAAGCAGAGGTCAGAGTGTGGTGAGGAAGGCGGCACCAGCCCGAGGACA

GCAGGCACCCTGAGAGGCTGGAAAAGAAAGAACACAGGCAGACATCCTCTCTGAAACCTCCAGGAAAGACCAGACTTGGCCAACGTGCCGATGCCA

GCCCTGCCCCCCAGGTACTGTGAGAG**AGTAAA**GCTGCACTCTGATTTGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.8 3' UTR sequence of the BKB_1 receptor cDNA ends isolated by 3' RACE.

3'RACE products were isolated from IL-1β-treated JTC-19 cells according to Methods and Materials. Nucleotide sequence for the largest product is shown. The sequence starts with the STOP codon, TGA, shown in boldface. Polyadenylation sites are shown in boldface and underlined. Of the six recombinants sequenced, only one corresponded to the utilisation of the downstream polyadenylation site (AGTAAA). Three clones utilised the upstream polyadenylation site (CATAAA), in these cases the poly(A) tail started at the C nucleotide (underlined) located 28 bp downstream of the polyadenylation site. The remaining two clones were PCR artifacts.

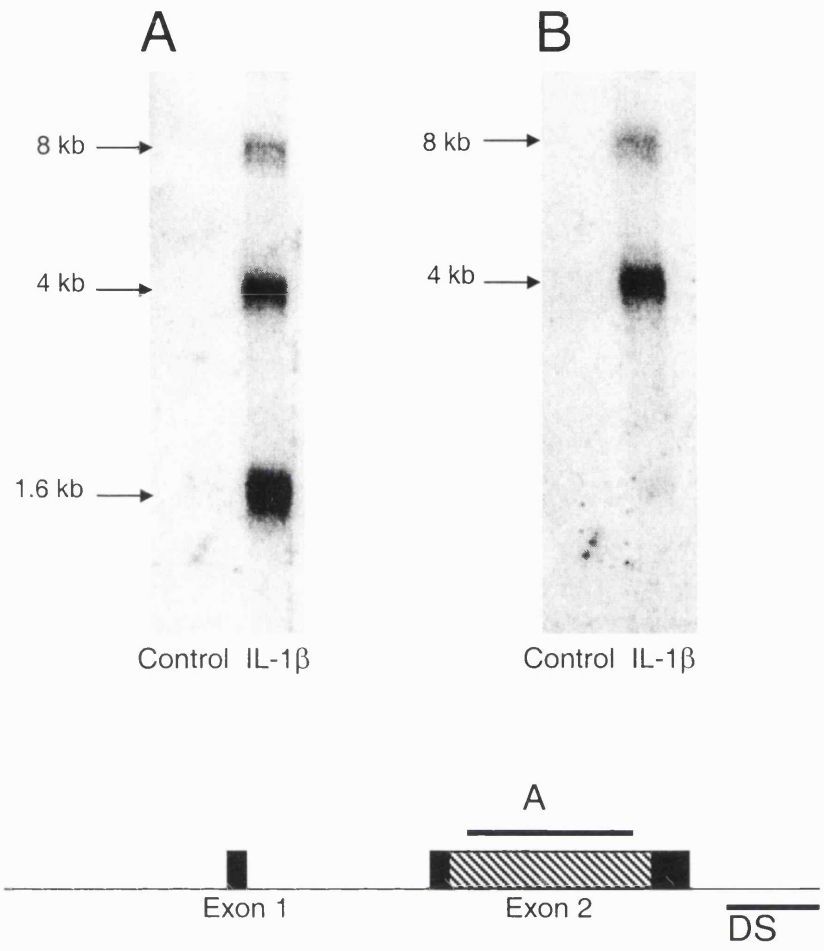


Figure 3.9 Detection of BKB_1 receptor mRNA transcripts using probes spanning different regions of the BKB_1 receptor gene.

A Northern blot analysis showing the BKB_1 receptor mRNA transcripts from control and IL-1β-treated (10ng/ml) JTC-19 cells hybridised with probe A. In panel **B** the same blot was hybridised with a probe located downstream of the poly(A) addition site in the BKB_1 receptor gene (DS probe). The diagram below the Northern blot analysis shows a schematic representation of the rat BKB_1 receptor gene structure with the probe locations. The boxes area represent the exons, with the hatched box representing the ORF. The probe used for Northern analysis in panel (**A**) is labelled A and that used in panel (**B**) is labelled DS.

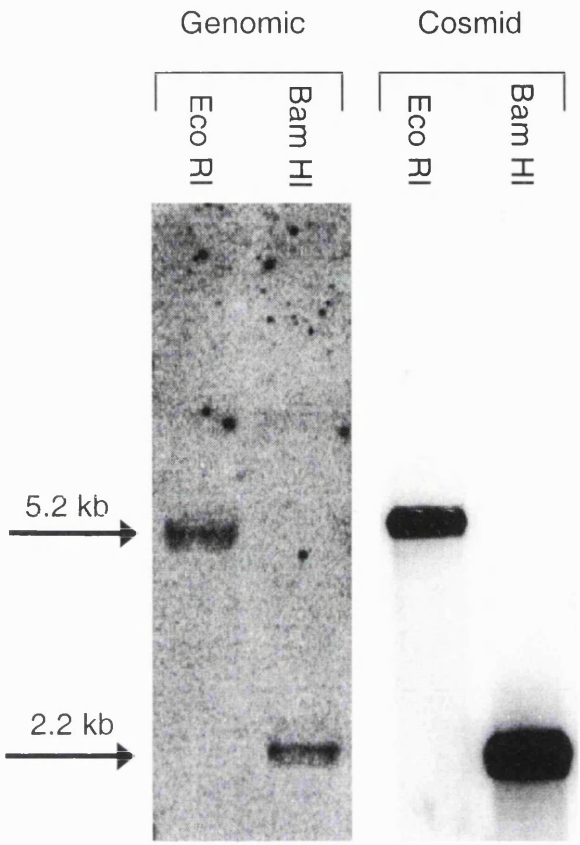


Figure 3.10 Southern blot analysis of genomic and cosmid DNA restriction digests.

The figure shows the results obtained from Southern blot analysis of restriction digests of rat genomic DNA and DNA from the cosmid clone, RB1. 6.5 μg of rat genomic DNA and 1 μg of RB1 cosmid DNA were digested with Eco RI or Bam HI restriction enzymes as indicated. Digested products were separated by electrophoresis and Southern blotted (see Methods and Materials). Southern blots were hybridised with probe A. Hybridisation to a 5.2 kb Eco RI fragment and a 2.2 kb Bam HI fragment in both genomic and cosmid DNA are shown by arrows.

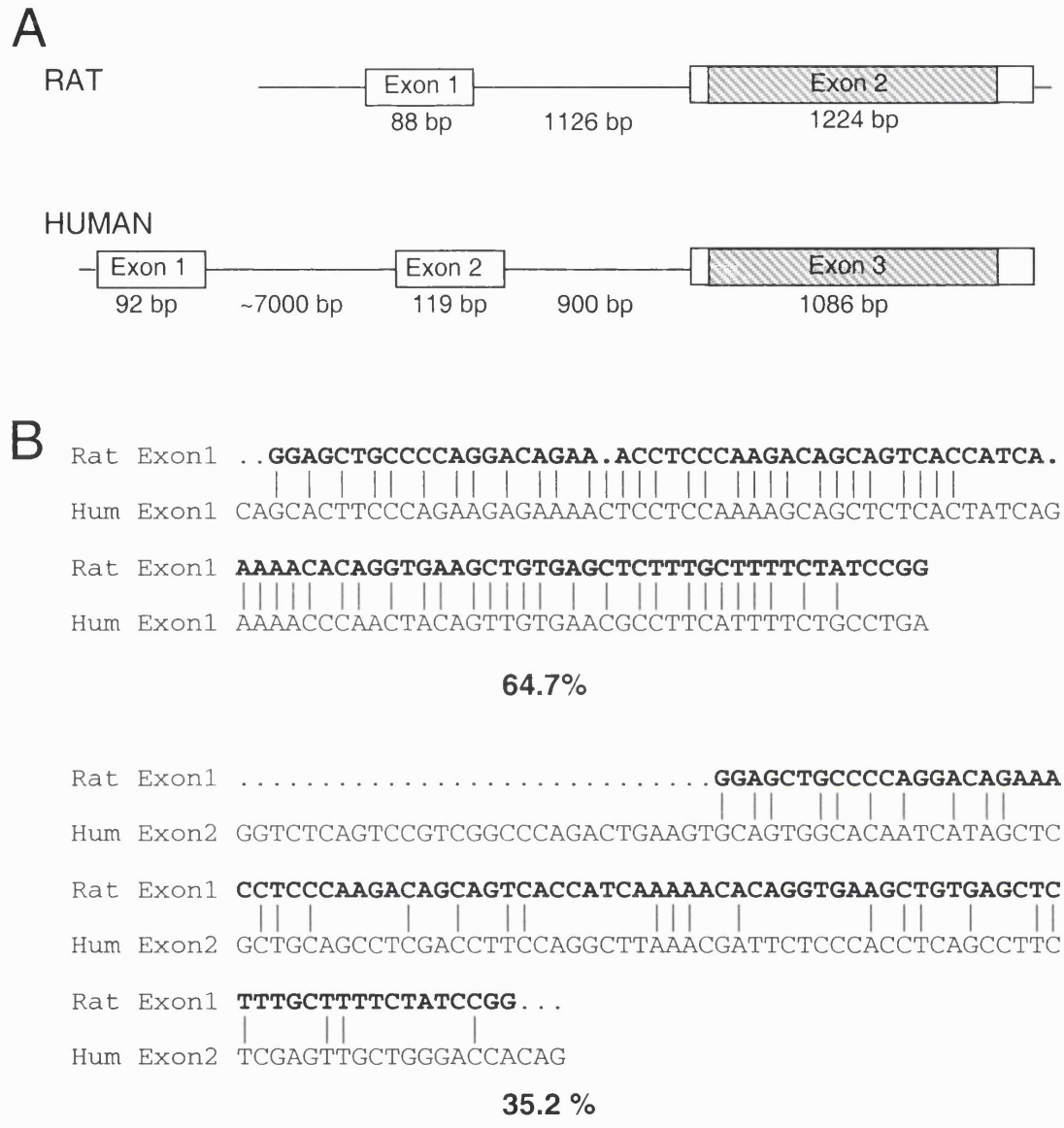


Figure 3.11 Structural organisation of the rat and human BKB_1 receptor genes.

A Shows a schematic diagram representing the structural organisation of the rat and human (Yang and Polgar, 1996) BKB_1 receptor genes. Locations of exons and introns and their sizes are shown. Exons are indicated by solid boxes, the coding region is shown by a hatched box inside exon 2 of the rat gene and exon 3 of the human gene. **B** Upper panel shows an alignment of exon 1 of the rat with exon 1 of the human. Lower panel shows an alignment of exon 1 of the rat with exon 2 of the human. The rat sequence is shown above in boldface and the human sequence is shown below, lines join identical nucleotides. The percentage of identity between exon 1 of the rat gene with the respective human exons are shown below the alignment.

3.3 Discussion

This chapter describes the elucidation of the exon-intron structure of the rat BKB_1 receptor gene. This was achieved by isolation and characterisation of cDNA and genomic clones. Using these sequences as specific probes, a distinct expression pattern for the BKB_1 receptor gene in a number of tissues and the rat cell line, JTC-19, has been determined.

The rat BKB_1 receptor cDNA clone contains a single ORF encoding a G-protein coupled receptor of 338 amino acids, and is identical to that recently described by Ni and colleagues (1998b). The structure of the rat receptor shows a high degree of similarity to the mouse BKB_1 receptor (Hess *et al.*, 1996; Pesquero *et al.*, 1996). In contrast, both rodent receptors show marked differences with the human and rabbit receptor sequences. The large insertion of basic amino acids in the first intracellular loop together with the shortened C-terminal cytoplasmic tail, may be reflected in differences in coupling of the rat and human receptors to heterotrimeric G-proteins (Strader *et al.*, 1994; Wong and Ross, 1994; Capeyrou *et al.*, 1997). Significant divergence of the BKB_1 and BKB_2 receptors has been noted previously (Menke *et al.*, 1994). Despite their limited homology to each other, the BKB_1 and BKB_2 receptors are obviously functionally related since they are both activated by kinins. Indeed, the human receptor genes are located close together on human chromosome 14q32 (Ma *et al.*, 1994a; Kammerer *et al.*, 1995; Pesquero *et al.*, 1994; Chai *et al.*, 1996; Bachvarov *et al.*, 1998a). The BKB_1 and BKB_2 receptors are most likely derived by divergent evolution from the same ancestral gene which may resemble the chicken ornithokinin receptor (Schroeder *et al.*, 1997). The structural differences between the BKB_1

receptors from different species represents further divergent evolution within this subgroup of receptors.

Following the isolation of the rat BKB_1 receptor, the pharmacological properties of the rat and human receptors were examined by investigating the ability of a range of agonists and antagonists to inhibit the binding of [^{3}H]desArg^{10}kallidin (This work was carried out by other colleagues in the laboratory and is reported in Jones *et al.,* 1999). These studies showed that the rat BKB_1 receptor has higher affinity for des-Arg^{10}kallidin (K_I value 1.6 ± 0.2 nM) than desArg^{9}BK (K_I value 15 ± 5 nM), although Ni *et al.,* (1998b) report that these ligands have equal affinity for the rat receptor. Significantly, at the human receptor, desArg^{10}kallidin was about ten times more potent than at the rat receptor (K_I value 0.19 ± 0.05 nM), with 5,000 fold greater affinity compared to desArg^{9}BK (K_I value 1620 ± 153). In addition, the potential kininase I derivative of T-kinin, desArg^{11}T-kinin had relatively high affinity for the rat BKB_1 receptor (K_I value 46 ± 14 nM), with 30 fold lower activity at the human receptor. Therefore, it would appear that due to differences in the kinin systems between rodents and humans (see Chapter 1 section 1.1), the human receptor has evolved greater sensitivity towards kallidin and desArg^{10}kallidin and the rodent receptor has evolved sensitivity towards bradykinin and T-kinin derivatives. Other studies have shown that the mouse BKB_1 receptors exhibit a 2 to 3 fold greater selectivity for desArg^{9}BK over desArg^{10}kallidin (Hess *et al.,* 1996; Pesquero *et al.,* 1996). The region of the receptor responsible for these binding affinities has not been elucidated. Whether these different pharmacological properties can be explained by differences between the species in the first intracellular loop and the C-terminus remains to be determined. Studies carried out

on chimeric BKB_1 and BKB_2 receptor proteins have shown that the TM VI of the BK receptor affects the binding of desArg^{10}kallidin (Leeb *et al.,* 1997). Alignment of the BKB_1 receptor protein sequences over TM VI and the third extracellular loop has shown the location of a number of amino acids that are different between the rodent receptors compared to the rabbit and human receptors (Jones *et al.,* 1999).

The findings presented in this chapter show that the rat BKB_1 receptor gene structure consists of two exons, this differs from that seen in the human receptor gene which spans three exons (Bachvarov *et al.,* 1996; Yang and Polgar, 1996). However the coding domain of both the rat and human receptors are located on a single exon. Preliminary observations of the human and rat BKB_1 receptor exon-intron sizes suggested that exon 1 of the rat is equivalent to exon 2 of the human BKB_1 receptor and that the rat BKB_1 receptor gene was incompletely characterised. However, alignment of the non-coding sequence presented here shows that exons 1 of the rat and human receptor genes are homologous indicating that the differences in gene structure are species-specific. The BKB_1 receptor genomic structure for other organisms remains to be determined. The data show the presence of two adjacent TISs. In contrast, a recent report by Ni and co-workers (1998b) describes the existence of a TIS located 20 bps upstream from the start sites reported in this study. Similar discrepancies have been reported for the identification of the TIS in the human BKB_1 receptor gene (Bachvarov *et al.,* 1996; Yang and Polgar, 1996). These data suggest that there may be subtle differences in the transcription of the gene in different cell types. No evidence was found from primer extension or 5'RACE for the existence of a TIS at the beginning of the coding exon, analagous to that suggested for the human BKB_1 receptor in IMR-90 cells (Yang and Polgar, 1996). Analysis of 5'RACE products shows the existence of an

alternative splice acceptor site, a finding recently reported by Ni *et al.,* (1998b). The downstream splice acceptor site is equivalent to the exon-intron junction observed in the human BKB_1 receptor gene (Bachvarov *et al.,* 1996; Yang and Polgar, 1996). However, it is not known if similar splice variants exist in the human BKB_1 receptor mRNA. Analysis of the expression of these splice variants is described in Chapter 4. Investigation of the 3' cDNA ends of the rat BKB_1 receptor gene by 3' RACE revealed the existence of alternate polyadenylation usage, although alternative transcripts generated by these sites were not detected by Northern blot analysis. However, it is possible that variations in polyadenylation of the transcripts generated by these sites render them indistinguishable by Northern blot studies. In addition, hybridisation experiments using a 3' genomic fragment showed that the large mRNA transcripts of 4 and 8 kb are generated by alternative 3' processing of the BKB_1 receptor gene.

The BKB_1 receptor gene expression is induced under some pathophysiological conditions. In particular, *in vivo* induction of the BKB_1 receptors has been characterised in the mediation of inflammatory and cardiovascular responses following treatment with a sub-lethal dose of LPS (Campos *et al.,* 1996; Regoli *et al.,* 1981; Siebeck *et al.,* 1989, 1996, 1997; Tokumasu *et al.,* 1995; Nicolau *et al.,* 1996). The present study shows that the rat BKB_1 receptor mRNA is up-regulated by LPS in a distinct tissue expression pattern. In all the tissues tested the mRNA was up-regulated from basal levels. In uterus, bladder and lung the final levels of BKB_1 receptor mRNA were particularly high compared with the other tissues. Whether this increase in mRNA results in an increase in BKB_1 receptor protein has not been shown directly. However, it is well established for the majority of genes that the difference in mRNA expression levels are paralleled by differences in protein composition (Latchman, 1995). The basal levels of BKB_1

receptor mRNA in uterus were 10 fold higher than in any of the other tissues tested. The significance of BKB_1 receptor mRNA under normal conditions remains unclear but one can speculate that the BKB_1 receptor is important in the function of the uterus and may be regulated by oestrogen as has been shown for the BKB_2 receptor (Madeddu *et al.,* 1997b). However, another study showed high levels of receptor mRNA in the rat prostate with no effect of the sex hormones on BKB_1 receptor mRNA levels (Ni *et al.,* 1998b). A separate study has shown a similar tissue expression pattern for BKB_1 receptor mRNA as that reported in this chapter, with additional tissues showing increased receptor mRNA in duodenum and undetectable levels of receptor mRNA in brain, spinal cord and DRG (Jones *et al.,* 1999). Other studies have analysed the BKB_1 receptor mRNA levels in rats and mice by RT-PCR following LPS treatment (Pesquero *et al.,* 1996; Ni *et al.,* 1998b; Marin-Castano *et al.,* 1998). The induction of BKB_1 receptor mRNA resulted in increased functional receptor expression in the rat nephron (Marin-Castano *et al.,* 1998). Thus, the BKB_1 receptor up-regulation produced by LPS in different *in vitro* and *in vivo* systems is caused by mRNA induction. However, within this up-regulation there is a great deal of heterogeneity, this could be due to a number of effects including tissue-specific regulation, the ability of the tissues to generate an inflammatory response or the local concentration of LPS between tissues.

Data presented in this chapter shows that the rat fibroblast cell line, JTC-19, and the mouse fibroblast cell line, NIH-3T3 increase BKB_1 receptor mRNA levels following treatment with IL-1β in a similar way to that observed with the human fibroblast cell lines, IMR-90 and WI38 (Bachvarov *et al.,* 1996; Phagoo *et al.,* 1997,1999; Zhou *et al.,* 1998; Shanstra *et al.,* 1998; Bastian *et al.,* 1998). More recent studies have shown that rat VSMCs, and the rat cell line, A10, express inducible BKB_1 receptor mRNA (Ni *et*

al., 1998a,b). BKB_1 receptor mRNA levels in A10 cells are induced 3-4 fold following the addition of inducing agents. However, the 16 fold induction observed following IL-1β treatment in JTC-19 cells resembles the increases observed in rat tissues. In contrast, expression of BKB_1 receptor mRNA is non-inducible in rat hepatoma H4 cells.

In summary, these data show the isolation, characterisation and expression of the rat BKB_1 receptor gene. The identification of JTC-19 cells as a cellular model for analysis of BKB_1 receptor up-regulation will allow detailed analysis of the mechanisms of BKB_1 receptor gene expression.

Chapter 4

Expression and regulation of the rat BKB_1 receptor gene

4.1 Introduction

There is ample evidence showing that IL-1β and LPS induce the responsiveness to desArg^{9}BK both *in vitro* (Deblois *et al.*,1988, 1989, 1991; Bouthillier *et al.*,1987) and *in vivo* (Regoli *et al.*,1981; Deblois *et al.*,1989, 1991; Bouthillier *et al.*,1987; Marceau *et al.*,1984; Davis and Perkins 1994b; Perkins and Kelly, 1994; Campos *et al.*,1995, 1996). In addition, other cytokines including IL-2, IL-8 and TNF-α have been shown to increase BKB_1 receptor expression (Davis and Perkins, 1994b; Phagoo *et al.*, 1997; Bastian *et al.*, 1998; Ni *et al.*, 1998a). In contrast, other *in vitro* and *in vivo* studies have shown that TNF-α does not increase BKB_1 receptor responsiveness (Deblois *et al.*, 1988, 1991; Davis and Perkins, 1994b; Perkins and Kelly, 1994). Thus, the physiological role of TNF-α in the induction of BKB_1 receptors remains unclear. Recent studies have shown that the kinins, BK and desArg^{10}kallidin can autoregulate human BKB_1 receptor mRNA levels in IMR-90 cells (Schanstra *et al.*,1998; Phagoo *et al.*,1999). In addition, complex autocrine interactions between the cytokines, LPS and the kinins may contribute to the increased levels of BKB_1 receptor expression *in vivo*

(Dinarello and Krueger, 1986; Tiffany and Burch, 1989; Dinarello, 1992; Vassalli, 1992; Tsukagoshi *et al.,* 1999). A central role for IL-1 was initially proposed to explain the action of diverse agents that can increase the BKB_1 receptor (Deblois *et al.,* 1991). This is supported by a number of *in vitro* and *in vivo* studies which have shown an important role for IL-1 in the IL-8, IL-2 and BK mediated up-regulation of the BKB_1 receptor following examination of the effects of IL-1Ra (Davis and Perkins, 1994b, 1996; Bastian *et al.,* 1998; Phagoo *et al.,* 1999). However, *in vivo* studies using IL-1Ra failed to inhibit the LPS-induced hypotensive responses to $desArg^9BK$ in rabbits suggesting that these effects were not mediated by IL-1 (Whalley *et al.,* 1993). Molecular determination of the effects of IL-1Ra on the LPS-mediated up-regulation of the BKB_1 receptor remains to be determined. More recently, the identification of a conserved cAMP response element (CRE) site in the human and rat BKB_1 receptor 5' flanking sequences suggests a possible role for cAMP signalling pathways in the BKB_1 receptor mRNA up-regulation (Ni *et al.,* 1998a,b). Moreover, activation of cAMP signalling pathways by the addition of cAMP activators or LPS has been shown to induce IL-1β mRNA expression (Chandra *et al.,* 1995).

The previous chapter established that BKB_1 receptor mRNA levels are increased following the addition of IL-1β or LPS. A number of stages intervene between the initial synthesis of the primary RNA transcripts by the action of RNA polymerases and the eventual production of the mRNA. It is therefore conceivable that any of these stages may be subject to regulation. Many studies have shown that cytokines and LPS can control gene regulation by both transcriptional (reviewed in Bankers-Fulbright et al., 1996; Sweet and Hume, 1996; Collins et al., 1995) and post-transcriptional mechanisms (Chu *et al.,*1994; Ristimaki *et al.,*1994; Newton *et al.,*1997a; Croft *et al.,*1999; Barrios-

Rodiles *et al.*,1999). In addition, studies in the previous chapter consistently showed the induction of three BKB_1 receptor mRNA transcripts of greatly differing length, generated by alternative 3' processing. Moreover, the existence of alternative splice acceptor sites and alternative polyadenylation sites were identified, resulting in more subtle differences in mRNA transcript size. Use of alternative polyadenylation signals have been shown to cause 3' heterogeneity in many mammalian genes (reviewed in Leff *et al.*, 1986). Interestingly, differences in the 3' UTR have been shown to serve a role in the post-transcriptional regulation of a number of genes (Chu *et al.*,1994; Lu and Menon, 1996; Miyamoto *et al.*,1996; Newton *et al.*,1997a). Furthermore, multiple mRNA species have been shown to be differentially regulated by events such as development, infection and differentiation (Murtagh *et al.*,1991; Mann *et al.*,1993). To fully understand the mechanisms of BKB_1 receptor gene expression, it is important to determine the significance of these BKB_1 receptor transcripts in gene regulation.

This chapter examines the effect of the cytokines IL-1β and TNF-α, the bacterial endotoxin LPS, the rat BKB_1 receptor agonists, and cAMP activators on BKB_1 receptor gene expression in the rat embryonic lung cell line, JTC-19. Furthermore, the contribution of transcriptional and post-transcriptional mechanisms in the IL-1β and LPS-mediated up-regulation of the BKB_1 receptor gene are studied.

4.2 Results

4.2.1 Expression of BKB_1 receptor in JTC-19 cells

A radioligand binding assay was performed on JTC-19 cells to show that the increase in BKB_1 receptor mRNA levels correlated to an increase in receptor protein. Radioligand binding was analysed in control, IL-1β-treated and LPS-treated (4 hours) cells (see Methods and Materials). As shown in Figure 4.1 the specific binding of the agonist, [^{3}H]desArg^{10}kallidin, showed a 6 fold increase following the addition of IL-1β and an 11 fold increase following the addition of LPS. Saturation binding isotherms were not performed, so true B_{max} values were not determined. Therefore, a change in the binding affinity of the BKB_1 receptors for [^{3}H]desArg^{10}kallidin cannot be excluded to explain the increase in binding.

4.2.2 cAMP activators increase BKB_1 receptor mRNA levels

A role for the cAMP signalling pathway was investigated by direct activation with dbcAMP, or indirectly by using an inducer of endogenous cAMP, forskolin. Poly(A)$^+$ RNA was purified from JTC-19 cells treated with dbcAMP or forskolin for 2 hours. BKB_1 receptor mRNA levels were determined by Northern blot analysis (Figure 4.2A). Figure 4.2B shows that the addition of dbcAMP or forskolin increased BKB_1 receptor mRNA levels 20 and 8 fold respectively, compared to control cells. The role of new protein synthesis in the dbcAMP-mediated up-regulation of BKB_1 receptor mRNA levels was investigated by pre-treating the cells with the translational inhibitor, cycloheximide, prior to the addition of dbcAMP (Figure 4.3). Figure 4.3B shows that in the presence of cycloheximide, dbcAMP treatment increases BKB_1 receptor mRNA

levels relative to the dbcAMP-mediated up-regulation in the absence of cycloheximide. Thus, protein synthesis, including dbcAMP-induced expression of IL-1β, is not required for the dbcAMP-induced BKB_1 receptor gene expression.

4.2.3 BKB_1 receptor agonists increase BKB_1 receptor mRNA levels

To determine whether an autoregulatory mechanism increases rat BKB_1 receptor mRNA levels, JTC-19 cells were treated with the rat BKB_1 receptor agonists, $desArg^9BK$ or $desArg^{10}$kallidin in the absence or presence of LPS for 4 hours. Poly(A)$^+$ RNA was extracted and BKB_1 receptor mRNA levels were measured by Northern blot analysis (Figure 4.4A). Figure 4.4B shows that the BKB_1 receptor mRNA levels are not up-regulated following the addition of BKB_1 receptor agonists *per se*. However, in the presence of LPS, the BKB_1 receptor agonists $desArg^9BK$ and $desArg^{10}$kallidin increased the BKB_1 receptor mRNA levels 1.4 and 1.2 fold, respectively, above the induction observed with LPS alone. These data suggest that an up-regulated population of BKB_1 receptors is a prerequisite for the inducing effects of the rat BKB_1 receptor agonists.

4.2.4 BKB_1 receptor mRNA expression patterns following IL-1β, TNF-α or LPS treatment

In vitro studies have shown that the responsiveness to BKB_1 receptor agonists following the administration of inducing agents was observed following a delay of several hours (Bouthillier *et al.*,1987; Deblois *et al.*,1988, 1989). Similar time delays have been reported in cultured cell lines (Schneck *et al.*,1994; Menke *et al.*,1994; Levesque *et al.*,1995a,b; Galizzi *et al.*,1994). In the present study IL-1β, TNF-α and LPS were

examined for their ability to up-regulate BKB_1 receptor mRNA levels in JTC-19 cells. Dose response curves for these compounds showed that the concentrations used in this study were super-maximal for induction of the BKB_1 receptor mRNA (data not shown). IL-1β (10 ng/ml), was administered to JTC-19 cells for various lengths of time up to 24 hours and poly(A)$^+$ RNA was analysed by Northern blotting (see Methods and Materials). As shown in Figure 4.5A, three mRNA transcripts of 1.6, 4 and 8 kb were detected, with the 4 and 8 kb transcripts only observed following the addition of the inducing agent. Figure 4.5B shows that BKB_1 receptor mRNA levels were up-regulated at the earliest measured time point of 30 minutes following IL-1β treatment. Maximum mRNA levels were reached 2 hours post-IL-1β treatment corresponding to a 15 fold increase compared to basal levels. Even after 24 hours post-IL-1β treatment, mRNA levels were still 5 fold higher than basal levels. TNF-α (10 ng/ml) was administered to JTC-19 cells for varying lengths of time up to 10 hours. Poly(A)$^+$ RNA was extracted from the cells and BKB_1 receptor mRNA levels were determined by Northern blot analysis (Figure 4.6A). Maximum mRNA levels were attained 2 hours post-TNF-α treatment resulting in a 30 fold increase in BKB_1 receptor mRNA compared to basal levels (Figure 4.6C). At 10 hours post-treatment the mRNA levels were still 7 fold higher than basal levels. In contrast to IL-1β and TNF-α, maximum mRNA levels were attained 1 hour post-LPS treatment (Figure 4.7). This corresponded to a 27 fold increase in BKB_1 receptor mRNA levels compared to basal levels (Figure 4.7B).

The expression analyses described above were carried out as separate studies. However, cultured cells may exhibit changes in their responsiveness as a function of culture time. Therefore, the fold induction of these inducing agents cannot be accurately compared.

Consequently, Northern blot analysis was performed on JTC-19 cells of equivalent passage number treated with either IL-1β, TNF-α or LPS for times corresponding to peak levels of BKB_1 receptor mRNA induction. As can be seen from Figure 4.8, the up-regulation of the 1.6 kb transcript is greatest following treatment with LPS. These data showed a 15 fold induction with IL-1β, a 19 fold induction with TNF-α and a 25 fold induction with LPS (Figure 4.8C). The fold induction for LPS and IL-1β are comparable to those observed in Figures 4.5 and 4.7. In contrast, the levels of BKB_1 receptor mRNA induced by TNF-α showed greater variability. The variability observed with TNF-α could result from the the cell culture time or the stability of the compound.

Previous experiments have not examined the biological stability of these inducing agents. Therefore, Northern blot analysis was carried out to determine whether the inducing agents were liable to degradation. IL-1β, TNF-α and LPS were administered for double the maximal up-regulation times. The reagents were then re-administered half way through this period. At the same time control samples were treated with the inducing agents for maximal induction times. $Poly(A)^+$ RNA was purified and BKB_1 receptor mRNA levels were analysed by Northern blot studies (Figure 4.9). Quantitative analysis in Figure 4.9B shows that a repeat application of IL-1β or LPS does not increase BKB_1 receptor mRNA levels above those determined for the maximal times determined in Figures 4.5 and 4.7. In contrast, re-administration of TNF-α over 4 hours resulted in higher BKB_1 receptor mRNA levels compared to the levels at 2 hours (the maximal time of induction determined from the timecourse in Figure 4.6). This result suggests that the TNF-α-induced BKB_1 receptor mRNA levels are affected by the stability of the mediator. Based on these observations, and on the established role of IL-

1β and LPS in mediating increased BKB_1 receptor responsiveness, these studies have focused on analysis of the IL-1β and LPS-mediated mechanisms of BKB_1 receptor up-regulation.

4.2.5 IL-1β does not mediate the major effects of LPS-induced BKB_1 receptor mRNA levels

To assess whether the LPS-induced BKB_1 receptor mRNA levels are mediated by the autocrine release of IL-1, the effects of IL-1Ra were analysed on IL-1β and LPS-induced BKB_1 receptor mRNA levels. Poly(A)$^+$ RNA was purified from control and treated JTC-19 cells in the absence or presence of IL-1Ra and BKB_1 receptor mRNA levels were determined by Northern blot analysis (Figure 4.10A). The results in Figure 4.10B show that IL-1Ra reduced the IL-1β-induced BKB_1 mRNA levels by 67%. In contrast, IL-1Ra reduced the LPS-mediated up-regulation of BKB_1 receptor mRNA levels by only 12%. Therefore, these data indicate that the major response of the LPS-induced increase in BKB_1 receptor mRNA is not due to the secondary release of IL-1. In addition, these data suggest that the majority of the LPS response does not use the IL-1 receptor to transduce signals in these cells.

4.2.6 IL-1β and LPS do not alter BKB_1 receptor mRNA stability

To examine whether IL-1β and LPS increase the BKB_1 receptor mRNA levels by altering mRNA stability, the effect of the transcription inhibitor, actinomycin D, was assessed. Cells were pre-treated with IL-1β for 2 hours or with LPS for 1 hour. This

pre-treatment was followed by the addition of actinomycin D to both treated and control cells. RNA was isolated from the cells at several time points following the addition of actinomycin D. Poly(A)$^+$ RNA was purified and BKB_1 receptor mRNA levels were measured by Northern blot analysis (Figures 4.11A and 4.12A). In order to visualise all the bands from control cells, 8 μg of poly(A)$^+$ RNA were used in Northern blot analysis. The graphs in Figures 4.11B and 4.12B represent the stability of the most abundant 1.6 kb transcript. Under control conditions the BKB_1 receptor mRNA transcript decayed with a half-life of 59 minutes ± 4 minutes. Following treatment with IL-1β or LPS the half life of the 1.6 kb transcript was not altered. Therefore, these data indicate that the mechanism of BKB_1 receptor mRNA up-regulation by LPS and IL-1β at the peak time of induction is independent of changes in mRNA stability. As reported previously, the 4 and 8 kb transcripts are only detectable following induction. Nevertheless, it is possible that low levels of expression of these transcripts may exist which are beyond the limits of detection by Northern blot analysis. To determine whether the larger transcripts exhibit equivalent stability to the 1.6 kb transcript following induction, the mRNA half-lives of the 4 and 8 kb transcripts were examined. Half-lives of the large transcripts were determined from the Northern blots of IL-1β-induced BKB_1 receptor mRNA expression presented in Figure 4.11. The graph in figure 4.13 represents the stability of the 4 and 8 kb transcripts. The half-lives of the 4 and 8 kb transcripts are both approximately 1 hour. In addition the half-lives of the large transcripts following LPS treatment of JTC-19 cells using the Northern blot analysis in Figure 4.12 were also approximately 1 hour (data not shown).

4.2.7 Pre-treatment with a transcription inhibitor prevents IL-1β and LPS-induced BKB_1 receptor gene expression

An experiment, in which actinomycin D was added prior to treatment with LPS or IL-1β, was carried out to determine the contribution of transcription mechanisms in the BKB_1 receptor up-regulation. This is the converse of the previously described experiment. mRNA was purified and Northern blot analysis was carried out to determine the BKB_1 receptor mRNA levels (Figure 4.14A). Quantitative analysis in Figure 4.14B shows that pre-treatment with actinomycin D completely abolished the IL-1β and LPS-mediated BKB_1 receptor mRNA up-regulation. Thus, these data indicate that an increase in transcription rather than mRNA stabilisation is primarily responsible for the IL-1β and LPS-mediated increase in BKB_1 receptor mRNA. Nuclear run-on experiments were performed to show a direct effect of IL-1β and LPS on the rate of transcription of the BKB_1 receptor gene. However, hybridisation signals were not detected. One explanation for the absence of a hybridisation signal is the low levels of expression of BKB_1 receptor mRNA, even under induced conditions, relative to the total RNA expression levels.

4.2.8 Pre-treatment with a translation inhibitor super-induces BKB_1 receptor mRNA levels

To determine if new protein synthesis is required for BKB_1 receptor mRNA transcriptional up-regulation, JTC-19 cells were pre-treated with the translation inhibitor, cycloheximide, prior to treatment with IL-1β (2 hours) or LPS (1 hour). mRNA was purified and BKB_1 receptor mRNA levels were analysed by Northern blot

studies (Figures 4.15). Figure 4.15B shows that in the presence of cycloheximide, the BKB_1 receptor mRNA levels from control, IL-1β and LPS-treated JTC-19 cells were increased relative to their counterparts in the absence of the translation inhibitor. A number of mechanisms have been attributed to the super-inducible effects of cycloheximide and are discussed in section 4.3. Failure of the translation inhibitor to block IL-1β and LPS-mediated increases in the BKB_1 receptor mRNA levels demonstrates that protein synthesis is not required for the up-regulation of mRNA levels.

4.2.9 BKB_1 receptor mRNA splice variants are expressed in control and treated rat tissues and JTC-19 cells

The biological role of the splice variants described in Chapter 3 (section 3.2.6.2) remains unclear. It is possible that differential expression of the variants in different tissues or under different cell conditions exists, or that the variants may have different stabilities or translation efficiencies. The transcripts generated using the alternative splice sites are indistinguishable by Northern blot analysis, therefore RT-PCR was performed to analyse the expression of these transcripts *in vivo* from control and LPS-treated rats and from LPS-treated JTC-19 cells. Primers were designed to amplify products of 170 bp and 129 bp corresponding to the upstream and downstream splice acceptor sites, respectively. Amplification products were separated on a 6% polyacrylamide gel and visualised according to Methods and Materials. Figure 4.16 shows that both splice variant cDNA products were expressed in control and LPS-treated rat heart, lung, kidney, bladder and uterus tissues. In addition, both PCR products were expressed in LPS-treated JTC-19 cells. These findings suggest that the

alternative splice variants are not differentially expressed in tissues from control and LPS-treated rats.

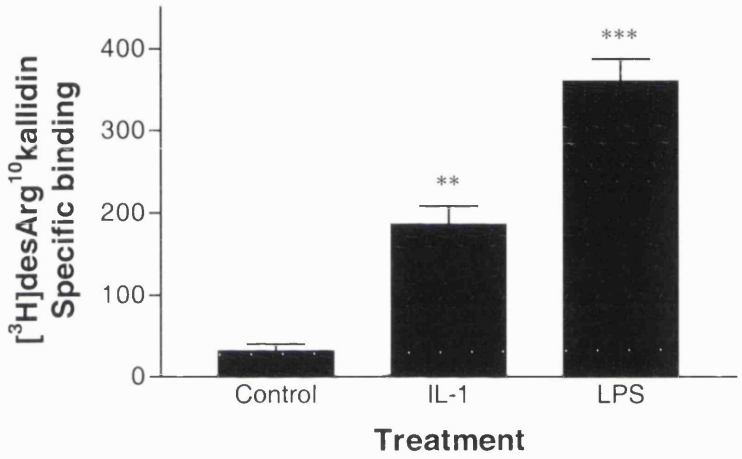


Figure 4.1 [^{3}H]desArg^{10}kallidin binding in control and treated JTC-19 cells.

Confluent JTC-19 cells grown in 24-well plates were cultured with medium only (control), or IL-1β-treated (10 ng/ml), or LPS-treated (10 μg/ml) for 4 hours prior to performing binding assays. Radioligand binding assays were carried out at room temperature as described in Methods and Materials. The specific binding was determined and plotted against the appropriate mediator. Each value is the mean ± SEM (n=3). * indicates $p < 0.05$, ** indicates $p < 0.01$ (compared with binding of control cells).

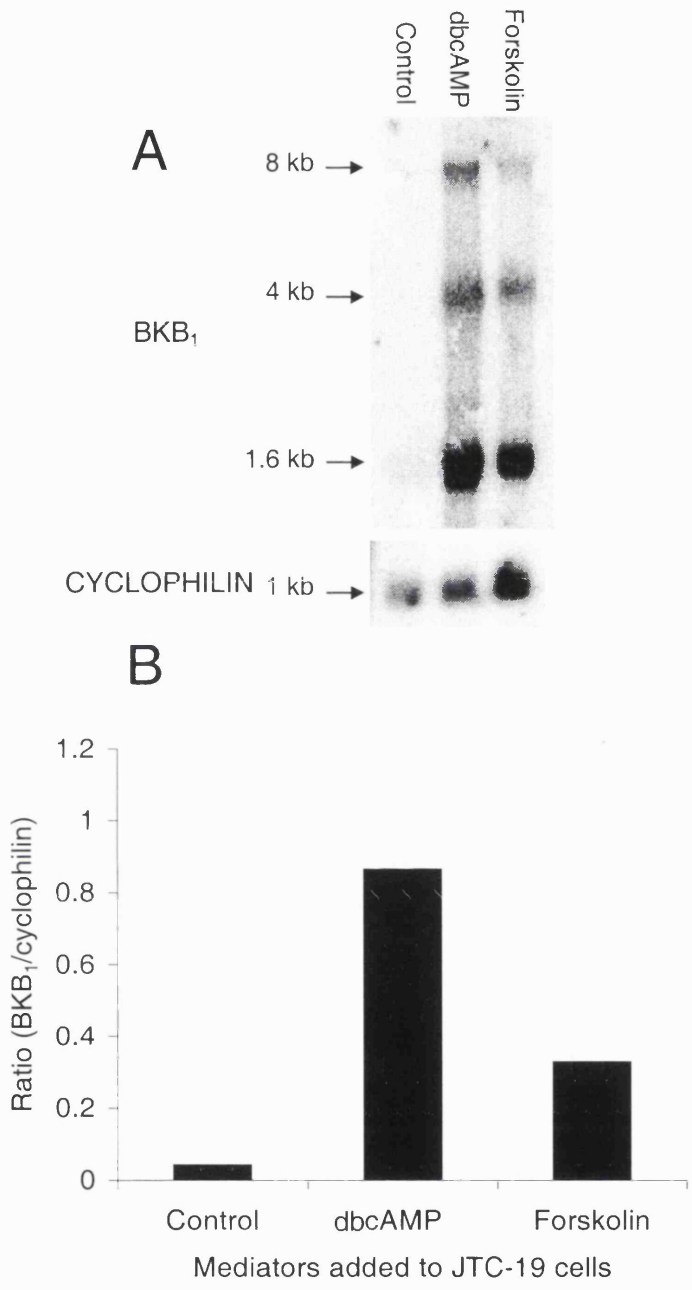


Figure 4.2 Effect of dbcAMP and forskolin on BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from control, and dbcAMP-treated (1 mM) or forskolin-treated (10 μM) JTC-19 cells for 2 hours. The same blot was stripped and re-probed with cyclophilin as shown in lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

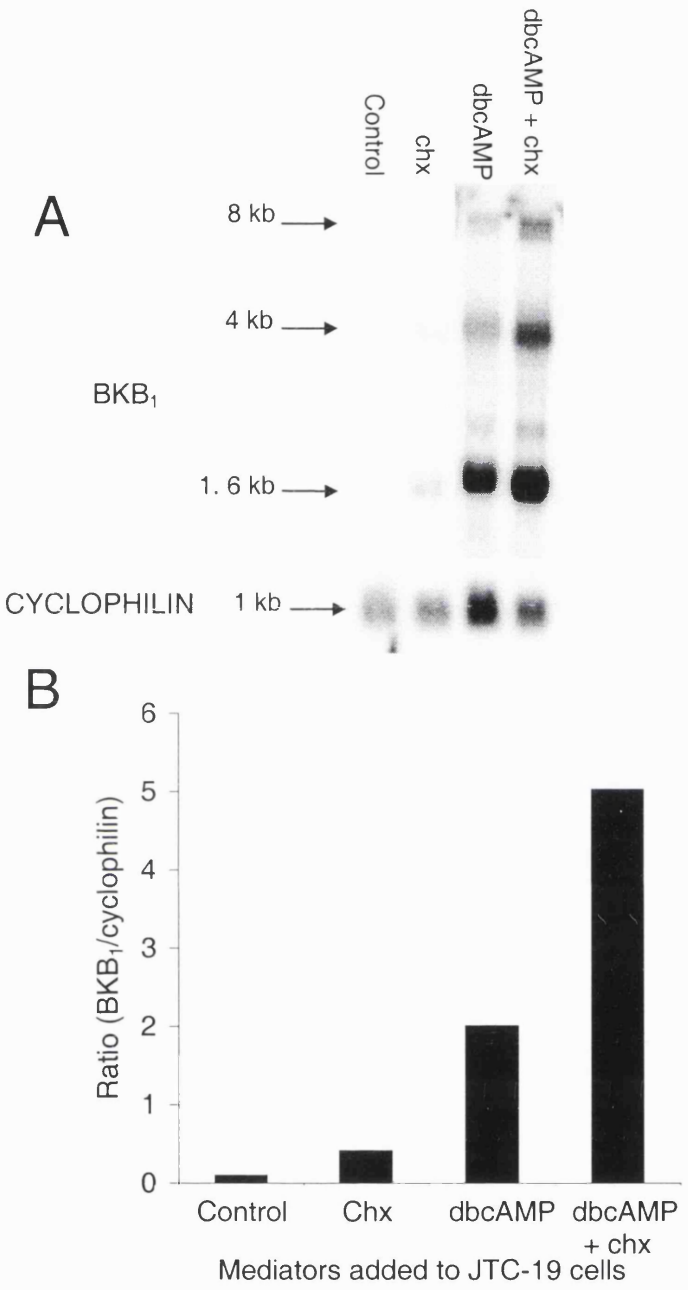


Figure 4.3 Effect of cycloheximide pre-treatment on dbcAMP-induced BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells pre-treated with cycloheximide (10 μg/ml) for 30 minutes, followed by treatment ± dbcAMP (1 mM) for 2 hours. The same blot was stripped and re-probed with cyclophilin as shown in the analysis in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

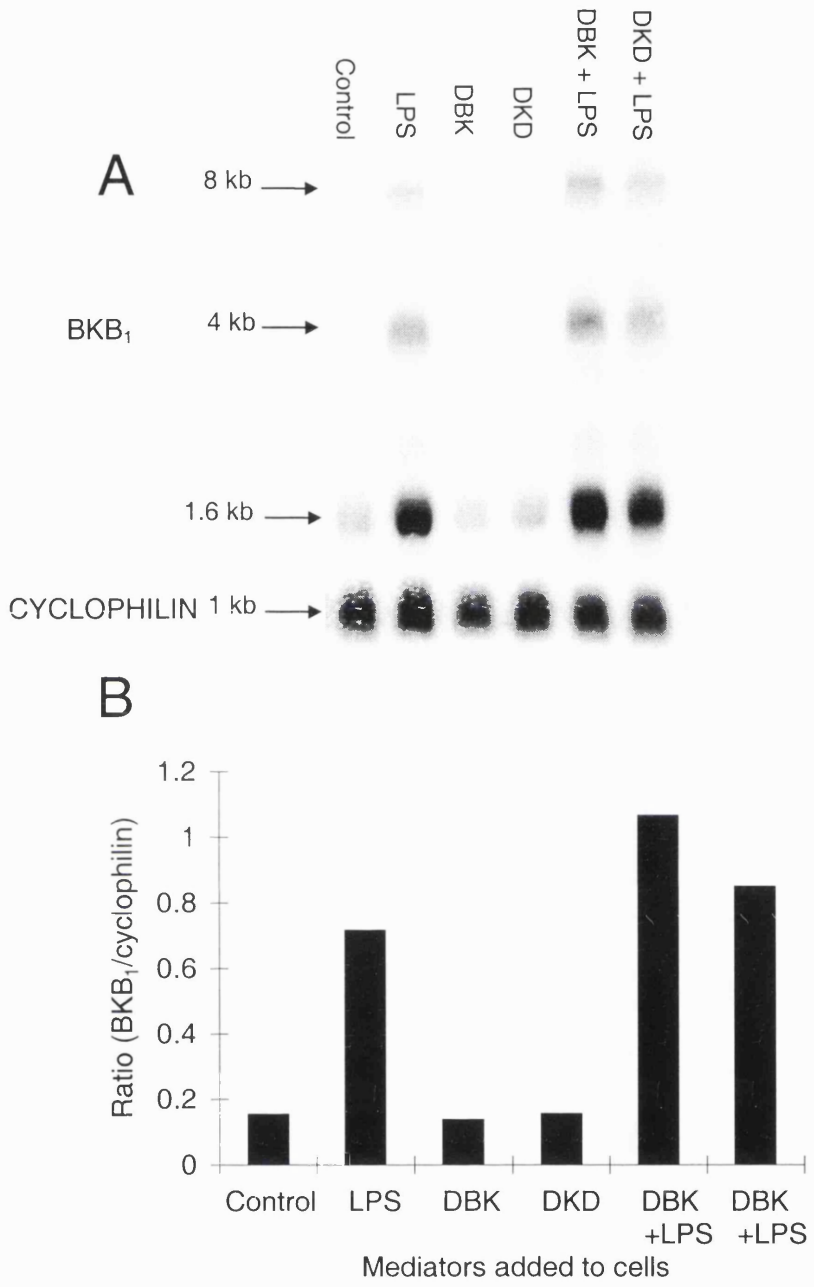


Figure 4.4 Effect of BKB_1 receptor agonists on BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells treated with the BKB_1 receptor agonists desArg^{9}BK (DBK) or desArg^{10}kallidin (DKD) (0.1 μM) in the absence or presence of LPS (10 μg/ml) for 6 hours. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the mediators added to JTC-19 cells.

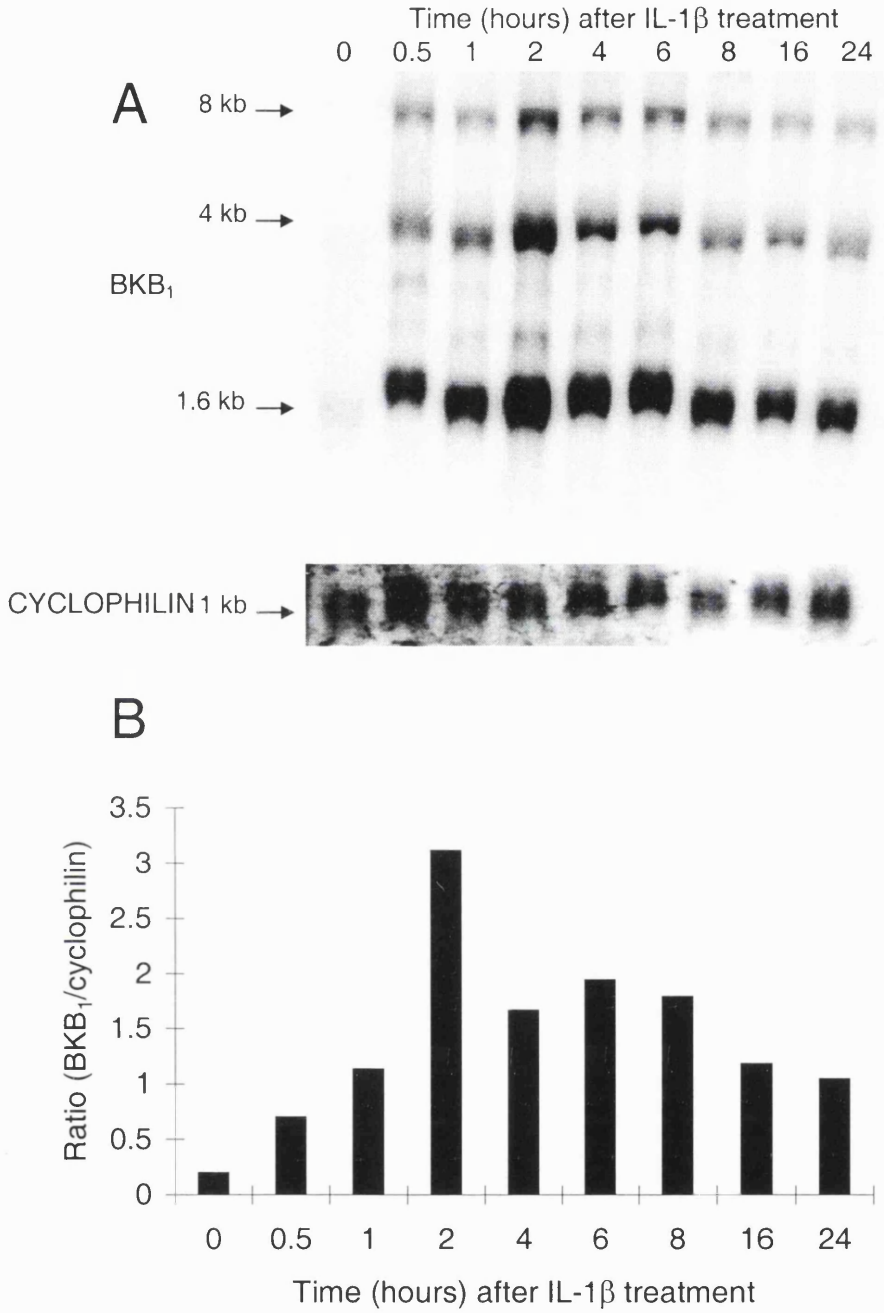


Figure 4.5 IL-1β-induced expression of BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells treated with IL-1β (10 ng/ml) for 0-24 hours. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the time course with IL-1β. The Northern blots shown are representative of two separate experiments.

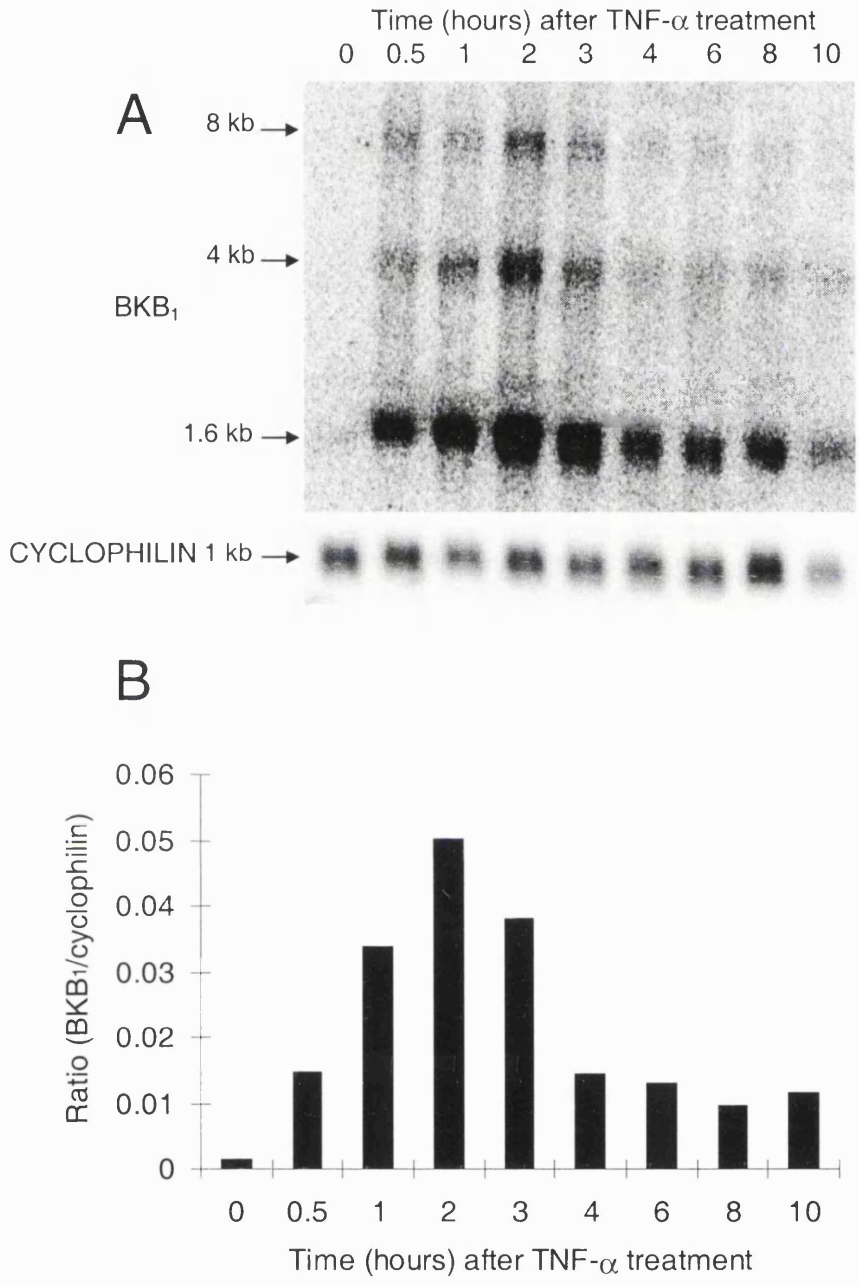


Figure 4.6 TNF-α-induced expression of BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells treated with TNF-α (10 ng/ml) for 0-10 hours. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the time course with TNF-α. The Northern blots shown are representative of two separate experiments.

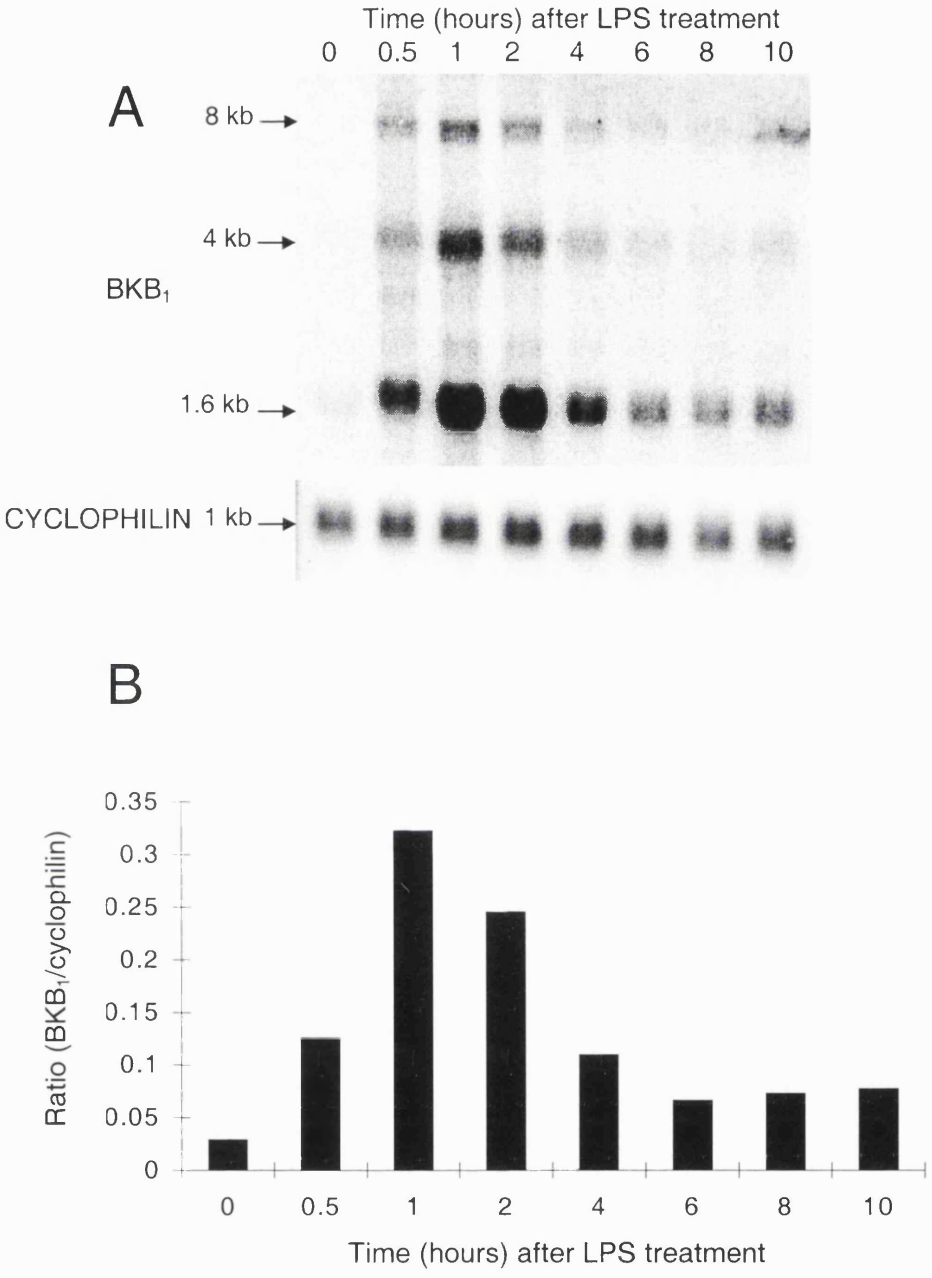


Figure 4.7 LPS-induced expression of BKB_1 receptor levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells treated with LPS (10 μg/ml) for 0-10 hours. The same blot was stripped and re-probed with cyclophilin as shown the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the time course with LPS. The Northern blots shown are representative of two separate experiments.

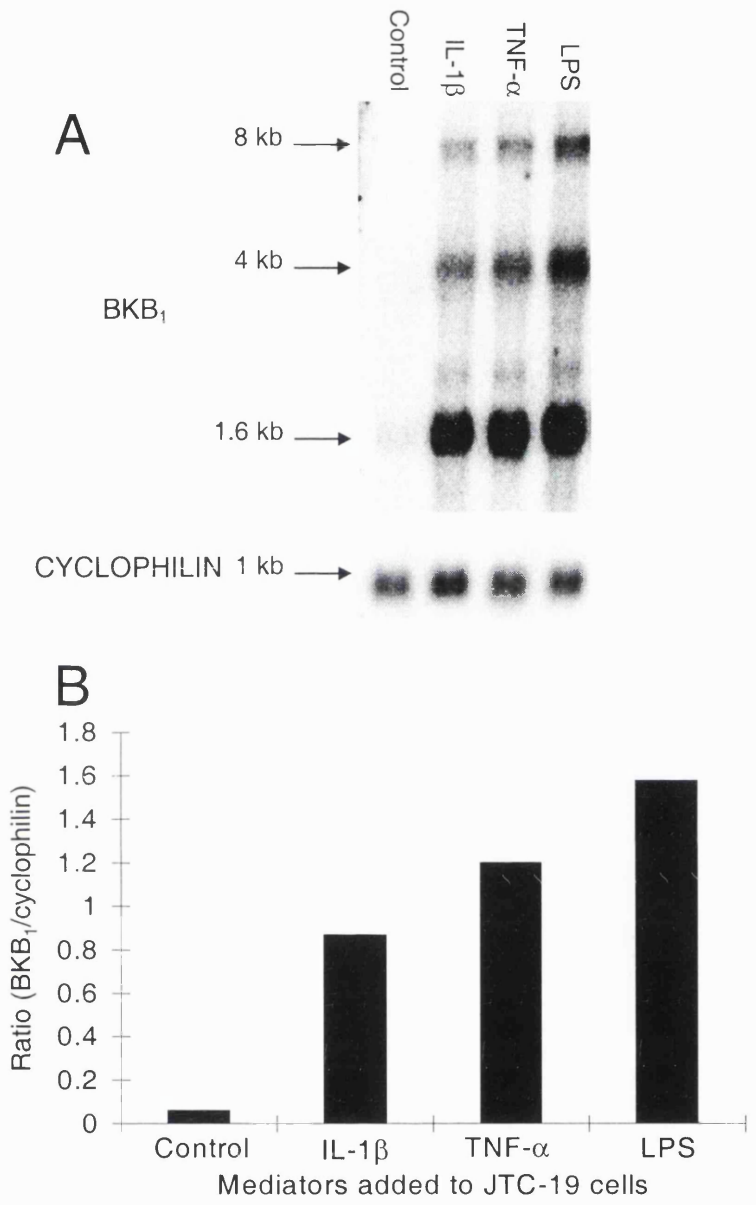


Figure 4.8 Comparison of IL-1β, TNF-α and LPS-induced expression of BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from IL-1β-treated (10 ng/ml), TNF-α-treated (10 ng/ml) or LPS-treated (10 μg/ml) JTC-19 cells for maximal induction times (2 hours for IL-1β and TNF-α, and 1 hour for LPS). The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

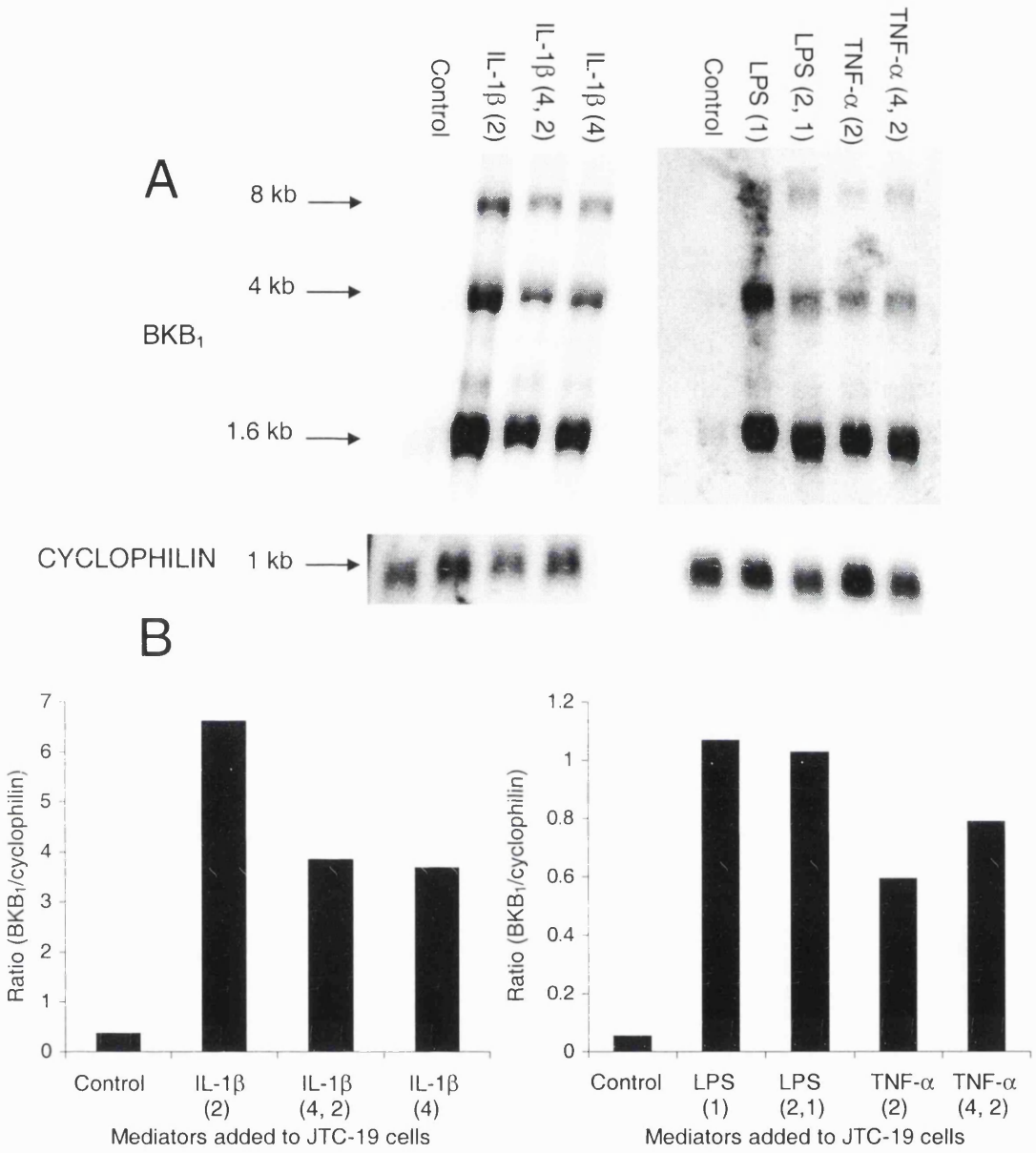


Figure 4.9 Analysis of BKB_1 receptor mRNA levels following re-administration of IL-1β, TNF-α or LPS.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from control, IL-1β-treated (10 ng/ml), TNF-α-treated (10 ng/ml) or LPS-treated (10 μg/ml) JTC-19 cells. The number in brackets refer to the time (hours) of treatment of one or two doses (applied at separate times) of the inducing agents. The same blots were stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

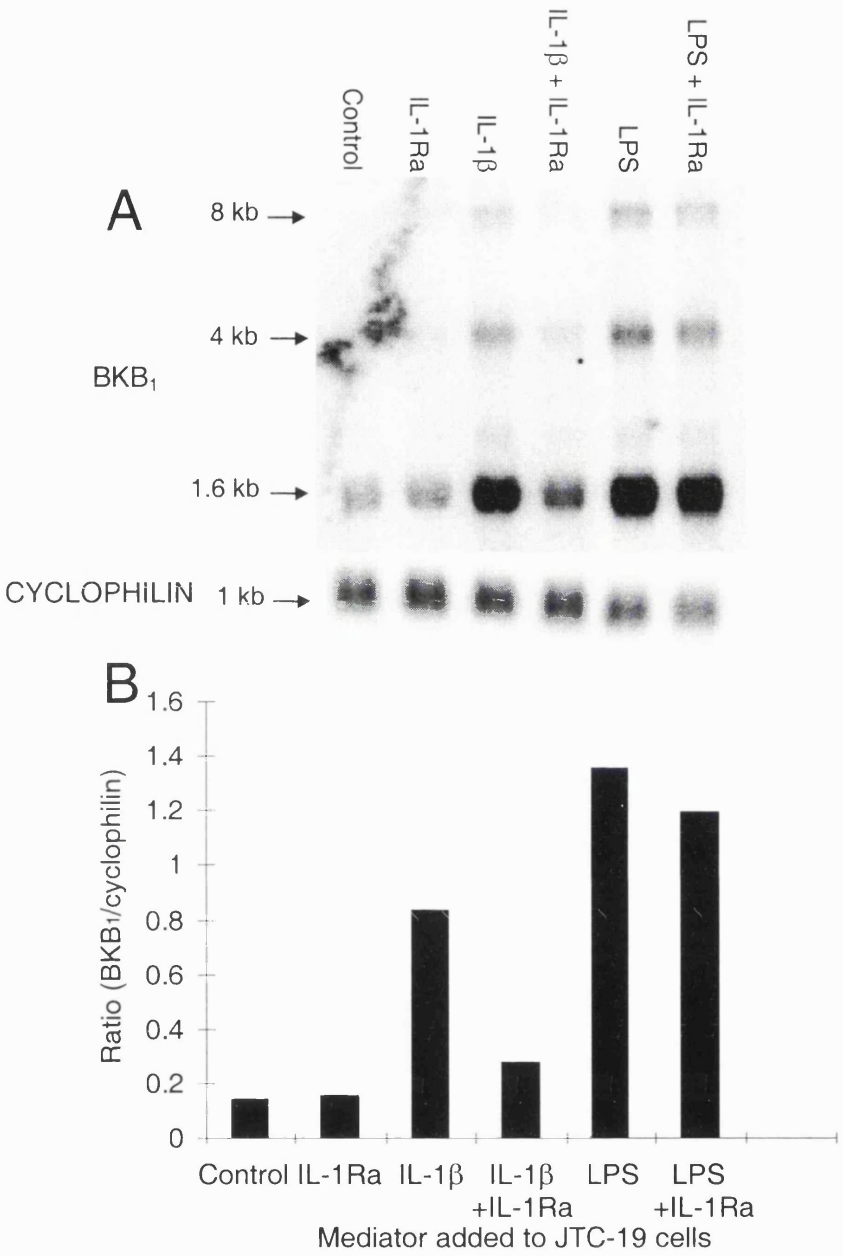
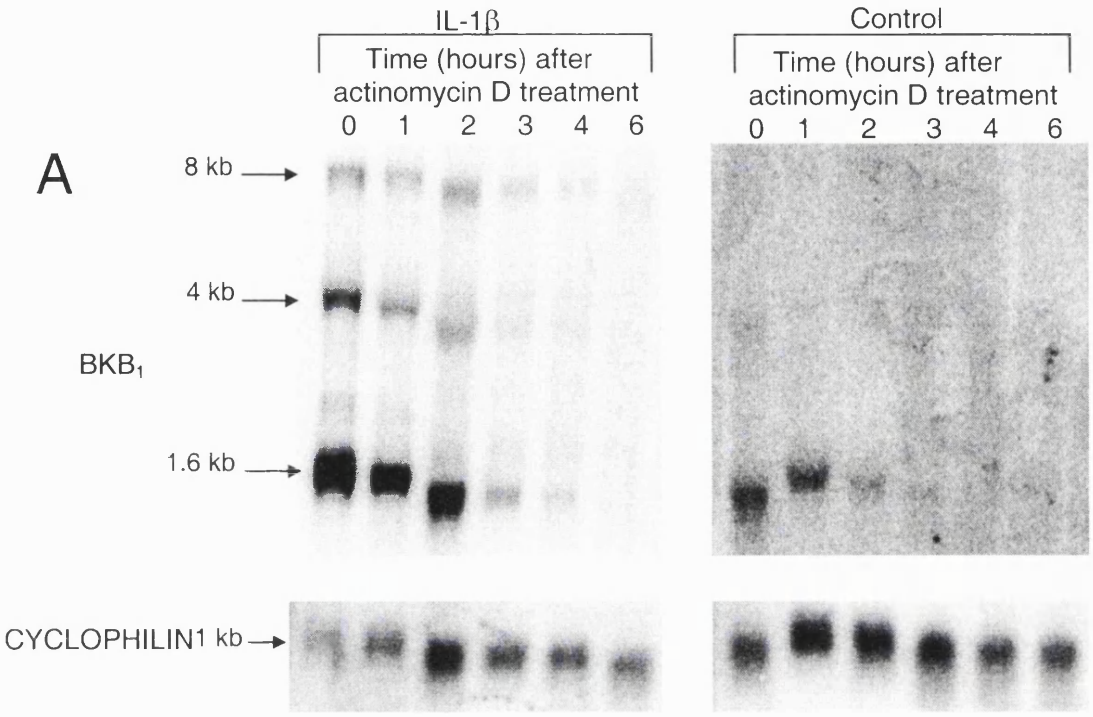


Figure 4.10 Effect of IL-1Ra on IL-1β and LPS-induced BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells pre-treated (30 minutes) in the absence or presence of IL-1Ra (50 ng/ml) before treatment with IL-1β (1 ng/ml) or LPS (1 μg/ml) for 1 hour. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

Figure 4.11 Analysis of BKB_1 receptor mRNA stability in control and IL-1β-treated JTC-19 cells.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells cultured in medium only (control) or pre-treated with IL-1β (10 ng/ml) for 2 hours. Following the addition of actinomycin D (5 μg/ml) to the cultures, mRNA was isolated at 0, 1, 2, 3, 4 and 6 hours. The blots were stripped and re-probed with cyclophilin as shown in the lower panels. In order to visualise all the bands, 8 μg of poly(A)$^+$ RNA from the control samples were loaded, while 3 μg of poly(A)$^+$ RNA from the IL-1β pre-treated samples were loaded. **B** The amount of radioactivity in the 1.6 kb transcript was quantified with the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted as a percentage of radioactivity at time 0 against the time course of addition of actinomycin D. The Northern blots are representative of two separate experiments.


A
IL-1β
Time (hours) after actinomycin D treatment
0 1 2 3 4 6
Control
Time (hours) after actinomycin D treatment
0 1 2 3 4 6
8 kb
4 kb
BKB1
1.6 kb
CYCLOPHILIN1 kb

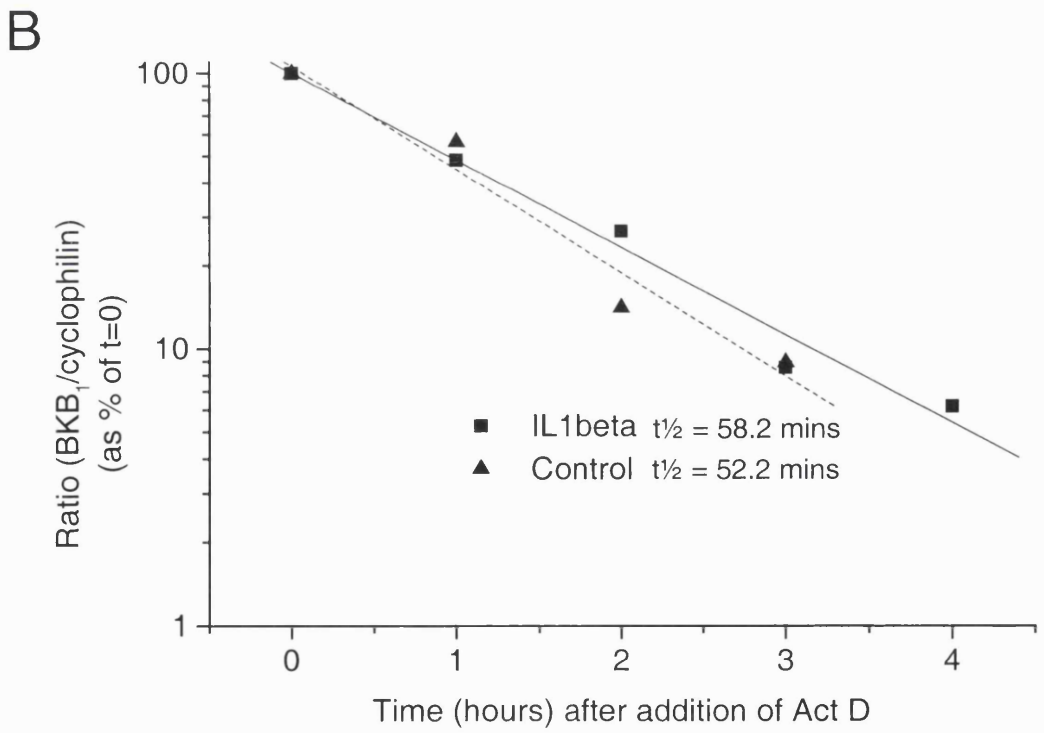
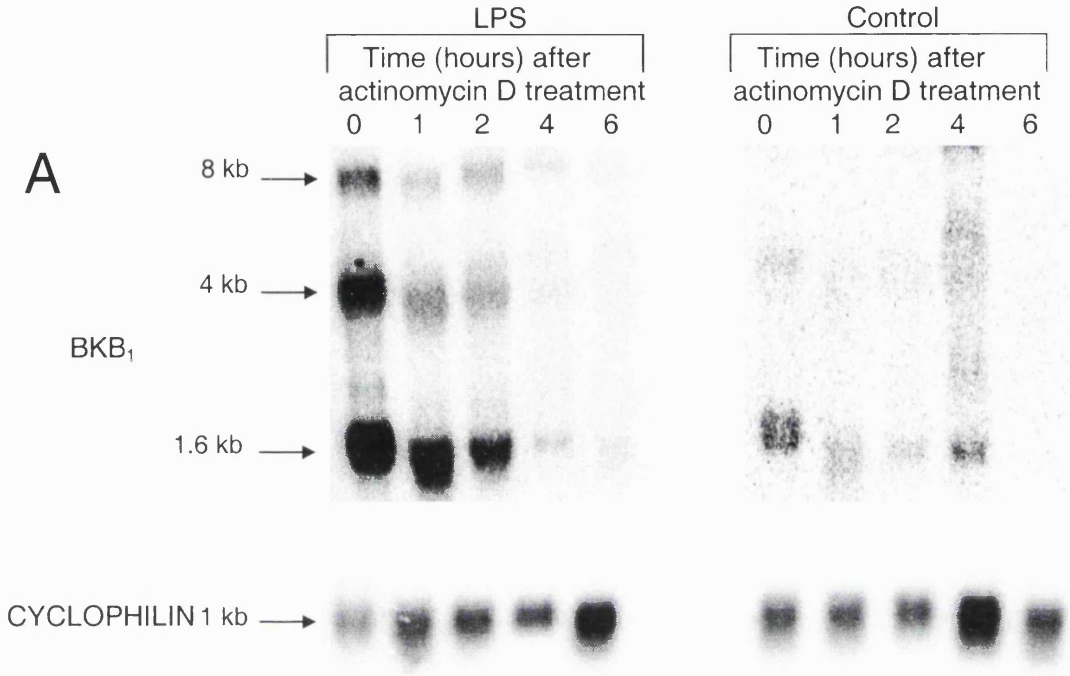
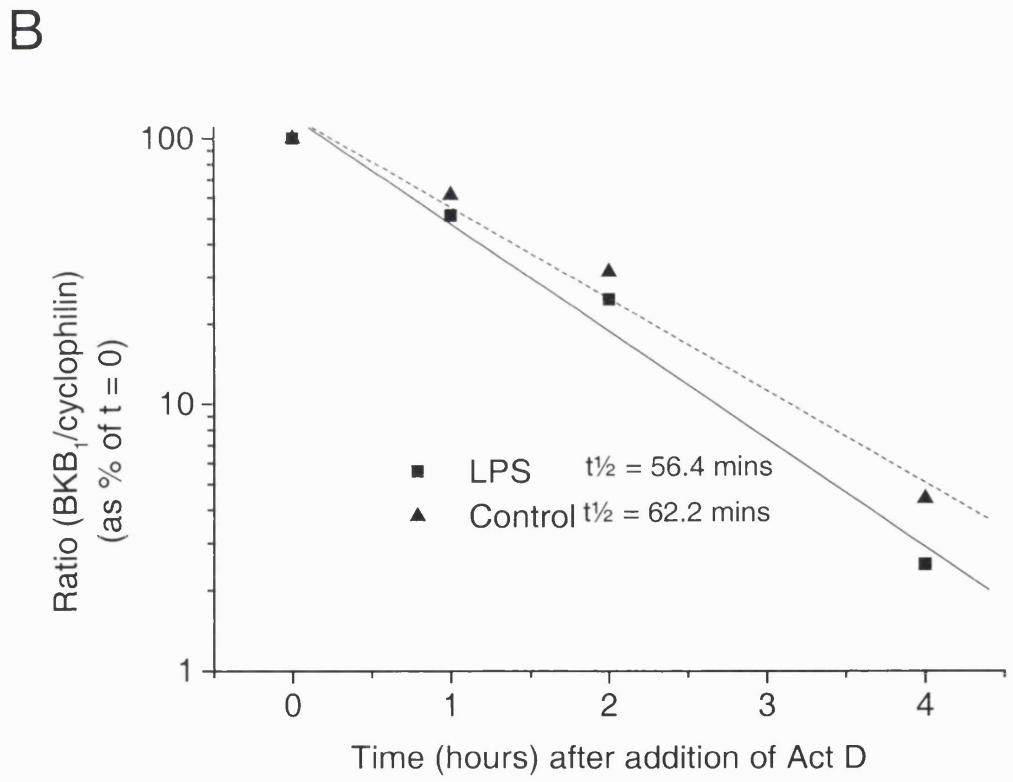

B
100
10
1
Ratio (BKB1/cyclophilin) (as % of t=0)
IL1beta t½ = 58.2 mins
Control t½ = 52.2 mins
0 1 2 3 4
Time (hours) after addition of Act D

Figure 4.12 Analysis of BKB_1 receptor mRNA stability in control and LPS-treated JTC-19 cells.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells cultured in medium only (control) or pre-treated with LPS (10 μg/ml) for 1 hour. Following the addition of actinomycin D (5 μg/ml) to the cultures, mRNA was isolated at 0, 1, 2, 4 and 6 hours. The same blots were stripped and re-probed with cyclophilin as shown in the lower panels. In order to visualise all the bands, 8 μg of poly(A)$^+$ RNA from the control samples were loaded, 3 μg of poly(A)$^+$ RNA from the LPS pre-treated samples were loaded. **B** The amount of radioactivity in the 1.6 kb transcript was quantified with the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted as a percentage of radioactivity at time 0 against the time course of addition of actinomycin D. The Northern blots shown are representative of two separate experiments.


A
LPS
Time (hours) after actinomycin D treatment
0 1 2 4 6
Control
Time (hours) after actinomycin D treatment
0 1 2 4 6
8 kb
4 kb
BKB1
1.6 kb
CYCLOPHILIN 1 kb


B
100
10
1
Ratio (BKB1/cyclophilin) (as % of t = 0)
LPS t½ = 56.4 mins
Control t½ = 62.2 mins
0 1 2 3 4
Time (hours) after addition of Act D

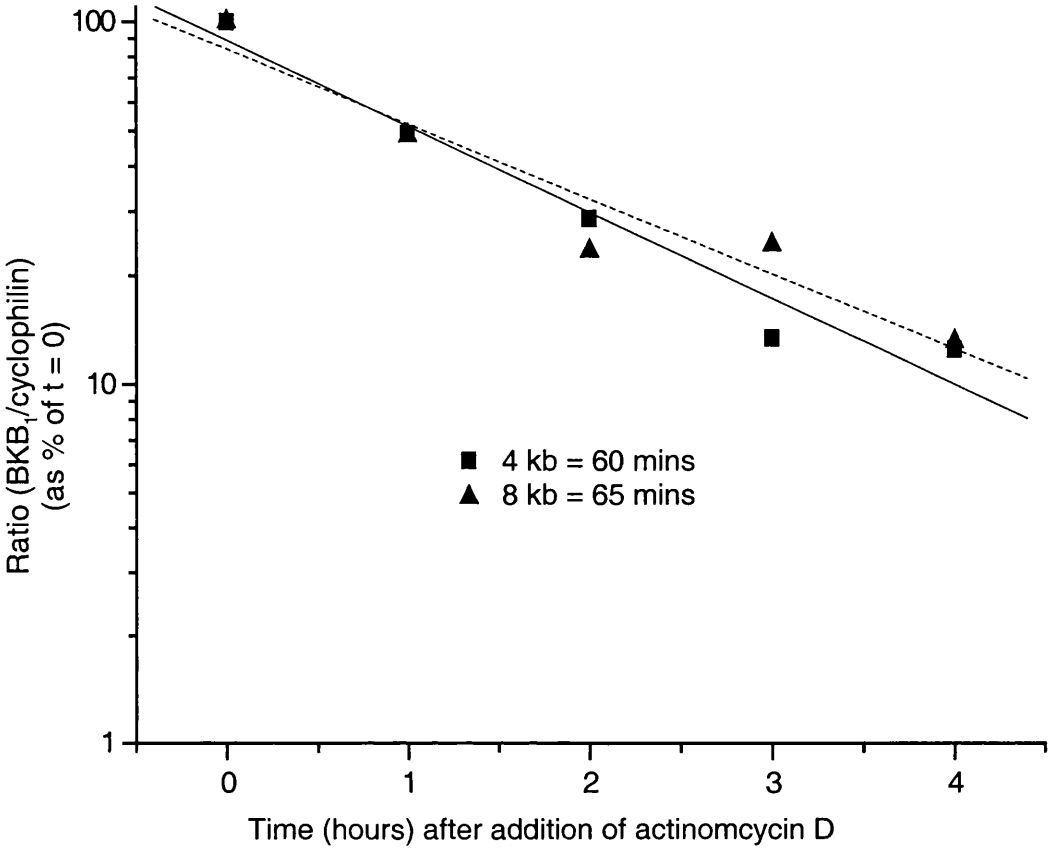


Figure 4.13 Analysis of BKB_1 receptor mRNA stabilities of the 4 and 8 kb transcripts in IL-1β-treated JTC-19 cells.

The amount of radioactivity in the 4 and 8 kb transcripts, shown in the Northern blot analysis in Figure 4.11, were quantified with the Storm phosphorimager and ImageQuaNT analysis software and plotted as a percentage of radioactivity at time 0. This data represent one of two experiments showing similar results.

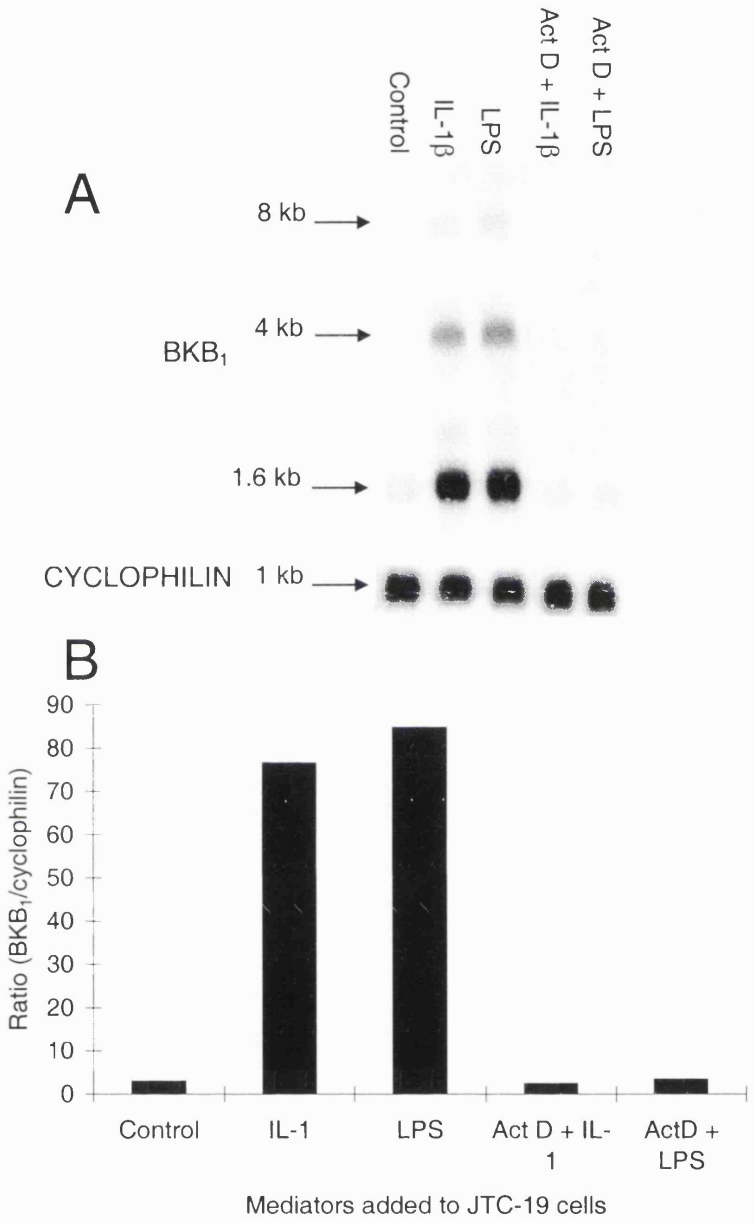


Figure 4.14 Effect of actinomycin D pre-treatment on IL-1β and LPS-induced BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 mRNA levels from JTC-19 cells pre-treated for 30 mins in the absence and presence of actinomycin D (Act D) (5 μg/ml) followed by treatment with IL-1β (10 ng/ml) for 2 hours or with LPS (10 μg/ml) for 1 hour. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

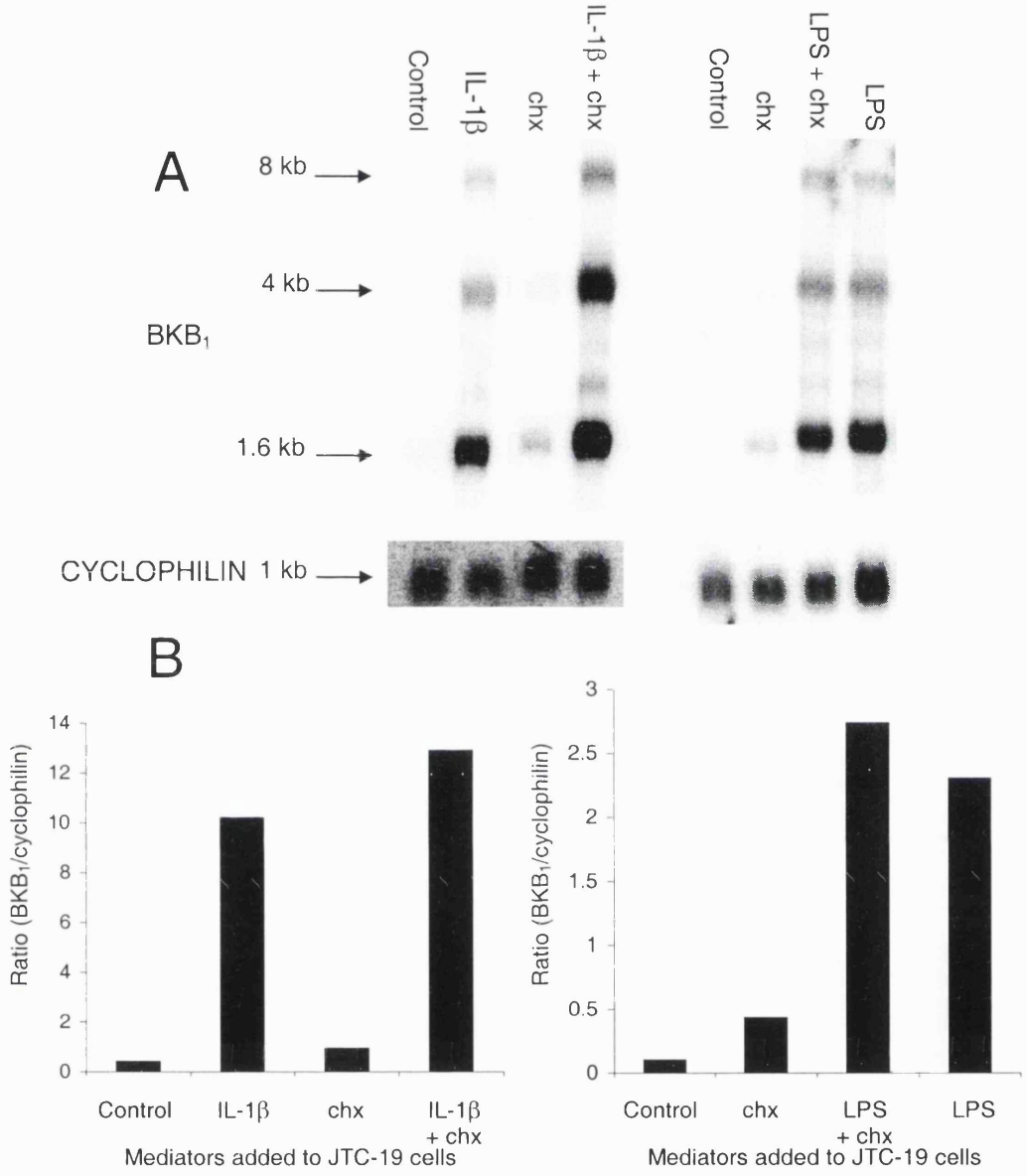


Figure 4.15 Effect of cycloheximide pre-treatment on IL-1β and LPS-induced BKB_1 receptor mRNA levels.

A Northern blots showing the BKB_1 receptor mRNA levels from JTC-19 cells. Cells were pre-treated with cycloheximide (chx) (10 μg/ml) for 30 minutes followed by treatment with IL-1β (10 ng/ml) for 2 hours, or treatment with LPS (10 mg/ml) for 1 hour. The same blots were stripped and re-probed with cyclophilin as shown in the lower panels. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediators. The Northern blots shown are representative of two separate experiments.

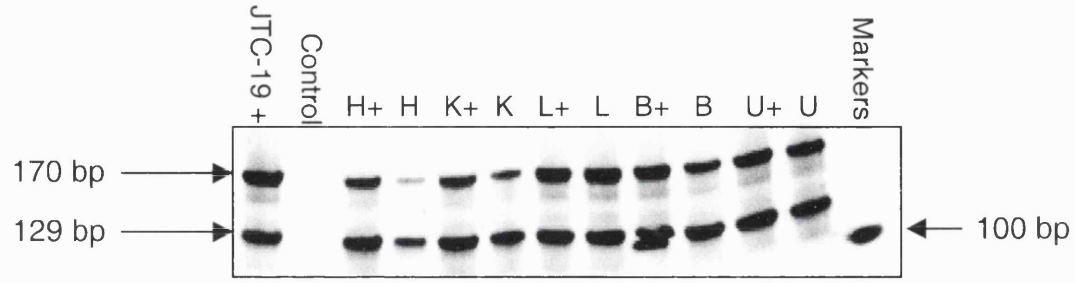


Figure 4.16 RT–PCR analysis of BKB_1 receptor mRNA splice variants.

Poly(A)$^+$ RNA was isolated from control and 4 hour LPS-treated (i.v. injection, 3 mg/kg) rat tissues and from 4 hour LPS-treated (10 μg/ml) JTC-19 cells. 500 ng of poly(A)$^+$ RNA was used for cDNA synthesis in the reverse transcription as described in Methods and Materials. PCR was conducted for 40 cycles using intron spanning primers. Amplification products were separated on a 4-20% polyacrylamide gel, stained with Vistra Green and visualised by fluorimager analysis. H= heart, K= kidney, L= lung, B= bladder, U= uterus. The presence of a + sign indicates LPS treatment.

4.3 Discussion

This chapter describes the inducing effects of IL-1β, TNF-α, LPS, the BKB_1 receptor agonists and cAMP activators on BKB_1 receptor mRNA levels in JTC-19 cells. Using IL-1β and LPS as inducing agents, the present study shows that BKB_1 receptor mRNA up-regulation is achieved through a transcriptional mechanism that is independent of new protein synthesis.

The data in this chapter show that the difference in BKB_1 receptor mRNA levels following treatment with LPS or IL-1β are paralleled by increases in the receptor binding in JTC-19 cells. The most obvious conclusion for this increase in BKB_1 receptor binding is an increase in receptor density; however, an increase in affinity of the receptor for the agonist cannot be eliminated. This interpretation is supported by radioligand binding studies carried out in rabbit smooth muscle cells (Galizzi *et al.*, 1994; Levesque *et al.*, 1995b; Schneck *et al.*, 1994) and IMR-90 cells (Bastian *et al.*, 1998: Phagoo et al., 1999), which show an increase in BKB_1 receptors with no change in ligand affinity following treatment with inducing agents.

The current study shows that dbcAMP, alone, is capable of inducing BKB_1 receptor mRNA levels with no involvement for protein synthesis in this pathway. However, the mechanisms of cAMP-induced BKB_1 receptor mRNA levels remains to be determined. The role of the CRE site in the rat BKB_1 receptor 5' flanking sequence and the signal transduction pathways activated by cAMP will be discussed in detail in Chapter 5.

The findings in this chapter show that the rat BKB_1 receptor agonists, $desArg^9BK$ and $desArg^{10}$kallidin increase the BKB_1 receptor mRNA levels only in the presence of LPS. Although the effects of BKB_1 receptor antagonists were not examined in this study, one can speculate that the effects are mediated by activation of the up-regulated BKB_1 receptors. Indeed, other studies have documented an autoregulatory mechanism for $desArg^{10}$kallidin via activation of the human BKB_1 receptors (Schanstra *et al.,* 1998; Phagoo *et al.,* 1999). In addition, in IMR-90 cells, BK stimulation of BKB_2 receptors increases BKB_1 receptor mRNA in a mechanism dependent on production of endogenous IL-1β (Phagoo et al., 1999). Thus, these mechanisms may contribute to the elevated levels of BKB_1 receptors at the sites of kinin and cytokine production *in vivo.*

The increase in physiological responses to BKB_1 receptor agonists in response to IL-1β and LPS treatment are well established (Regoli *et al.,*1981; Deblois *et al.,*1989, 1991; Bouthillier *et al.,*1987; Marceau *et al.,*1984; Campos *et al.,*1995, 1996; Davis and Perkins 1994b; Perkins and Kelly, 1994). In contrast, there are conflicting data regarding the role, if any, of TNF-α (Deblois *et al.,*1991; Davis and Perkins, 1994; Perkins and Kelly, 1994; Phagoo *et al.,*1997; Ni *et al.,* 1998a). This study shows that the cytokines IL-1β and TNF-α, and the endotoxin, LPS are capable of inducing BKB_1 receptor mRNA levels in JTC-19 cells in a time-dependent manner. The expression of BKB_1 receptor mRNA in JTC-19 cells was observed to peak at 2 hours for the cytokines and at 1 hour for LPS. In addition, LPS consistently exhibited a stronger inducing effect compared to IL-1β and TNF-α. Interestingly, the TNF-α-mediated up-regulation showed greater variability compared to LPS or IL-1β. Analysis of the stability of these inducing agents showed that TNF-α was more labile in comparison to IL-1β or LPS.

Therefore, one could speculate that the variability in the up-regulation of the BKB_1 receptor in response to TNF-α may be due to the instability of this reagent. The passage number of the cultured cells may also influence the effects of these mediators. Whether these effects contribute to the controversial role of TNF-α as an inducing agent for the BKB_1 receptor in other cell types remains to be determined. Molecular determination of the effects of IL-1Ra on the LPS-induced BKB_1 receptor mRNA levels show that the majority (88%) of the induction is mediated by a mechanism independent of the secondary release of IL-1. In addition, LPS utilises a distinct cell surface receptor to activate the signal transduction cascade leading to increased BKB_1 receptor mRNA levels.

At the onset of these studies the molecular mechanisms involved in the IL-1β and LPS-mediated up-regulation of the BKB_1 receptor had not been investigated. IL-1β and LPS-induced BKB_1 receptor mRNA levels show the presence of multiple transcripts of 1.6, 4 and 8 kb, expressed in the ratio 10:3.5:2. The most abundant transcript of 1.6 kb corresponds to use of the proximal poly(A) site. This is consistent with studies which show that in genes containing multiple poly(A) sites the upstream site, even if it is weaker, is preferentially selected (Batt *et al.*,1994; Schul *et al.*,1998). Two lines of evidence show that the increase in BKB_1 receptor mRNA levels following the addition of IL-1β or LPS are a consequence of transcriptional activation. Firstly, there is no change in the stability of the mRNA following the addition of IL-1β or LPS. Secondly, pre-treatment with a transcriptional inhibitor prevents the up-regulation of BKB_1 receptor mRNA. Attempts to measure the transcription rate directly by nuclear run-on analysis could not be achieved in these cells. Since this technique labels all the nascent

RNA transcripts, the most likely explanation for this result are the low relative levels of BKB_1 receptor expression. The data presented in this chapter show that the 1.6 kb BKB_1 transcript is rapidly degraded, thus allowing for rapid and precise adjustments of transcript levels following a change in the rate of transcription. However, the stability of the 4 and 8 kb transcripts could not be measured under control conditions due to non-detectable expression levels. Nevertheless, the equivalent mRNA half-lives and unchanged ratio of expression of these transcripts following induction, compared to the 1.6 kb transcript, indicates that they exhibit co-ordinated regulation. Moreover, the equivalent half-lives of these transcripts suggests that elements involved in their decay, if any, are located within the shared 1.6 kb sequence. A number of sequence elements have been identified that determine mRNA stability (Sachs, 1993). In particular, a number of genes contain the instability motif AUUUA within the 3' UTR (Shaw and Kamen, 1986), however this motif is absent from the 3' UTR of the 1.6 kb BKB_1 receptor transcript. These data have examined the mechanisms of regulation of the rat BKB_1 receptor gene at the point of maximal induction. Thus, the contribution of post-transcriptional mechanisms at other stages in the timecourse of up-regulation cannot be excluded.

Recent studies have examined the role of transcriptional and post-transcriptional mechanisms in the up-regulation of the human BKB_1 receptor gene in IMR-90 cells (Zhou *et al.*,1998; Schanstra *et al.*,1998). Zhou and co-workers (1998) showed that a combination of transcriptional and post-transcriptional mechanisms contribute to the IL-1β-mediated up-regulation. In contrast, Schanstra *et al.*, (1998) showed an increase in transcriptional activation of the human BKB_1 receptor gene in response to IL-1β and

desArg^{10}kallidin, with no change in mRNA stability. It is possible that differences in the induction time may explain these discrepancies.

Data presented in this study show that the protein synthesis inhibitor, cycloheximide, super-induces the basal and dbcAMP, IL-1β and LPS-induced BKB_1 receptor mRNA levels. These findings are consistent with an increased responsiveness to BKB_1 receptor agonists in rabbits following a pulse exposure to cycloheximide (Deblois *et al.*,1991). More recently, studies in IMR-90 cells and rat VSMCs have demonstrated a similar super-induction of BKB_1 receptor mRNA levels (Zhou *et al.*,1998; Ni *et al.*,1998a). Several mechanisms can account for the effects of these inhibitors. A post-transcriptional mechanism may either block the production of a rapidly generated protein, which under normal conditions maintains the mRNA at low levels, or it may prevent translation-dependent degradation of the mRNA (Shaw and Kamen, 1986). However, other studies have shown that the effects of cycloheximide can be attributable to transcriptional mechanisms (Newton *et al.*,1996, 1997c; Faggioli *et al.*, 1997). Zhou and co-workers (1998) have shown that cycloheximide-mediated up-regulation of the human BKB_1 receptor occurs as a result of increased mRNA stability with no change in transcription. However, the mechanism responsible for the cycloheximide-mediated up-regulation of the rat BKB_1 receptor gene remains to be determined. In addition, the increase in IL-1β and LPS-mediated BKB_1 receptor mRNA levels in the presence of cycloheximide shows that new protein synthesis is not required for the transcriptional increase in BKB_1 receptor gene expression. These data suggest that the BKB_1 receptor is an early response gene for dbcAMP, IL-1β and LPS.

Alternative 3' processing and alternative splice sites of the rat BKB_1 receptor generate multiple mRNA transcripts. The BKB_1 receptor alternative splice variants were expressed in a number of tissues from control and LPS-treated rats. Furthermore, a recent study by Ni *et al.*,(1998b) confirms the presence of the alternative splice variants in a number of LPS-treated rat tissues. A differential role, if any, for the 4 and 8 kb BKB_1 receptor mRNA transcripts compared to the 1.6 kb transcript remains elusive. Multiple protein factors are required for the formation of mRNA 3' ends (Zhao *et al.*,1999). Studies have shown that RNA polymerase II, through the conserved carboxyl terminal domain of its largest subunit, has properties which make it an authentic cleavage factor (Hirose and Manley, 1998; McCracken *et al.*,1997). An interesting corollary to these findings is that the efficiency of 3' processing might vary depending on the strength of the promoter and how well the polyadenylation factors are loaded onto the carboxyl terminal domain. Therefore, it could be hypothesised that following the increase in transcription of the rat BKB_1 receptor gene, there may be a subsequent change in the efficiency of 3' mRNA processing, resulting in the generation of the 4 and 8 kb transcripts. Moreover, the efficiency of transcription termination correlates with the strength of the polyadenylation signal (Edwalds-Gilbert *et al.*,1993; Greger and Proudfoot,1998). Consequently many regulated poly(A) sites are weak, either as a result of non-consensus signal sequences or due to an unfavourable sequence context (Edwalds-Gilbert *et al.*,1997). Interestingly, a number of studies show that sequences in the 3' UTR can effect the efficiency of translation (Jackson and Standart, 1990; Lu and Menon, 1996; Miyamoto *et al.*, 1996). Whether the alternative 3' UTRs in the rat BKB_1 receptor mRNA transcripts are differentially translated remains to be determined.

In conclusion, the data in this Chapter establish a time-dependent increase in BKB_1 receptor mRNA levels in JTC-19 cells following the addition of cAMP activators, IL-1β, TNF-α or LPS. These studies show that the major effects of the LPS-induced BKB_1 receptor mRNA levels in these cells occurs by a mechanism independent of endogenous IL-1 release. Furthermore, analysis of the mechanisms of BKB_1 receptor up-regulation following the addition of IL-1β and LPS shows that at the peak time of induction there is an increase in transcription with no change in mRNA stability. Analysis of the mechanisms of transcriptional regulation of the BKB_1 receptor gene are described in the following chapter.

Analysis of the BKB_1 receptor mRNA levels in the presence of actinomycin suggests that a transcriptional mechanism increases the BKB_1 receptor mRNA levels following the addition of IL-1β or LPS. However, due to close coupling of the related processes of transcription, termination and translation of mRNA, inhibition of transcription by actinomycin D may indirectly effect mechanisms of post-transcriptional regulation. Thus, to unambiguously distinguish the relative contributions of transcriptional and post-transcriptional mechanisms to regulation of the inducible expression of BKB_1 receptor mRNA, direct measurement of the transcription rate is required.

Chapter 5

Studies on the mechanisms of rat BKB_1 receptor transcriptional regulation

5.1 Introduction

Studies in the previous chapter have shown that both IL-1β and LPS increase BKB_1 receptor mRNA levels up to 30 fold, and that this up-regulation was primarily due to an increase in transcriptional activity. The first phase in transcriptional regulation is the processing of extracellular and/or intracellular signals, such that they lead to activation of transcription factors that bind to DNA. These DNA bound factors influence transcription through direct or indirect protein-protein interactions with components of the general transcription machinery, thereby resulting in an enhanced recruitment or stabilisation of the transcription complex to the template (Latchman, 1995). IL-1β and LPS have been shown to activate a large number of intracellular cascades, with most of the IL-1 and LPS induced immune and inflammatory genes regulated by NF-κB activation (reviewed in O'Neill, 1995; Sweet and Hume, 1996; Bankers-Fulbright *et al.*, 1996).

Whilst these studies were in progress a number of conflicting reports describing the mechanisms of transcriptional regulation of the human BKB_1 receptor gene were published (Ni *et al.,* 1998a; Zhou *et al.,* 1998; Yang *et al.,* 1998a; Schanstra *et al.,* 1998; Marceau *et al.,* 1998). Two separate NF-κB-like sequences were identified located at −55 to −64 bp (Ni *et al.,* 1998a) and −1115 to −1127 bp (Schanstra *et al.,* 1998) in the 5' flanking region. These sites were shown to confer IL-1β inducibility of reporter gene expression. Furthermore, reduced IL-1β-mediated BKB_1 receptor gene expression in the presence of an inhibitor of NF-κB activation, PDTC, verified a role for NF-κB. (Schanstra *et al.,* 1998). In addition, recent analysis of the rat BKB_1 receptor 5' flanking sequence showed that a 79 bp fragment spanning the conserved downstream NF-κB-like site increased reporter gene expression by 2.5 fold following the addition of LPS (Ni *et al.,* 1998b). In contrast, Zhou *et al.,* (1998) showed that PDTC failed to block the IL-1β-mediated up-regulation in IMR-90 cells. Moreover, other studies have shown a lack of inducibility using human BKB_1 receptor-directed promoter constructs spanning the NF-κB-like sites (Yang *et al.,* 1998a; Marceau *et al.,* 1998). Other putative transcription factor binding sites have been identified in the 5' flanking sequence including a conserved CRE site in the human and rat BKB_1 receptor genes (Ni *et al.,* 1998a,b). The presence of this site is consistent with the dbcAMP-mediated up-regulation of BKB_1 receptor mRNA levels shown in Chapter 4. Ni *et al.,* (1998a) have shown that the CRE site is important for basal, and LPS and IL-1β-induced transcriptional regulation of reporter gene expression. In addition, MAPK inhibitors and protein tyrosine kinase inhibitors have also been effective in reducing the IL-1β-mediated up-regulation of the human BKB_1 receptor mRNA levels (Larrivee *et al.,* 1998; Zhou *et al.,* 1998). Thus, these findings suggest that IL-1β and

LPS may increase the BKB_1 receptor through more than one transcriptional mechanism.

This chapter describes some of the mechansims of transcriptional regulation of the rat BKB_1 receptor gene. Specific inhibitors and activators of signal transduction pathways, were used to investigate the role of signalling pathways in the BKB_1 receptor up-regulation. Promoter activity of the 5' flanking sequences was determined by analysis of transiently transfected reporter gene constructs. In addition, the ability of transcription factors to bind to upstream elements were investigated using EMSAs.

5.2 Results

5.2.1 NF-κB is involved in the iL-1β, and LPS-induced BKB_1 receptor expression but not in the dbcAMP-induced BKB_1 receptor expression

The role of NF-κB activation in the BKB_1 receptor up-regulation was studied using a dominant-negative mutant of the IκB-α protein. In the last step of the NF-κB/IκB degradation cascade, phosphorylation of two amino-terminal sites in IκB-α results in dissociation and degradation of the inhibitory protein (Baeuerle and Baltimore, 1996). A form of IκB-α with the amino terminus deleted (IκB-mut) cannot be phosphorylated and therefore retains NF-κB in an inactive form within the cytoplasm (Brockman *et al.*, 1995). This construct was used to examine the role of the NF-κB signalling pathway in the IL-1β, LPS and dbcAMP-mediated up-regulation of the BKB_1 receptor gene. IκB-mut was co-transfected with pMCI-Neo. To eliminate the residual activity of NF-κB in untransfected cells, a stable clone was selected as described in Methods and Materials. Immunoprecipitation and immunoblotting analysis demonstrated that this FLAG epitope-tagged IκB-mut was expressed in the cytoplasm (Figure 5.1). The IκB-mut cell line and control JTC-19 cells were treated with LPS, IL-1β or dbcAMP followed by purification of poly(A)$^+$ RNA and Northern blot studies (Figure 5.2). Figure 5.2B shows that in the presence of NF-κB inactivation, the IL-1β-mediated up-regulation was reduced by 70% and the LPS-mediated up-regulation was reduced by 30%, compared to control treated cells. These findings suggest that the NF-κB activation pathway serves a larger role in the IL-1β-mediated up-regulation of the BKB_1 receptor compared to the LPS-mediated induction. In comparison, the increase in BKB_1 receptor mRNA levels following treatment with dbcAMP were unchanged in

the presence of NF-κB inactivation (Figure 5.2). Thus, these data show that an NF-κB activation pathway is involved in the LPS and IL-1β-mediated up-regulation of the rat BKB_1 receptor but not in the dbcAMP activated signalling pathway of induction.

5.2.2 PI 3-kinase is involved in the IL-1β and LPS-induced BKB_1 receptor expression

The studies above suggest that IL-1β and LPS activate multiple signalling pathways, including the NF-κB/IκB degradation pathway, resulting in BKB_1 receptor up-regulation. To further characterise the components of these signalling pathways the effects of protein kinase inhibitors were examined. Studies show that IL-1 and LPS can activate the MAPK cascades (O'Neill *et al.,* 1998; Paul *et al.,* 1997; Kyriakis *et al.,* 1996; Saklatvala *et al.,* 1999; Hall *et al.,* 1999; Carter *et al.,* 1999) and PI 3-kinase (Sizemore *et al.,* 1999; Marmiroli *et al.,* 1998; Reddy *et al.,* 1997; Donaldson *et al.,* 1996). Initial studies with MAPK inhibitors require further investigation since the dilutent, dimethyl sulphoxide (DMSO), was found to increase BKB_1 receptor mRNA levels. The role of PI 3-kinase in the IL-1β and LPS-mediated up-regulation of BKB_1 receptor mRNA levels was examined using the specific PI 3-kinase inhibitor, LY294002 (Vlahos *et al.,* 1994). JTC-19 cells were pre-treated with LY294002 for 15 minutes followed by treatment with IL-1β or LPS for 1 hour. Poly(A)$^+$ RNA was purified and BKB_1 receptor mRNA levels were measured by Northern blot analysis (Figure 5.3). As shown in Figure 5.3B, LY294002 inhibited the IL-1β-induced BKB_1 receptor mRNA levels by 65% and the LPS-induced BKB_1 receptor mRNA levels by 56% in response to LPS, with no effect on basal receptor levels. These results show,

for the first time, that activation of PI 3-kinase by IL-1β or LPS can increase BKB_1 receptor mRNA levels.

5.2.3 Alignment of the 5' flanking sequence of the rat and human BKB_1 receptor genes

To determine whether any consensus transcription factor binding sites are present within the 5' flanking sequence which might account for the observations of induction of the BKB_1 receptor gene, the 5' flanking region was analysed. The 5' flanking sequence from –2839 bp to +1 bp relative to the TIS was sequenced in its entirety (Appendix I). As noted previously, exon 1 of the rat and human BKB_1 receptor genes exhibit 65% identity suggesting they are equivalent. Therefore, the rat sequence from –1201 to +88 bp was aligned with the human 5' flanking sequence (Bachvarov *et al.*, 1996; Yang and Polgar, 1996) to determine the identity within this region (Figure 5.4). Alignment of the rat and human BKB_1 receptor gene sequences from –2839 to +88 bp exhibit 54% identity. The highest region of identity was observed in the sequence located –457 to +88 bp, corresponding to 66% identity. A number of reports have described elements within the 5' flanking sequence important for regulation of the human BKB_1 receptor gene (Ni *et al.*, 1998a; Schanstra *et al.*, 1998; Yang *et al.*, 1998a). These elements are underlined in Figure 5.4 and include a TATA box, a CRE site, two AP-1 sites, two polyomavirus enhancer A-binding protein-3 (PEA-3) sites, an NF-1-like site and two NF-κB-like sites. In addition, an AP-1 site, CRE site and NF-κB-like site located within the first 100 bp of 5' flanking sequence in the rat BKB_1 receptor described in the study by Ni and co-workers (1998b) are

highlighted in Figure 5.4. A search for transcription factor binding consensus sequences in the rat 5' flanking sequence using SignalScan (Prestridge, 1991) revealed the presence of other putative transcription factor binding sites. These include a non-classical TATA box, two SV40 protein-1 (SP-1) sites, a CCAAT/enhancer binding protein (C/EBP) site and an NF-κB site. A 174 bp stretch of pyrimidines between −1077 to −903 bp is also shown. Analysis of the shared putative transcription factor binding sites between the rat and human BKB_1 receptor genes, reveal a conserved TATA box, a CRE site, a NF-κB-like, a C/EBP site, a PEA-3 site and two AP-1 sites.

Alignment of the rat 5' flanking sequences from this study with the equivalent sequence reported by Ni *et al.,* (1998b) from −500 to +1 bp showed that they diverged from −412 bp upstream of the TIS (Figure 5.5). The reason for this discrepancy is unclear. However, alignment of the alternative rat sequences with the equivalent sequence of the human BKB_1 receptor revealed that the rat sequence reported in this study exhibits 85% identity with the human sequence. In contrast, the sequence reported by Ni and co-workers (1998b) shows only 42% identity with the human sequence. Thus, these data suggest that the rat 5' regulatory sequence reported in this study is the true orthologue of the human BKB_1 receptor gene.

5.2.4 Identification of positive and negative regulatory elements in the 5' flanking region of the BKB_1 receptor gene

To delineate the sequences essential for transcription of the BKB_1 receptor gene and identify important regulatory domains, 2.9 kb fragment from the 5' flanking sequence, including all of exon 1 was cloned upstream of the firefly luciferase reporter gene in

pGL3-Basic. The resulting construct was called pG2926luc and spans the sequence from −2838 to +88 bp. Restriction digestion of this fragment with Bam HI generated a 5' deletion of this construct spanning the sequence −643 to +88 bp, called pG731luc. Two further constructs were generated by PCR equivalent to those reported by Ni *et al.,* (1998b). Construct pG485luc contains 485 bp of sequence from −477 to +8 bp and construct pG79luc contains 79 bp of sequence from −71 to +8 bp. These constructs contain elements that drive luciferase gene expression and confer LPS inducibility of the reporter gene following transfection in A10 cells (Ni *et al.,* 1998b). Whereas pG79luc contains identical sequence to those described by Ni *et al.,* (1998b), construct pG485luc differs over the most 5' 66 bp. A schematic representation of these constructs is shown in Figure 5.6A. The promoter activity of these constructs were determined by transient transfection in JTC-19 cells as described in Methods and Materials. The luciferase activity data were normalised for each of the constructs by monitoring the transfection efficiency of the co-transfected *Renilla* luciferase and expressed as fold over expression of the promoterless pGL3-Basic vector (Figure 5.6). Constructs, pG2926luc, pG731luc and pG485luc expressed 5-10 fold activity above the promoterless vector. Construct pG79luc did not drive expression significantly above the promoterless vector suggesting that elements contained within the sequence −71 to +8 bp are not sufficient for transcription of the reporter gene. Therefore, sequence from −477 to −71 bp contains positive elements which can drive reporter gene expression. In addition, the higher activity of pG731luc compared to the other constructs suggests the presence of positive regulatory elements between −643 to −478 bp and negative regulatory elements between −2838 to −644 bp. Studies by Ni *et al.,* (1998b) show a similar ratio of activity for the 485 bp and 79 bp constructs in

A10 cells. However, these data are difficult to interpret since they are expressed relative to the 485 bp construct, with the fold induction over a promoterless vector not presented.

5.2.5 Cell type-specific regulation of the the BKB_1 receptor 5' flanking region

To determine whether the regulatory elements within the 5' flanking region of the BKB_1 receptor gene exhibit cell type-specific promoter activity, constructs pG79luc, pG485luc, pG731luc and pG2926luc were transiently transfected into the rat BKB_1 receptor expressing cell lines, JTC-19 and A10 cells and the non-BKB_1 receptor expressing liver hepatoma cell line, H4. Luciferase activities were measured as described in Methods and Materials (Figure 5.7). The activities of the constructs were higher in JTC-19 and A10 cells compared to H4 cells indicating the presence of cell type-specific elements required for activation and/or repression of the constructs. The increase in activity of pG485luc compared to pG79luc observed in JTC-19 and A10 cells, is absent in the H4 cells suggesting that there is a cell type-specific enhancer in the region from –477 to –72 bp. The activity of pG731luc exhibited the highest activity in all three cell types suggesting that the positive regulatory elements from –643 to –478 bp can function in all cell types. Furthermore, the negative regulatory elements located from –2838 to –644 bp, can reduce the activating function of this positive regulatory region in all three cell types.

5.2.6 IL-1β and LPS have no effect on reporter gene expression

5.2.6.1 IL-1β and LPS have no effect on rat BKB_1 receptor-directed reporter gene expression

To characterise the inducible elements within the 5' flanking region, constructs pG79luc, pG485luc, pG731luc and pG2926luc were transiently transfected into JTC-19 and A10 cells. 48 hours following transfection cells were treated with IL-1β or LPS for 3 hours. Luciferase activities were measured as described in Methods and Materials (Figure 5.8). As shown in Figure 5.8, treatment of the transfected cells with IL-1β or LPS did not increase luciferase activities above that found in cells treated with medium only. In contrast, Ni *et al.,* (1998b) showed that LPS caused a 2.5 fold increase in the activity of constructs equivalent to pG79luc and pG485luc in A10 cells. This discrepancy is difficult to explain. One possible explanation could be the difference in composition of the media in the presence of mediators. A number of studies have examined human and rat BKB_1 receptor gene expression in serum-free media to eliminate the inducible effects of serum (Ni *et al.,* 1998a,b; Yang *et al.,* 1998a). Therefore, reporter gene expression levels were determined in the presence or absence of serum. Construct pG485luc was transiently transfected into JTC-19 and A10 cells. 48 hours following transfection cells were treated with medium only or LPS for 3 hours in the presence or absence of 10% serum. Luciferase activities were determined as described in Methods and Materials (Figure 5.9). These data show that serum has no effect on reporter gene expression. To account for variability in the translation efficiency of the luciferase reporter gene compared to endogenous BKB_1 receptor gene expression, luciferase activities were analysed at different times

following the addition of mediators. These data showed the same expression profiles as shown in Figure 5.10 following treatment with IL-1β or LPS for 0.5, 2, 4 and 6 hours, with no increase in luciferase activity following the addition of mediators (data not shown). In addition, the same expression profiles were observed using lipofectAMINE as an alternative transfection reagent (data not shown).

5.2.6.2 IL-1β and LPS have no effect on human BKB_1 receptor-directed reporter gene expression

In addition to the reporter constructs described above, the activity of two human BKB_1 receptor-directed reporter gene constructs were determined. One of these constructs contains 5' flanking sequence spanning the upstream NF-κB-like site, (+ NF-κB), the other construct contains 5' flanking sequence without the NF-κB-like site, (– NF-κB) These constructs have been shown to drive reporter gene expression, with + NF-κB inducing a five fold increase in expression following the addition of IL-1β or desArg^{10}kallidin to IMR-90 cells (Schanstra *et al.,* 1998). The constructs were transiently transfected into JTC-19 cells as described in Methods and Materials. Normalised luciferase activities were determined following a 3 hour treatment with IL-1β or LPS and expressed as fold over basic (Figure 5.10). These results show that the constructs exhibited promoter activity 6 fold over basic, however no increase in activity was observed following the addition of mediators. Similar results were obtained following transient transfection of the constructs in A10 cells (data not shown). However, due to the low transfection efficiency of IMR-90 cells, the data reported in the study by Schanstra *et al.,* (1998) could not be replicated. Thus, the inability to reproduce the inducible activity of the rat and human BKB_1 receptor-

directed reporter gene constructs in JTC-19 cells suggests that the transfection procedure itself may be critical to the sensitivity of the response.

5.2.7 Inhibition of LPS-induced BKB_1 receptor gene expression following transient transfection

To assess the effects of the transfection procedure on BKB_1 receptor gene expression, receptor mRNA levels were examined in transfected and non-transfected cells. JTC-19 cells were transiently transfected with pG731luc as described in Methods and Materials. 48 hours following transfection, non-transfected and transfected cells were treated with or without LPS for 3 hours. mRNA was isolated and BKB_1 receptor mRNA levels were determined by Northern blot analysis (Figure 5.11). As shown in Figure 5.11B, following transfection and in the absence of LPS, the endogenous BKB_1 receptor mRNA levels are equivalent to the levels in non-transfected JTC-19 cells. However, following LPS treatment, BKB_1 receptor mRNA levels were found to be reduced by 43% in transfected cells compared to non-transfected cells. Thus, these data show that the native BKB_1 receptor promoter is affected by the transfection procedure.

Furthermore, to assess a possible role for IL-1β and LPS-mediated chromatin re-modelling in the regulation of the BKB_1 receptor gene, the 5' regulatory region was examined for DNase I-hypersensitive sites. Preliminary analysis did not reveal the presence of DNase I-hypersensitivity in the 5' flanking region following treatment with IL-1β or LPS (data not shown). However, this may have been due to the location and/or size of the probe and requires further investigation.

5.2.8 Specific protein-DNA interactions within the 5' regulatory region of the BKB_1 receptor gene.

The ability of the conserved AP-1 site, CRE site and downstream NF-κB-like site, and the upstream NF-κB site to interact with transcription factors were examined by EMSAs using probes that span these sites.

5.2.8.1 Nuclear proteins bind to the NF-κB-like site and NF-κB site in the BKB_1 receptor regulatory region

Following the determination of a role for NF-κB in the IL-1β and LPS-mediated up-regulation of the BKB_1 receptor, the ability of the downstream NF-κB-like site (−64 to −57 bp) and the upstream NF-κB site (−1192 to −1183 bp) to interact with nuclear proteins were examined. EMSAs were used to study the binding of nuclear proteins to the NF-κB-like site from control, IL-1β or LPS-treated JTC-19 cells. Nuclear extracts were harvested from control and treated JTC-19 cells as described in Methods and Materials. Initially, a probe spanning the 5' regulatory sequence from –64 to –49 bp (rd NF-κB) containing the NF-κB-like site was end-labelled and used in binding studies. This demonstrated no complex formation with any of the nuclear extracts. To confirm the presence of NF-κB in the nuclear extracts an oligonucleotide containing a consensus NF-κB site was synthesised and used in an EMSA (Figure 5.12). Nuclear extracts from control JTC-19 cells demonstrate binding (lanes 1, 3 and 4) which is increased in IL-1β and LPS treated JTC-19 nuclear extracts (lanes 5,7,8,9,11 and 12) indicating the accumulation of NF-κB in the nucleus. The specificity of the observed DNA-protein interaction was confirmed by the ability of

excess unlabelled NF-κB oligonucleotide to inhibit binding (specific competitor) shown in lanes 2, 6 and 10 and the addition of a mutant oligonucleotide that did not block binding shown in lanes 3, 7 and 11. The addition of excess unlabelled rd NF-κB oligonucleotide in lanes 8 and 12 reduced the DNA-protein interaction, however no reduction in the DNA-interaction was observed from control nuclear extracts (lane 4). These data show that the NF-κB-like site has affinity for nuclear proteins from the IL-1β and LPS-treated nuclear extracts when added in 700 fold excess but binding is undetectable when the oligonucleotide, rd NF-κB, is used as a probe. Figure 5.13 shows the result of competition experiments with unlabelled oligonucleotide spanning the rat upstream (ru) NF-κB like site located. As described above, the labelled consensus NF-κB oligonucleotide demonstrated binding in nuclear extracts from IL-1β and LPS treated JTC-19 cells. The specificity of the reaction was determined as described above using excess unlabelled specific competitor in lanes 2 and 6 and non-specific competitor in lanes 3 and 7. The addition of excess unlabelled ru NF-κB oligonucleotide in lanes 4 and 8 partially blocked binding suggesting that the site has affinity for NF-κB when added in 700 fold excess. Although the ru NF-κB oligonucleotide inhibition was not as specific as the consensus NF-κB oligonucleotide (lanes 2 and 6 versus lanes 4 and 8), the inhibition displayed by ru NF-κB oligonucleotide appeared to be greater than that observed with the rd NF-κB oligonucleotide (Figure 5.12 lanes 8 and 12 versus Figure 5.13 lanes 4 and 8).

5.2.8.2 The AP-1 site in the BKB1 receptor regulatory region does not bind to nuclear proteins

A probe spanning the AP-1 site from –79 to –67 bp was synthesised and used in the EMSA. This did not exhibit binding activity with any of the nuclear extracts. A consensus AP-1 oligonucleotide was synthesised and used as a probe to confirm the presence of AP-1 protein dimers. Nuclear extracts from control, IL-1β and LPS-treated JTC-19 cells demonstrated binding as shown in Figure 5.14 lanes 1, 5 and 9. The specificity of the interaction was confirmed by the ability of excess unlabelled probe to inhibit the binding, lanes 2, 6 and 10, and the addition of the mutant oligonucleotide in lanes 3, 7 and 11, that did not block binding. Competition with unlabelled oligonucleotide from the AP-1 site in the rat 5' regulatory region did not block binding, lanes 4, 8 and 12. These data suggest that this AP-1 site is not functional in JTC-19 cells.

5.2.8.3 CREB binds to the CRE site in the BKB_1 receptor regulatory region

The increase in BKB_1 receptor mRNA levels following the addition of cAMP activators suggests the involvement of CREB in the regulation of the BKB_1 receptor gene (see Chapter 4, Figure 4.2). Using a probe spanning the CRE site from –53 to –40 bp, binding activity was observed in control, IL-1β and LPS-treated JTC-19 nuclear extracts (Figure 5.15 lanes 1, 6 and 11). The binding complexes were entirely competed with non-labelled probe (lanes 2, 7 and 12) and by an oligonucleotide containing a consensus CRE site (lanes 4, 9 and 14) but not by a mutant

oligonucleotide, (lanes 3, 8 and 13) indicating the specificity of binding. An antibody against the CREB protein was pre-incubated with the nuclear extracts to further characterise the nuclear protein and this resulted in a supershift of the complex (lanes 5, 10 and 15). Thus, these findings show that the CRE site binds to CREB in control, IL-1β and LPS-treated JTC-19 cells.

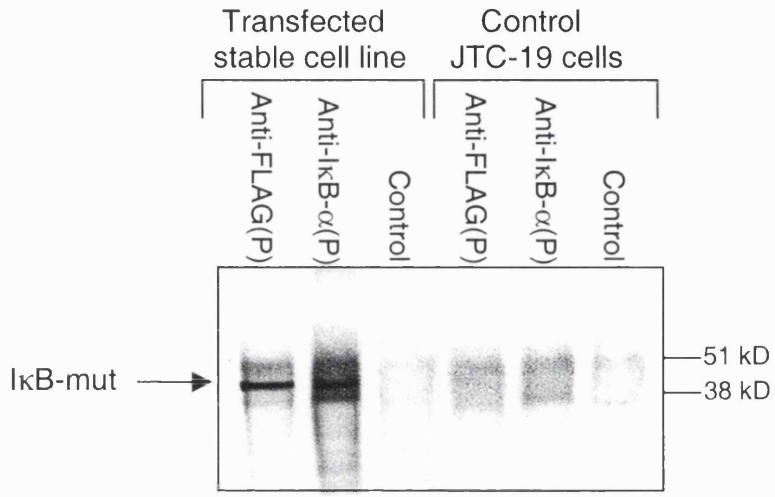


Figure 5.1 Immunoblot analysis of IκB-mut expression.

JTC-19 cells were transfected with plasmids encoding FLAG-IκB-mut and G418 resistance. A clonal cell line was isolated following selection with G418. Cellular extracts from this cell line and control JTC-19 cells were isolated. 500 μg of each protein sample were analysed by immunoprecipitation with either a FLAG-polyclonal antibody (anti-FLAG(P)), an IκB-α polyclonal antibody (anti-IκB-α(P)) or no antibody (control) as described in Methods and Materials. Samples were resolved by polyacrylamide gel electrophoresis, followed by Immunoblot analysis with a FLAG-monoclonal antibody.

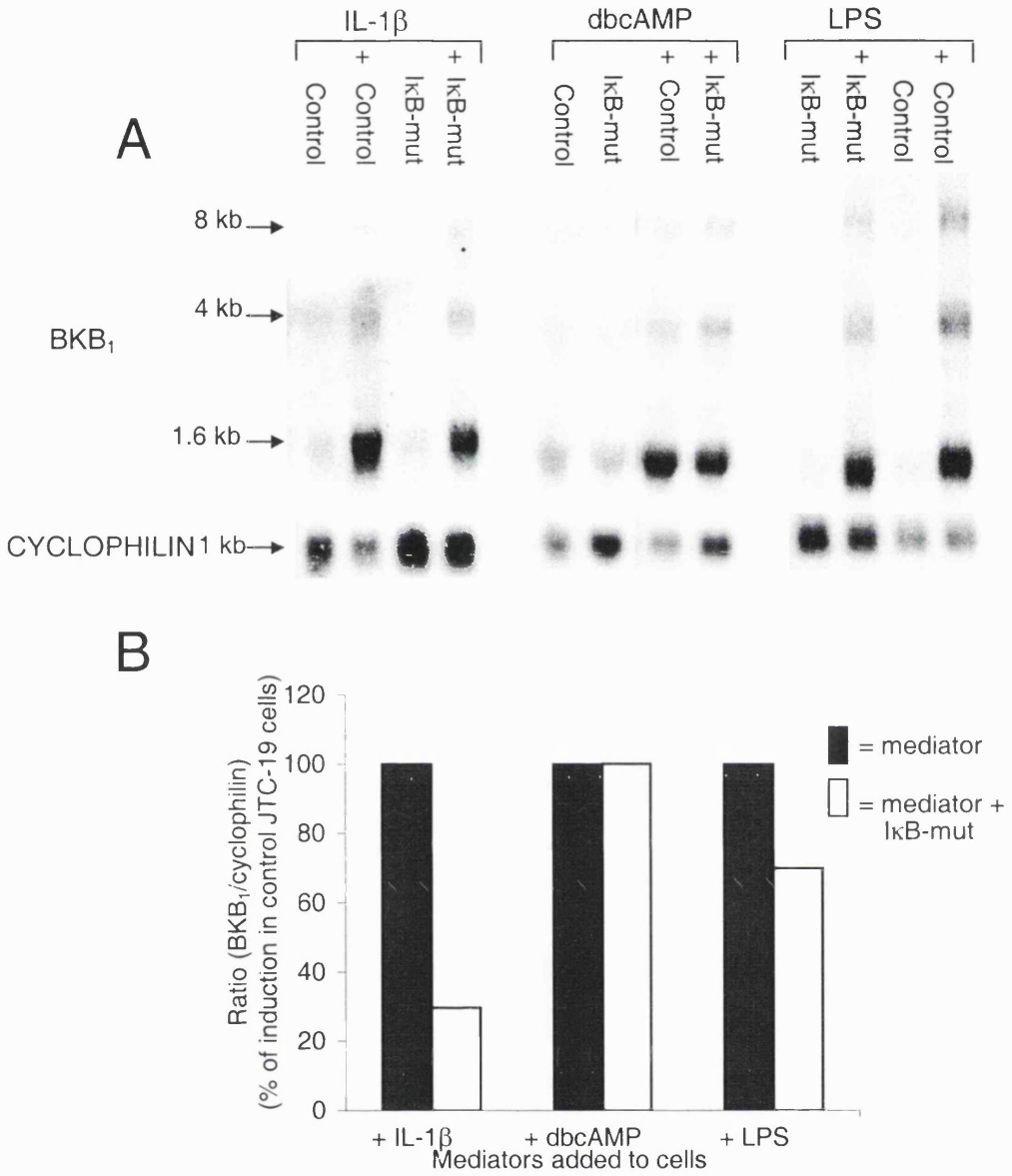


Figure 5.2 Effect of NF-κB inactivation on IL-1β, dbcAMP and LPS-induced BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells (control) and the stable IκB-mut expressing cell line (IκB-mut) with medium only, IL-1β-treated (10 ng/ml) for 2 hours, dbcAMP-treated (1 mM) for 2 hours or LPS-treated (10 μg/ml) for 1 hour. The blots were stripped and re-probed with cyclophilin as shown in the analysis in the lower panel. The presence of a + sign indicates the presence of the mediator. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted as a percentage of the up-regulation observed in JTC-19 cells.

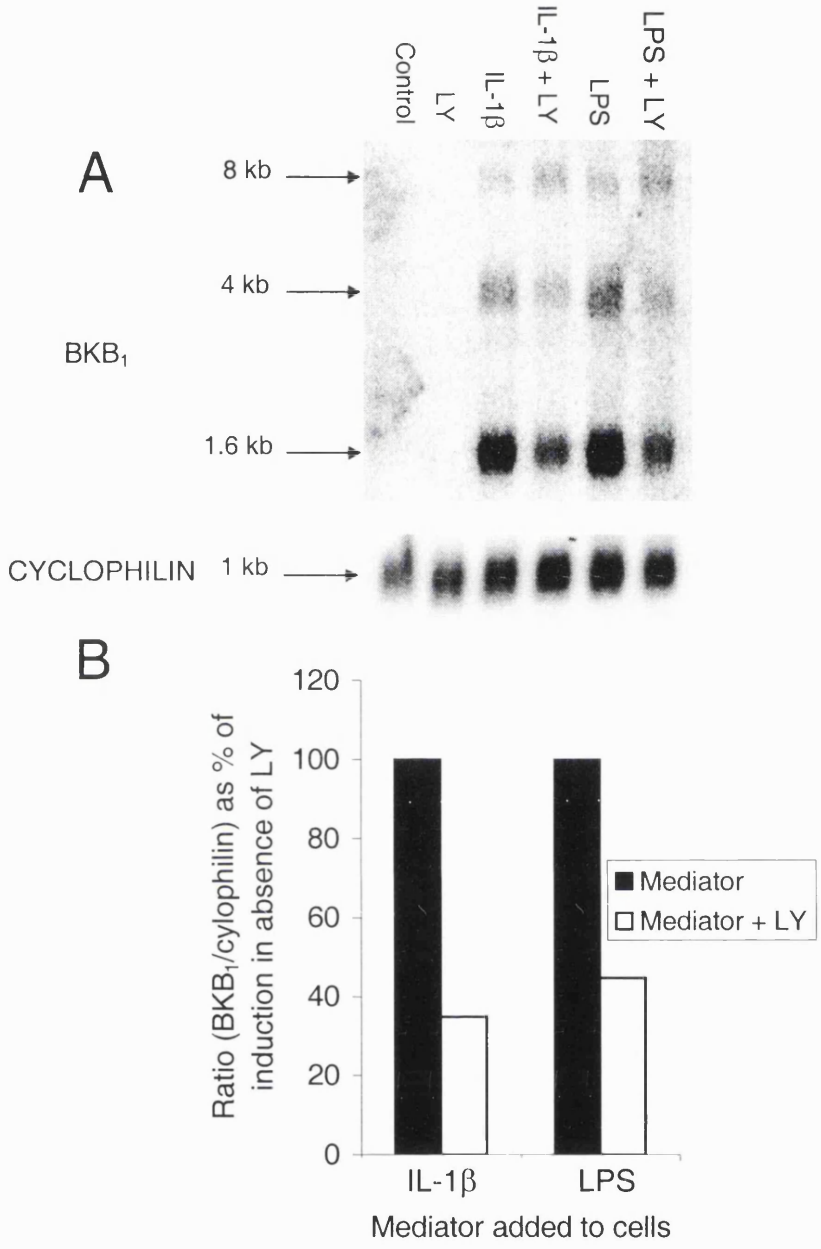
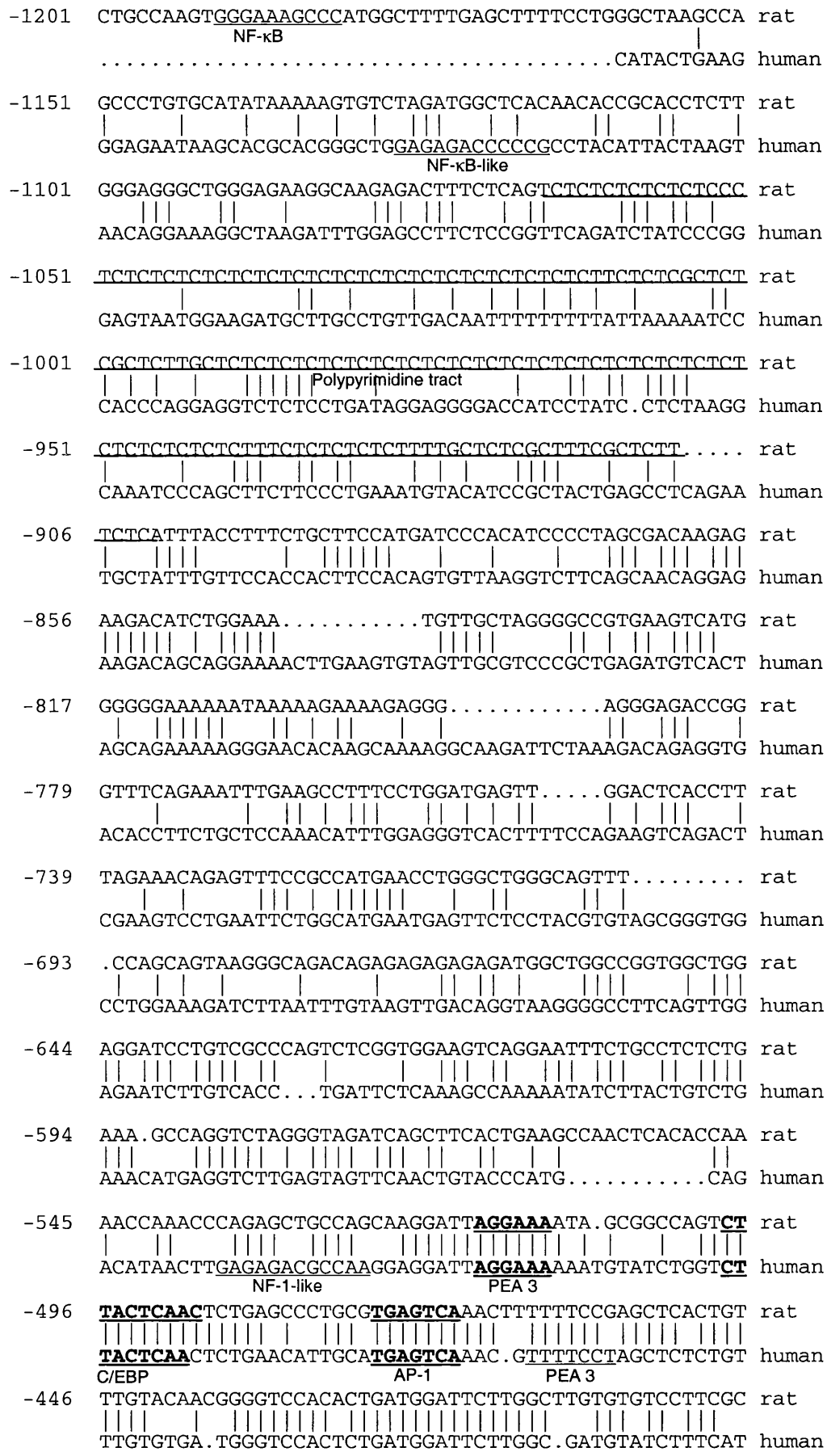


Figure 5.3 Effect of PI 3-kinase inhibition on IL-1β and LPS-induced BKB_1 receptor mRNA levels.

A Northern blot analysis showing the BKB_1 receptor mRNA levels from JTC-19 cells pre-treated in the absence or presence of LY294002 (LY) (20 μM) before treatment with IL-1β (10 ng/ml) or LPS (10 μg/ml) for 1 hour. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted as a percentage of the up-regulation in the absence of the inhibitor.

FIGURE 5.4


-1201 CTGCCAAGTGGGAAAGCCCATGGCTTTTGAGCTTTTCCTGGGCTAAGCCA rat
NF-κB
.....CATACTGAAG human
-1151 GCCCTGTGCATATAAAAAGTGTCTAGATGGCTCACAACACCGCACCTCTT rat
GGAGAATAAGCACGCACGGGCTGGAGAGACCCCCGCCTACATTACTAAGT human
NF-κB-like
-1101 GGGAGGGCTGGGAGAAGGCAAGAGACTTTCTCAGTCTCTCTCTCTCTCCCC rat
AACAGGAAAGGCTAAGATTTGGAGCCTTCTCCGGTTCAGATCTATCCCGG human
-1051 TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTCTCTCGCTCT rat
GAGTAATGGAAGATGCTTGCCTGTTGACAATTTTTTTTTATTAAAAATCC human
-1001 CGCTCTTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT rat
Polypyrimidine tract
CACCCAGGAGGTCTCTCCTGATAGGAGGGGACCATCCTATC.CTCTAAGG human
-951 CTCTCTCTCTCTTTCTCTCTCTCTTTTGCTCTCGCTTTCGCTCTT..... rat
CAAATCCCAGCTTCTTCCCTGAAATGTACATCCGCTACTGAGCCTCAGAA human
-906 TCTCATTTACCTTTCTGCTTCCATGATCCCACATCCCCTAGCGACAAGAG rat
TGCTATTTGTTCCACCACTTCCACAGTGTTAAGGTCTTCAGCAACAGGAG human
-856 AAGACATCTGGAAA.....TGTTGCTAGGGGCCGTGAAGTCATG rat
AAGACAGCAGGAAAACTTGAAGTGTAGTTGCGTCCCGCTGAGATGTCACT human
-817 GGGGGAAAAAATAAAAAGAAAAGAGGG.....AGGGAGACCGG rat
AGCAGAAAAAGGGAACACAAGCAAAAGGCAAGATTCTAAAGACAGAGGTG human
-779 GTTTCAGAAATTTGAAGCCTTTCCTGGATGAGTT.....GGACTCACCTT rat
ACACCTTCTGCTCCAAACATTTGGAGGGTCACTTTTCCAGAAGTCAGACT human
-739 TAGAAACAGAGTTTCCGCCATGAACCTGGGCTGGGCAGTTT..... rat
CGAAGTCCTGAATTCTGGCATGAATGAGTTCTCCTACGTGTAGCGGGTGG human
-693 .CCAGCAGTAAGGGCAGACAGAGAGAGAGAGATGGCTGGCCGGTGGCTGG rat
CCTGGAAAGATCTTAATTTGTAAGTTGACAGGTAAGGGGCCTTCAGTTGG human
-644 AGGATCCTGTCGCCCAGTCTCGGTGGAAGTCAGGAATTTCTGCCTCTCTG rat
AGAATCTTGTCACC...TGATTCTCAAAGCCAAAAATATCTTACTGTCTG human
-594 AAA.GCCAGGTCTAGGGTAGATCAGCTTCACTGAAGCCAACTCACACCAA rat
AAACATGAGGTCTTGAGTAGTTCAACTGTACCCATG.....CAG human
-545 AACCAAACCCAGAGCTGCCAGCAAGGATTAGGAAAATA.GCGGCCAGTCT rat
ACATAACTTGAGAGACGCCAAGGAGGATTAGGAAAAAATGTATCTGGTCT human
NF-1-like
PEA 3
-496 TACTCAACTCTGAGCCCTGCGTGAGTCAAACTTTTTTCCGAGCTCACTGT rat
TACTCAACTCTGAACATTGCATGAGTCAAAC.GTTTTCCTAGCTCTCTGT human
C/EBP
AP-1
PEA 3
-446 TTGTACAACGGGGTCCACACTGATGGATTCTTGGCTTGTGTGTCCTTCGC rat
TTGTGTGA.TGGGTCCACTCTGATGGATTCTTGGC.GATGTATCTTTCAT human

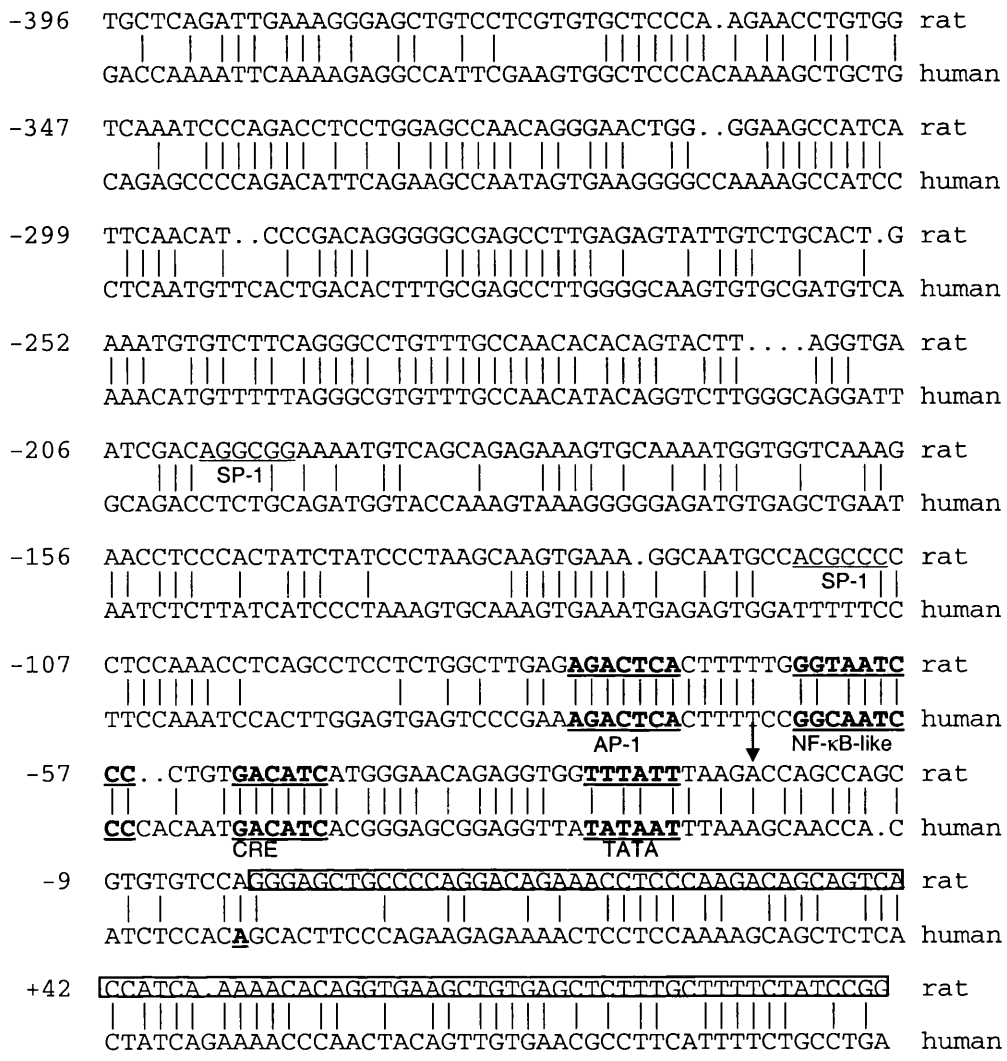


Figure 5.4 Nucleotide sequence alignment of exon 1 and the 5' flanking regions of the rat and human BKB_1 receptor genes.

Alignment of rat and human (Yang and Polgar, 1996) receptor genes from −1201 to + 88 bp. Homologous bases between the rat and human BKB_1 receptor genes are shown by vertical lines. Nucleotide numbering of the rat sequence starts with +1 at the TIS. Exon 1 of the rat is shown by the boxed region. The arrow on the rat sequence indicates the start site shown by Ni *et al.,* (1998b). The A residue shown in bold shows the TIS for the human sequence (Yang and Polgar, 1996). Regulatory elements which have been shown to be important for human BKB_1 receptor expression are underlined and labelled, those that are conserved in the rat sequence are shown in boldface. Other putative regulatory elements within the rat sequence are underlined and labelled.

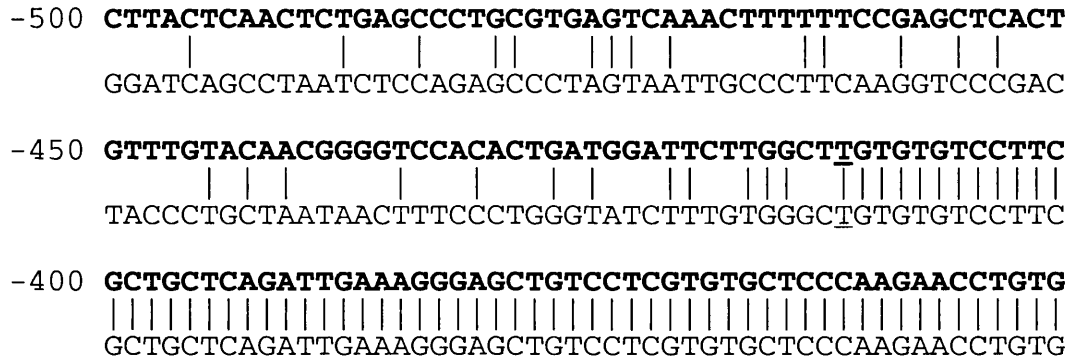


Figure 5.5 Nucleotide sequence alignment of rat BKB_1 receptor 5' flanking regions.

Nucleotide sequences were aligned for the 5' flanking region from −500 to −350 bp for the rat sequence from this study, shown in boldface, with the rat BKB_1 receptor sequence reported by Ni *et al.,* (1998b) shown below. Homologous bases are shown by vertical lines, the point of divergence between the two sequences at position −412 bp is underlined.

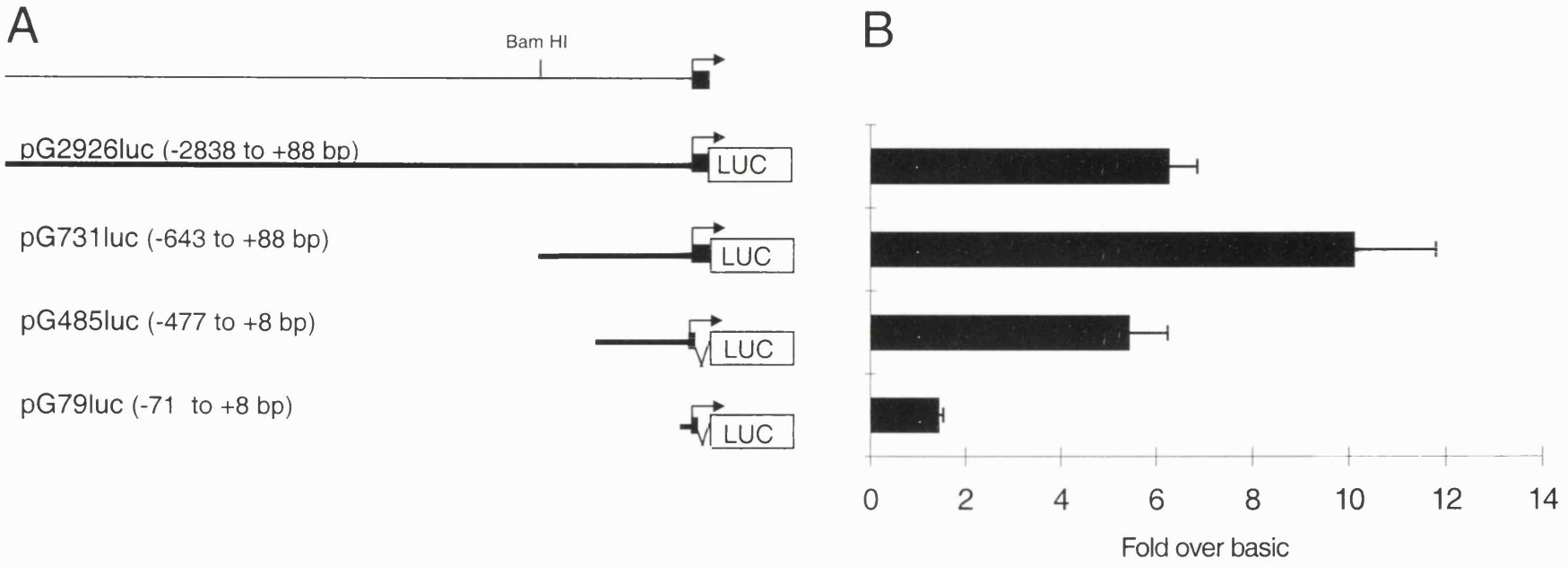


Figure 5.6 Luciferase reporter assays with the rat BKB_1 receptor-directed reporter gene constructs.

A represents a map of the constructs pG79luc, pG485luc, pG731luc and pG2926luc spanning the 5' flanking region of the rat BKB_1 receptor gene cloned in front of the the firefly luciferase gene. The arrow indicates the position of the TIS, the solid box represents exon 1. **B** shows the activity of the constructs from panel A following transient transfection into JTC-19 cells as described in Methods and materials. All data are corrected for transfection efficiency according to *Renilla* luciferase expression driven by co-transfected pRL-SV40. Results are expressed as expression over the promoterless pGL3-Basic vector. Each value is the mean ± SEM (n=3). The data shown are representative of three separate experiments.

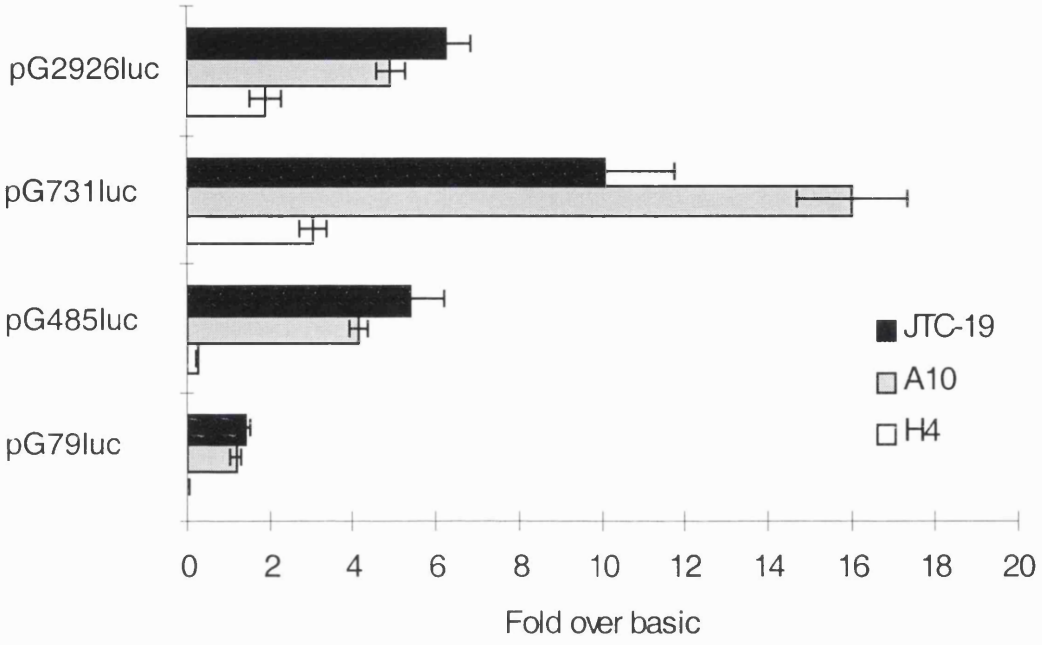


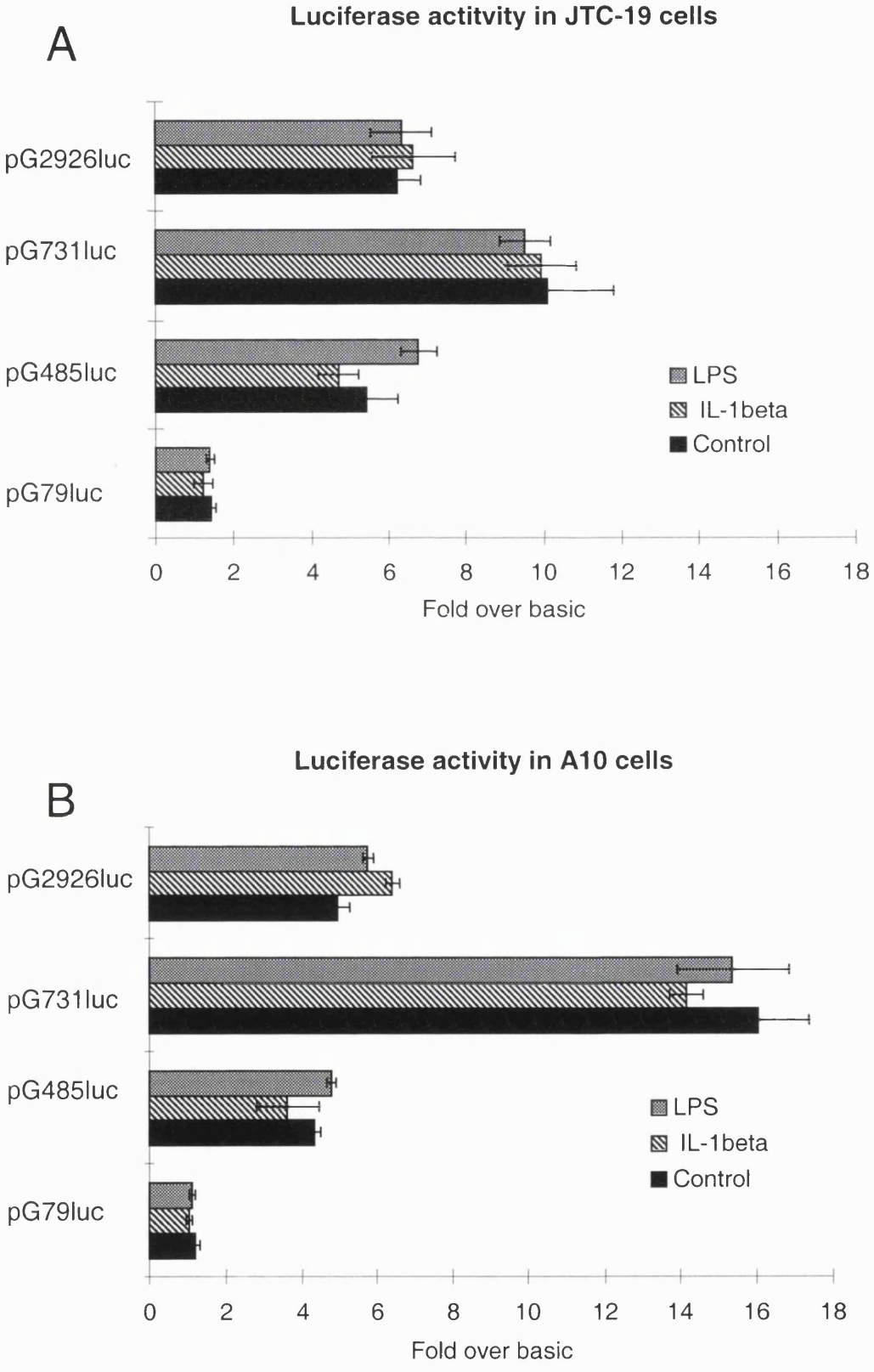
Figure 5.7 Luciferase reporter assays with the rat BKB_1 receptor-directed reporter gene constructs in different cell types.

JTC-19, A10 and H4 cells were transiently transfected using the constructs pG79luc, pG485luc, pG731luc and pG2926luc as described in Methods and Materials. All data are corrected for transfection efficiency according to *Renilla* luciferase expression driven by co-transfected pRL-SV40. Results are expressed as expression over the promoterless pGL3-Basic vector. Each value is the mean ± SEM (n=3). The data shown are representative of three separate experiments.

Figure 5.8 Luciferase reporter assays of the rat BKB_1 receptor-directed reporter gene constructs following IL-1β or LPS treatment.

Constructs, pG79luc, pG485luc, pG731luc and pG2926luc were transiently transfected into JTC-19 cells (**A**) and A10 cells (**B**) as described in Materials and Methods. 48 hours following transfection cells were treated for 3 hours with medium only or IL-1β (10 ng/ml) or LPS (10 μg/ml) followed by analysis of luciferase activities. All data are corrected for transfection efficiency according to *Renilla* luciferase expression driven by co-transfected pRL-SV40, results are expressed as expression over the promoterless pGL3-Basic vector. Each value is the mean ± SEM (n=3). The data shown are representative of three separate experiments.

FIGURE 5.8


Luciferase actitvity in JTC-19 cells
A
pG2926luc
pG731luc
pG485luc
pG79luc
LPS
IL-1beta
Control
0
2
4
6
8
10
12
14
16
18
Fold over basic
Luciferase activity in A10 cells
B
pG2926luc
pG731luc
pG485luc
pG79luc
LPS
IL-1beta
Control
0
2
4
6
8
10
12
14
16
18
Fold over basic

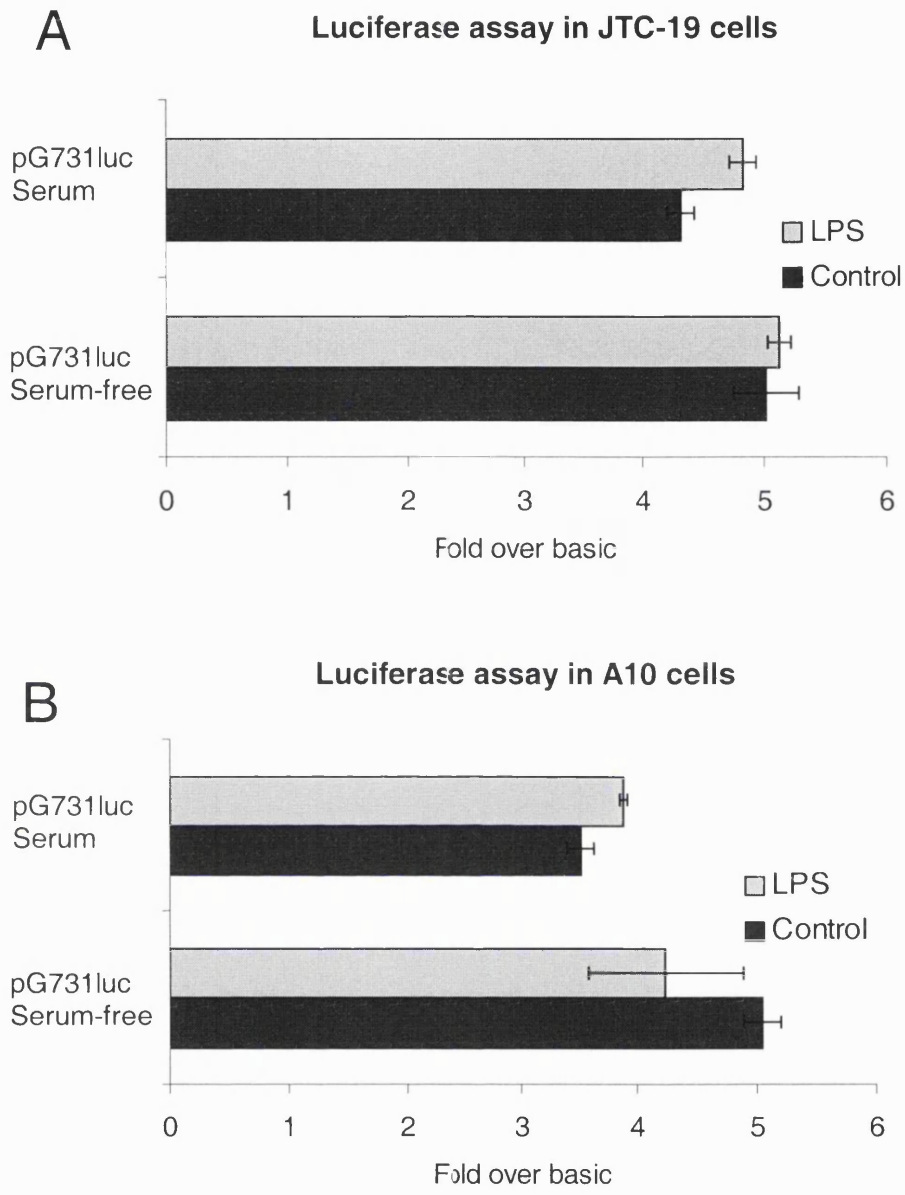


Figure 5.9 Effect of serum on luciferase reporter assays with a rat BKB_1 receptor-directed reporter gene construct.

Construct pG485luc was transiently transfected into JTC-19 cells (**A**) and into A10 cells (**B**). 48 hours following transfection, cells were treated for 3 hours with serum free media ± LPS (10 μg/ml) or with media containing 10% serum ± LPS (10 μg/ml), followed by analysis of luciferase activities. All data are corrected for transfection efficiency according to *Renilla* luciferase expression driven by co-transfected pRL-SV40, results are expressed as expression over the promoterless pGL3-Basic vector. Each value is the mean ± SEM (n=3). The data shown are representative of three separate experiments.

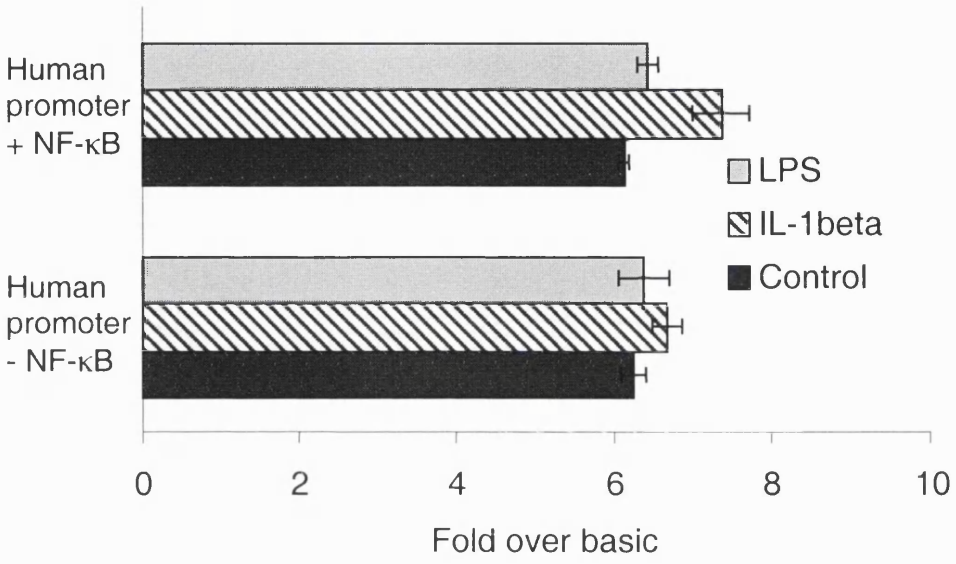


Figure 5.10 Luciferase reporter assays with the human BKB_1 receptor-directed reporter gene constructs.

JTC-19 cells were transiently transfected using the human BKB_1 receptor-directed reporter gene constructs with and without the upstream NF-κB-like sequence, donated as a kind gift by Dr. Bascands and reported in Schanstra *et al.*, (1998). 48 hours following transfection cells were treated for 3 hours with medium only, or IL-1β (10 ng/ml) or LPS (10 μg/ml) followed by analysis of luciferase activities. All data are corrected for transfection efficiency according to *Renilla* luciferase expression driven by co-transfected pRL-SV40, results are expressed as expression over the promoterless pGL3-Basic vector. Each value is the mean ± SEM (n=3). The data shown are representative of three separate experiments.

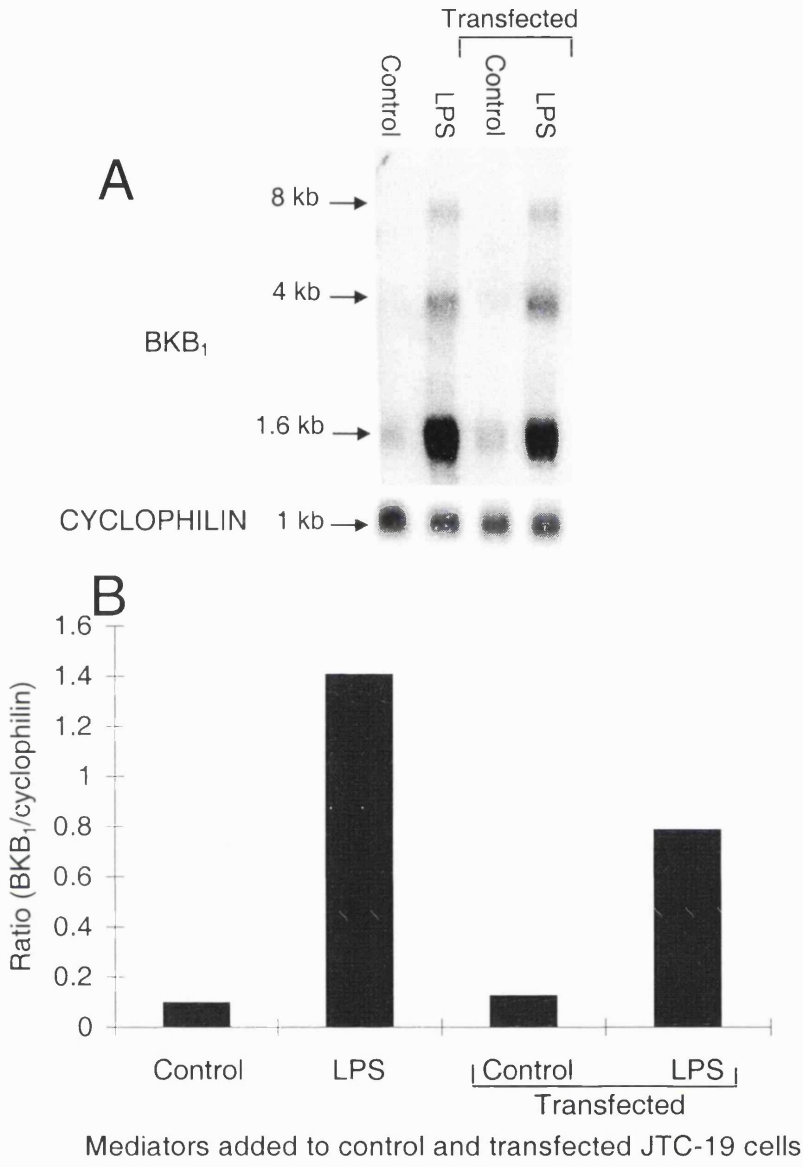


Figure 5.11 Analysis of BKB_1 receptor mRNA levels following transfection.

JTC-19 cells were transiently transfected using the rat construct pG731luc. 48 hours following transfection cells were treated with medium only (transfected control) or with LPS (10 μg/ml) for 3 hours. At the same time non-transfected cells were treated with with medium only (control) or LPS (10 μg/ml). **A** Northern blot analysis showing the BKB_1 receptor mRNA levels from normal and transfected JTC-19 cells ± LPS. The same blot was stripped and re-probed with cyclophilin as shown in the lower panel. **B** The amount of radioactivity in the 1.6 kb transcript was quantified using the Storm phosphorimager and ImageQuaNT analysis software and normalised to the respective cyclophilin band. The normalised signal was plotted against the appropriate mediator. The Northern blots shown are representative of two separate experiments.

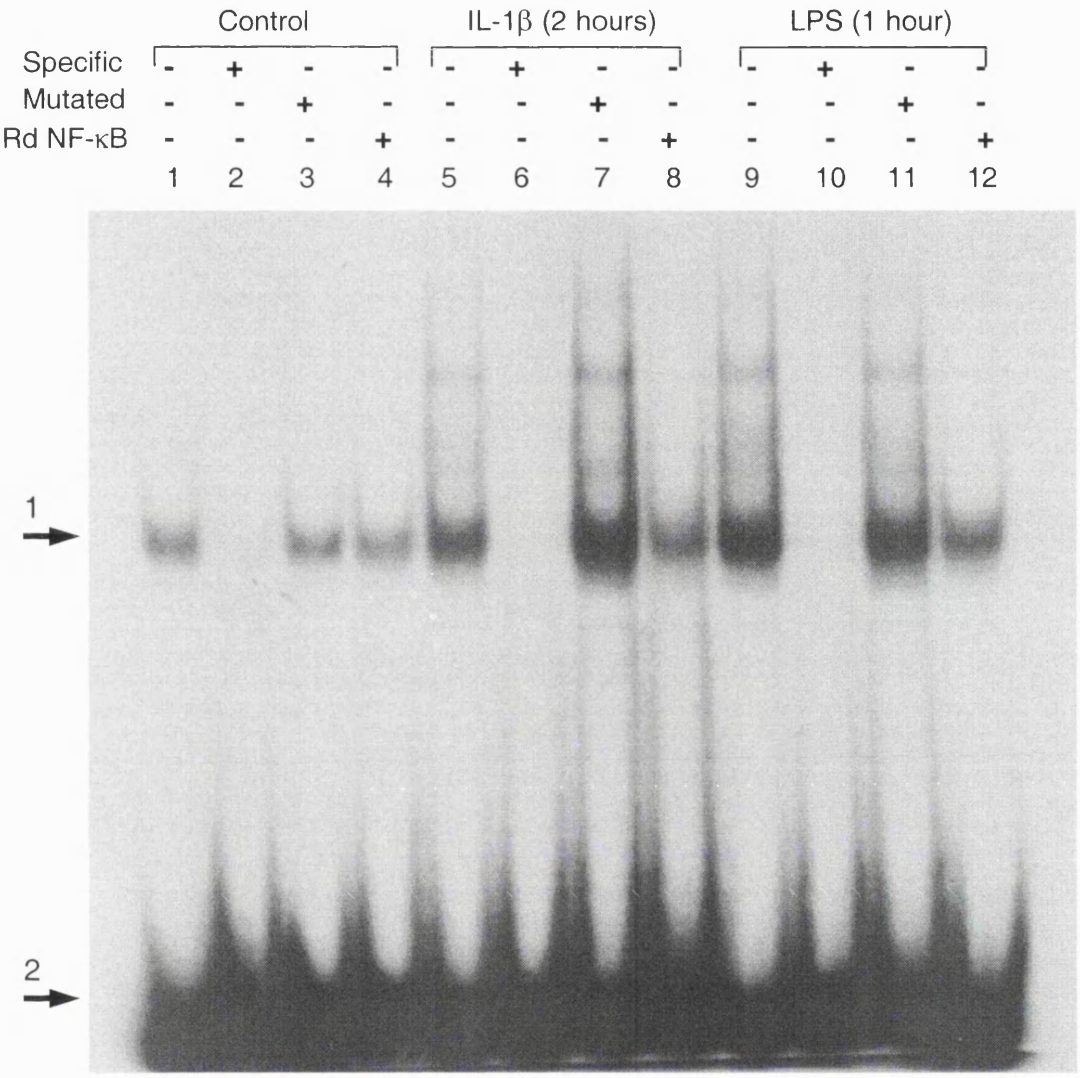


Figure 5.12 EMSA analysis of the nuclear protein binding activity of the NF-κB-like site in the BKB_1 receptor regulatory region.

Nuclear proteins were extracted from control (lanes 1-4), 2 hour IL-1β-treated (10 ng/ml) (lanes 5-8) and 1 hour LPS-treated (10 μg/ml) (lanes 9-12) JTC-19 cells. Binding to the ^{32}P labelled consensus NF-κB oligonucleotide was determined by EMSAs as described in Methods and Materials. The induced complex is indicated by the arrow 1. The presence of competitors is shown by a + sign above the appropriate lane; Specific= unlabelled NF-κB oligonucleotide (700-fold excess), Mutated= unlabelled oligonucleotide mutated in the NF-κB site (700-fold excess), rd NF-κB= unlabelled dowsntream NF-κB-like sequence in the 5' flanking region of the rat BKB_1 receptor gene (700-fold excess). Arrow 2 shows unbound labelled probe.

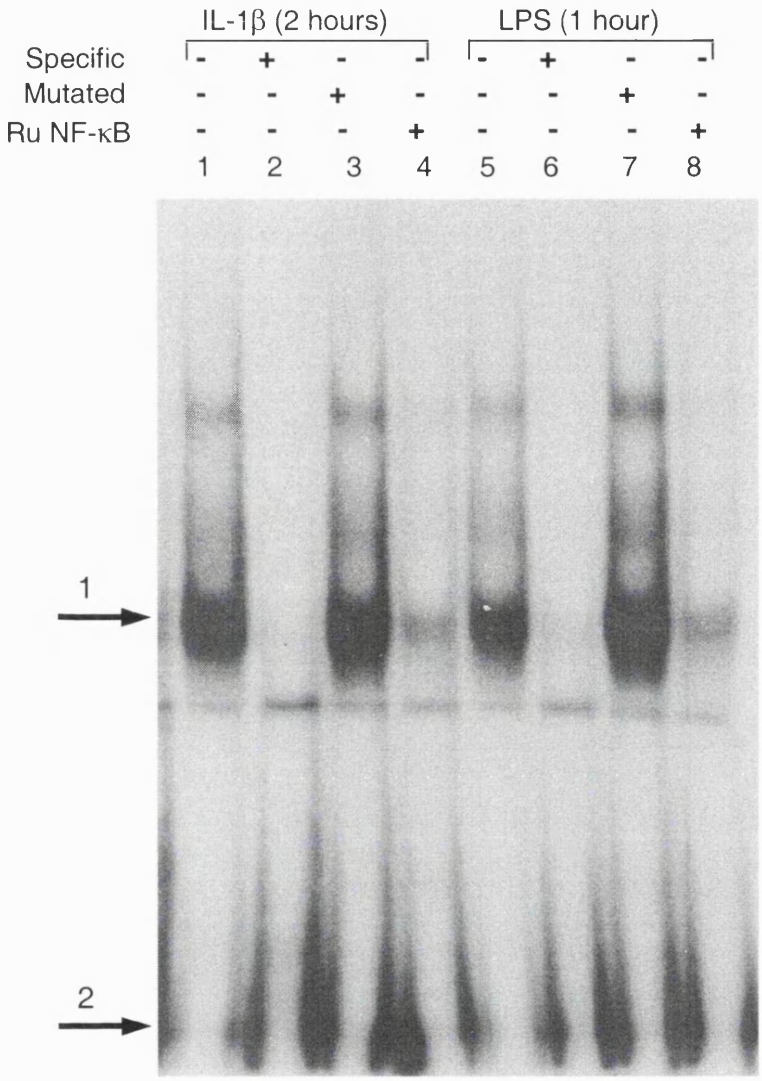


Figure 5.13 EMSA analysis of the nuclear protein binding activity of the NF-κB site in the BKB_1 receptor regulatory region.

Nuclear proteins were extracted from 2 hour IL-1β-treated (10 ng/ml) (lanes 1-4) and from 1 hour LPS-treated (10 μg/ml) (lanes 5-8) JTC-19 cells. Binding to the ^{32}P labelled consensus NF-κB oligonucleotide was determined by EMSAs as described in Methods and Materials. The induced complex is indicated by the arrow 1. The presence of competitors is shown by the + sign above the appropriate lane; Specific= unlabelled NF-κB oligonucleotide (700 fold excess), Mutated= unlabelled oligonucleotide mutated in the NF-κB site (700 fold excess), ru NF-κB= unlabelled upstream NF-κB sequence in the 5' flanking sequence of the rat BKB_1 receptor gene (700 fold excess). Arrow 2 shows unbound labelled probe.

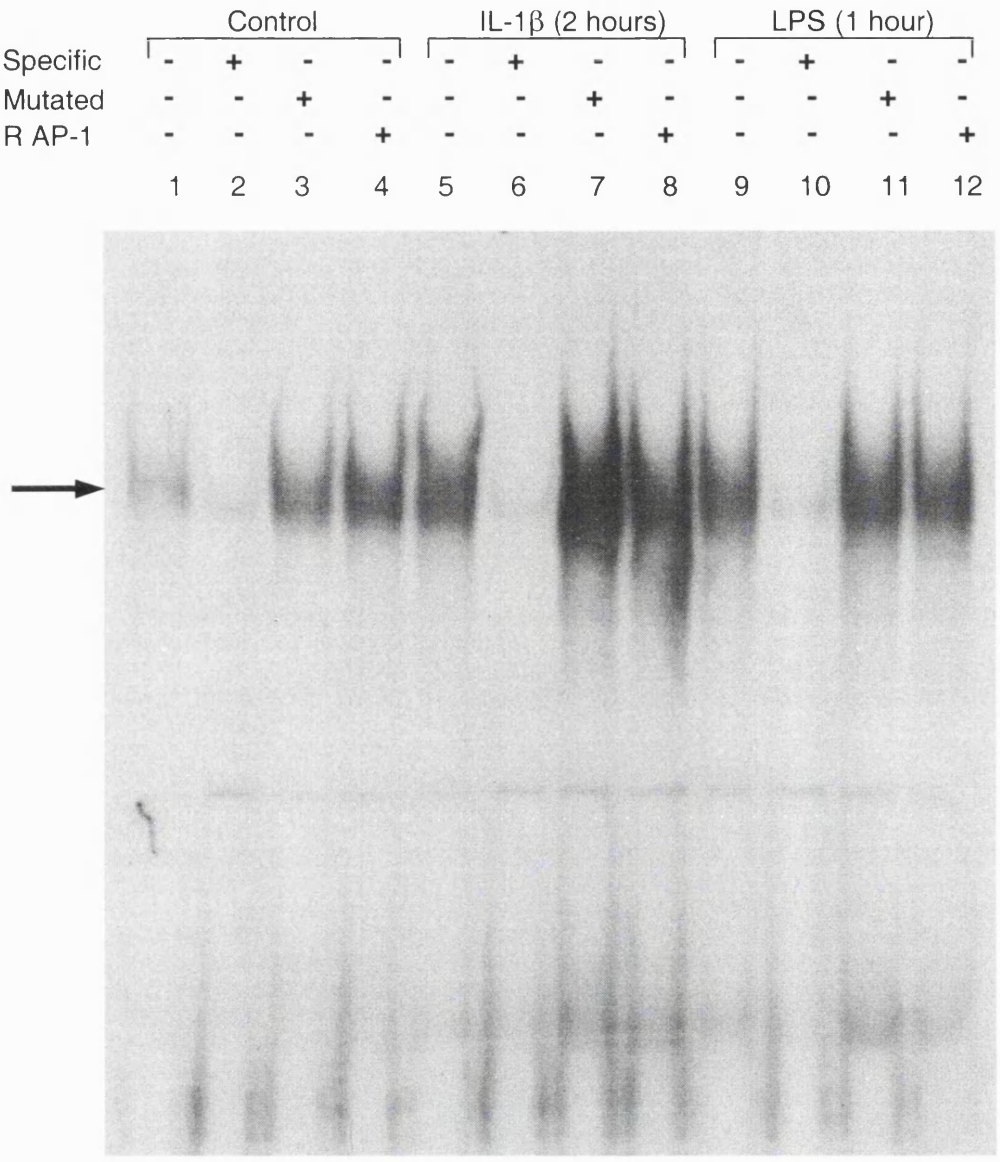


Figure 5.14 EMSA analysis of the nuclear protein binding activity of the AP-1 site in the BKB_1 receptor regulatory region.

Nuclear proteins were extracted from control (lanes 1-4), 2 hour IL-1β-treated (10 ng/ml) (lanes 5-8) and 1 hour LPS-treated (10 μg/ml) (lanes 9-12) JTC-19 cells. Binding to the ^{32}P labelled consensus AP-1 oligonucleotide was determined by EMSAs as described in Methods and Materials. The induced complex is indicated by the arrow. The presence of competitors is shown by a + sign above the appropriate lane; Specific= unlabelled AP-1 oligonucleotide (700-fold excess), Mutated= unlabelled oligonucleotide mutated in the AP-1 site (700-fold excess), R AP-1= unlabelled AP-1 like sequence in the 5' flanking region of the rat BKB_1 receptor gene (700-fold excess).

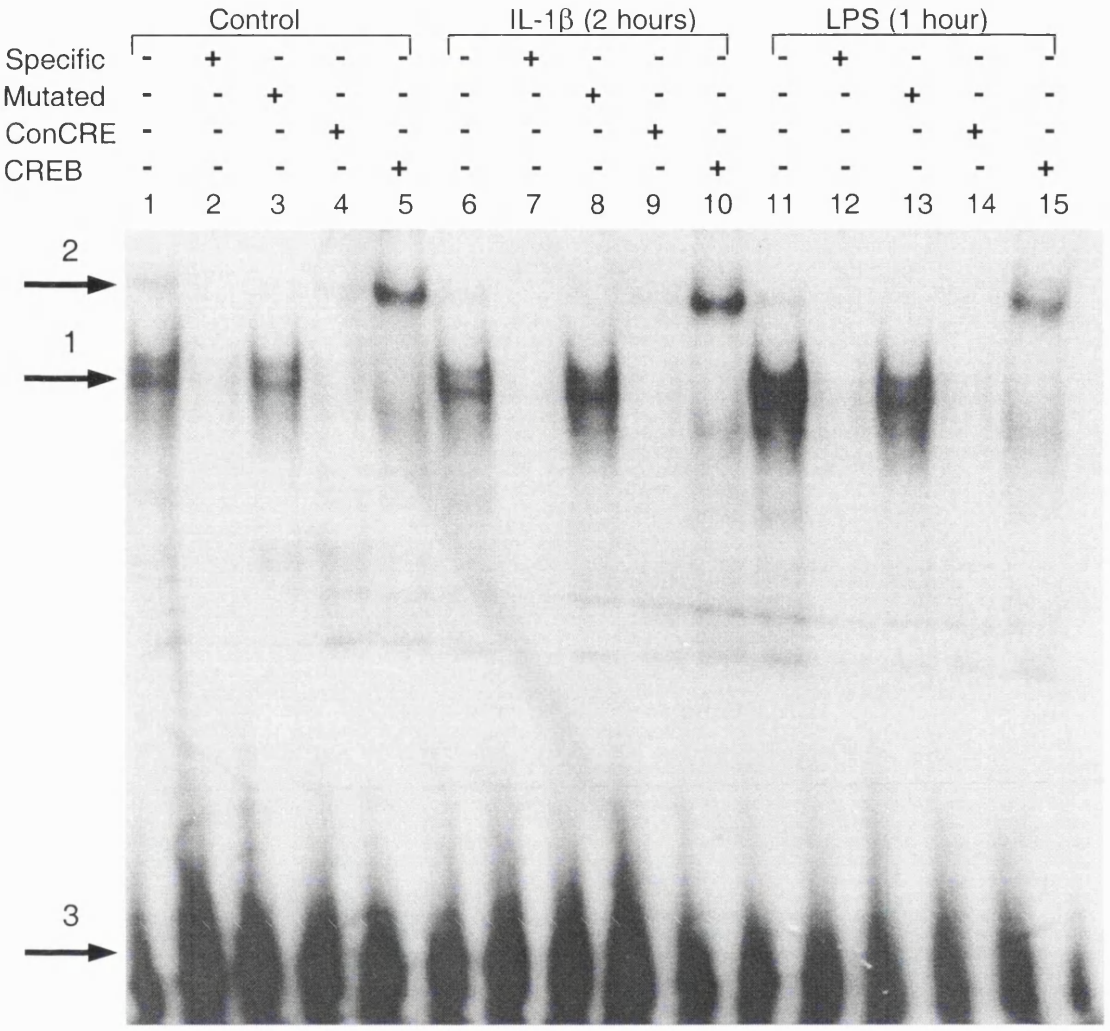


Figure 5.15 EMSA analysis of CREB binding activity to the CRE site in the BKB_1 receptor regulatory region.

Nuclear proteins were extracted from control (lanes 1-5), 2 hour IL-1β-treated (10 ng/ml) (lanes 6-10) and 1 hour LPS-treated (10 μg/ml) (lanes 11-15) JTC-19 cells. Binding to the ^{32}P labelled CRE oligonucleotide, located in the 5' flanking region of the rat BKB_1 receptor gene was determined by EMSs as described in Methods and Materials. The presence of competitors and antibody is shown by a + sign above the appropriate lane; Specific= unlabelled CRE oligonucleotide, the same as that used for labelling (1,000-fold excess), Mutated= unlabelled oligonucleotide mutated in the CRE site (1,000-fold excess), cons CRE= unlabelled consensus CRE oligonucleotide, (1,000-fold excess), CREB= CREB antibody. The induced complex is indicated by the arrow 1. Arrow 2 indicates the supershift complex where the nuclear proteins were incubated with CREB antibody. Arrow 3 shows the unbound labelled probe.

5.3 Discussion

The previous chapter showed that IL-1β and LPS significantly induce rat BKB_1 receptor mRNA levels via a transcriptional mechanism. This chapter further characterises the molecular mechanisms of transcriptional regulation of the rat BKB_1 receptor gene.

A cell line expressing a dominant negative IκB-α protein was used to analyse the role of NF-κB activation in the up-regulation of BKB_1 receptor mRNA. This IκB-mut protein has been shown to prevent the IL-1β, TNF-α and LPS-mediated increase in expression of reporter constructs containing NF-κB binding sites (Brockman *et al.*, 1995; Krushel *et al.*, 1999). The present study has verified expression of IκB-mut in the stable cell line, however, confirmation of the decrease in NF-κB binding has not been shown directly. These data show that part of the IL-1β and LPS-mediated up-regulation of BKB_1 receptor mRNA uses the NF-κB activation pathway. Moreover, these findings indicate that multiple signalling pathways, including the NF-κB activation pathway, contribute to the IL-1β and LPS-induced BKB_1 receptor mRNA levels. Studies in this chapter have identified a conserved downstream NF-κB-like site and an upstream NF-κB site within the 5' flanking region. EMSA analysis shows that these sites exhibit binding affinity for nuclear proteins when used in competition assays. Characterisation of the nuclear proteins binding to the NF-κB site has not been determined in this study. Increased binding was observed in extracts from IL-1β and LPS-treated cells consistent with studies showing an increase in NF-κB nuclear localisation following IκB degradation (Baeuerle and Baltimore, 1996). However, a

change in the binding affinity of the NF-κB site following the addition of inducing agents cannot be eliminated. Similarly, EMSA analysis of the human NF-κB-like sites has shown high affinity binding to nuclear proteins from IL-1β, TNF-α, desArg^{10}kallidin or LPS-treated cells (Schanstra *et al.,* 1998; Ni *et al.,* 1998a). Schanstra *et al.,* (1998) showed that the upstream NF-κB-like site binding complexes contained p65, p50 and c-Rel forms of NF-κB.

Recent studies have demonstrated that IL-1 intracellular signalling involves PI 3-kinase (Donaldson *et al.,* 1996; Reddy *et al.,* 1997, Marmiroli *et al.,* 1998). Examination of the effect of an inhibitor of PI 3-kinase shows that this pathway plays an important role in the IL-1β and LPS-mediated up-regulation of the BKB_1 receptor. The downstream targets for activated PI 3-kinase in the IL-1β and LPS-mediated up-regulation of the BKB_1 receptor remain to be determined. Interestingly, IL-1 activated PI 3-kinase has been shown to activate NF-κB by phosphorylation in a parallel cascade to the IκB degradation (Marmiroli *et al.,* 1998; Sizemore *et al.,* 1999). Other studies have shown that one of the downstream targets of PI 3-kinase, the serine/threonine kinase Akt or PKB can activate the NF-κB/IκB degradation pathway by phosphorylation of IKK1 (Ozes *et al.,* 1999; Romashkova and Makarov, 1999). In addition, PI 3-kinase activated Akt/PKB induces Serine 133 phosphorylation of CREB and recruitment of CREB binding protein (CBP) (Du and Montminy, 1998). PI 3-kinase has also been shown to activate MAPK (Bondeva *et al.,* 1998) and various isoforms of PKC and PLC (Rameh and Cantley, 1999). Consequently, numerous signal transduction cascades involving PI 3-kinase are possible.

dbcAMP-induced expression of the BKB_1 receptor mRNA was demonstrated in Chapter 4, although the mechanism of regulation has not been determined. cAMP results in phosphorylation of CREB by activation of protein kinase A (PKA) which is regulated by changes in intracellular cAMP levels (Gonzalez and Montminy, 1989). Following binding of cAMP to the regulatory subunit of PKA, the catalytic subunit of PKA translocates to the nucleus, where phosphorylation of CREB occurs at serine 133 in a region called the kinase inducible domain (KID) (Hagiwara *et al.,* 1993). In addition, two glutamine rich regions within the CREB protein, called Q1 and Q2 are required for transactivation (Montminy, 1997). Phosphorylated CREB, bound to the CRE site, then recruits the co-activator, CBP a homologue of the EIA-associated protein p300 (Chrivia *et al.,* 1993; Kwok *et al.,* 1994; Eckner *et al.,* 1994; Parker *et al.,* 1996). Accumulating evidence suggests that CBP/p300 functions as a molecular integrator that co-ordinates complex signalling events at the transcriptional level (Kamei *et al.,* 1996; Goldman *et al.,* 1997; Giles *et al.,* 1998). Several mechanisms by which CBP/p300 regulates transcription have been shown, including interaction with components of the basal transcription machinery and chromatin re-modelling by the intrinsic histone acetyltransferase (HAT) activity (reviewed in Goldman *et al.,* 1997; De Cesare *et al.,* 1999). Activated PKA has also been shown to phosphorylate other members of the CREB/activating transcription factor (ATF) family such as, cAMP response element modulator (CREM) and ATF-1 (Sassone-Corsi, 1995), and other transcription factors including C/EBPβ (Trautwein *et al.,* 1994) and NF-κB (Muroi and Suzuki, 1993). Studies in the present chapter have shown that NF-κB is not involved in the dbcAMP-mediated up-regulation of the BKB_1 receptor mRNA. However, the role of specific transcription factors in the dbcAMP-mediated up-regulation of the BKB_1 receptor remains to be determined. A role for the dbcAMP-

mediated phosphorylation of CREB is supported by EMSA studies showing that the CRE site is capable of binding CREB *in vitro*. No change in binding activity was observed between control and IL-1β or LPS-treated nuclear extracts, however, the affinity of CREB for a CRE site is generally accepted not to be a regulated process (Andrisani, 1999). Investigation of CREB phosphorylation in control and treated conditions would determine whether IL-1β and LPS can regulate this process. In addition to the cAMP-PKA signalling pathway of CREB activation, CREB can also be phosphorylated by the MAPK pathways, ERK and p38 (Tan *et al.*, 1996; Xing *et al.*, 1996, 1998; DeCesare *et al.*, 1998), by Ca^{2+}/calmodulin-dependent protein kinases (Sheng *et al.*, 1991; Sun *et al.*, 1994), and by PI 3-kinase activation of Akt/PKB (Du and Montminy, 1998). CREB activation by these pathways results from phosphorylation of serine 133, as targetted by PKA. Whether IL-1β and LPS activate the cAMP signalling pathway and/or CREB phosphorylation and furthermore, whether these signal transduction components converge remains to be determined.

Sequence analysis of the 5' flanking region of the rat BKB_1 receptor gene identified a non-classical TATA box and numerous putative transcription factor binding sequences. Alignment of the rat and human 5' regulatory regions exhibited high identity, with a number of conserved transcription factor binding sites within the proximal 100 bp. The conservation of these binding sites suggests an important regulatory function for these elements. Similarly, rodent and human 5' flanking sequences for the cytokine inducible genes, COX-2 (Lukiw and Bazan, 1998) and E-selectin (Becker-Andre *et al.*, 1992) have retained important recognition sites. Transient transfection analysis of chimeric constructs spanning the 5' regulatory region of the BKB_1 receptor identified two regions containing positive regulatory

elements and a region containing negative regulatory elements. Furthermore, the positive regulatory elements from –477 to –72 bp exhibit cell type-specific regulation. In addition, reduced expression levels of the constructs in H4 cells implies the involvement of other cell type-specific factors which could act to repress or activate the BKB_1 receptor gene accordingly. Similarly, examination of the human BKB_1 receptor regulatory region has shown cell type-specificity (Yang *et al.,* 1998a; Ni *et al.,* 1998a).

Intriguingly, the reporter gene constructs presented in this study showed no inducibility following the addition of IL-1β or LPS. Initial observations suggested that regulatory elements located either upstream or downstream of the cloned reporter constructs are required to induce transcription of the gene. However, the lack of inducible activity of the human BKB_1 receptor-directed reporter gene constructs described by Schanstra *et al.,* (1998) indicated that this was not the case. Interestingly, treatment of transiently transfected cells with LPS showed only 43% of the maximal BKB_1 receptor mRNA levels compared with control LPS-treated cells. In addition, the transfection efficiency in these cells is approximately 50%. These observations suggest that the transient transfection procedure could either reduce the LPS responsiveness of the entire cell population, or alternatively, prevent the up-regulation of the BKB_1 receptor gene in the population of successfully transfected cells. One possible mechanism for these findings is that binding of transcription factors and/or co-activators to the more accessible DNA structure of the transfected DNA may transrepress the endogenous gene expression by competition and dilution of limiting factors below a threshold necessary for induction. A number of studies have established that although nucleosomes are deposited onto non-replicating DNA

in transfected cells, the overall structure is disorganised, suggesting the DNA is more open and accessible than cellular chromatin (Cereghini and Yaniv, 1984; Jeong and Stein, 1994; Reeves *et al.,* 1995; Archer, 1992). Furthermore, possible differences in chromatin structure between the transfected DNA and the endogenous gene, provide a tangible explanation for the lack of inducibility of the reporter constructs. Thus, according to this hypothesis the transfected DNA may reflect the chromatin re-modelled actively transcribed gene, or alternatively, chromatin re-modelling may recruit an activating transcription factor that cannot be acquired by the transient template. Indeed, for a number of genes, significant differences occur between the transfected DNA and the endogenous promoter that are thought to be a consequence of differences in chromatin structure (Kim *et al.,* 1993; Bulla *et al.,* 1992; Cannon et al. 1994; Kitabayashi *et al.,* 1992; Archer *et al.,* 1992). Further studies are required to investigate a possible role for chromatin-remodelling in the regulation of the BKB_1 receptor gene.

In summary, these data have shown a role for the transcription factors, NF-κB and CREB in the regulation of the BKB_1 receptor gene. The IL-1β and LPS-mediated increase in transcription of the BKB_1 receptor gene involves activation of PI 3-kinase with a bifurcation in the activated signal transduction cascades resulting in NF-κB-dependent and and NF-κB-independent pathways of regulation.

Chapter 6

General Discussion

The demonstration of a role for inducible BKB_1 receptors in pathophysiological states, has resulted in increased interest in regulation of this receptor subtype. Pharmacological studies have shown that BKB_1 receptor responsiveness is up-regulated in biological systems following the addition of bacterial materials and cytokines. A large number of these studies have been characterised in rodent models of tissue injury and inflammation. However, a question that has remained unresolved from these studies is; how do cytokines and bacterial agents increase BKB_1 receptor expression? Although *de novo* synthesis of the BKB_1 receptors has been demonstrated from pharmacological studies, little was known about the regulation process due to a lack of molecular tools such as antibodies and cDNA clones. This study has addressed the regulation of BKB_1 receptors by cloning the rat BKB_1 receptor gene and using this as a probe to analyse the structure of the gene and mechanisms of up-regulation following the addition of inducing agents and inhibitors.

Molecular cloning of the rat BKB_1 receptor gene

Cloning and characterisation of the rat BKB_1 receptor gene has allowed detailed analysis of the receptor protein coding sequence. To date, studies have reported the isolation of cDNA clones for the human (Menke *et al.,* 1994; Jones *et al.,* 1999), rabbit (MacNeil *et*

al., 1995), mouse (Pesquero *et al.,* 1996; Hess *et al.,* 1996) and rat BKB_1 receptors (this study, reported in Jones *et al.,* 1999; Ni *et al.,* 1998b). These studies have been vital in determining a detailed pharmacological profile for the receptors and have shown that the relative potencies of the agonists for the BKB_1 receptors vary between species. The availability of these clones opens new avenues for identifying the regions of the receptor which determine species-specific agonist affinites. One approach could be the synthesis of recombinant receptor proteins, such as chimeric receptors, whereby regions of the homologous receptors are exchanged between species, or constructs with more subtle single amino acids mutations, followed by analysis in heterologous expression systems. These studies may aid in development of novel potent antagonists for the BKB_1 receptor. In addition, this research will be useful in extrapolating data obtained from studies using rat models to determination of the effects in humans. Cloning of the rat receptor should also facilitate in the production of antipeptide antibodies. Although human BKB_1 receptor antibodies have been reported, in both cases the most effective peptides correspond to the carboxy terminal domain of the receptor (Hess *et al.,* 1996; Schanstra *et al.,* 1998). Since this region differs significantly between the rodent and human receptors these antibodies cannot detect the rodent receptors. The generation of rat antibodies would provide a powerful tool in the study of receptor protein up-regulation. Immunohistochemical studies would allow the determination of the cell types which express the BKB_1 receptor *in vivo.* These studies may also prove particularly interesting in elucidating the cellular location of the BKB_1 receptors that mediate hyperalgesia.

Expression of the BKB_1 receptor gene

Using the rat BKB_1 receptor as a specific probe, this study shows for the first time, a quantitative tissue expression pattern for the BKB_1 receptor mRNA. Although studies have shown a role for both BKB_1 and BKB_2 receptors in cardiovascular and renal function of normal dogs (Staszewska-Wooley et al., 1991; Rhaleb *et al.,* 1989; Lortie *et al.,* 1992; Nakhostine et al., 1993; Belichard et al., 1996), very little is known about the role of BKB_1 receptors in healthy tissues. The higher levels of constitutive BKB_1 receptor mRNA expression in the uterus may therefore be of particular interest in understanding the role of BKB_1 receptors in healthy tissues. Furthermore, these findings are an important consideration in the use of BKB_1 receptor antagonists as therapeutic agents. The studies in this thesis have confirmed the molecular up-regulation of the BKB_1 receptor following the *in vivo* administration of LPS. All the tissues examined up-regulated BKB_1 receptor mRNA although the degree of up-regulation varied markedly between tissues. Further investigation may determine whether tissue-specific activators or repressors control up-regulation. This report demonstrates that the rat embryonic cell line, JTC-19, is an excellent model system in which to study the BKB_1 receptor up-regulation. A recent study has shown that the rat vascular smooth muscle cell line, A10, can up-regulate BKB_1 receptor mRNA in response to inducing agents (Ni *et al.,* 1998b). However, evidence presented in this thesis shows that the increase in BKB_1 receptor mRNA levels in JTC-19 cells resembles the induction observed in highly up-regulated tissues *in vivo*. Therefore, JTC-19 cells should be useful for the analysis of other aspects of the role of BKB_1 receptors, such as signal transduction pathways and desensitisation mechanisms. In addition, the identification of a non-BKB_1 receptor expressing cell line reinforces a need for tissue/cell type-specific transcriptional regulation.

This study shows that the inducing agents, IL-1β, TNF-α and LPS increase BKB_1 receptor mRNA levels in a time-dependent manner, with LPS being the most potent mediator of BKB_1 receptor expression. This thesis has shown that the major effects of LPS are not mediated by the secondary release of IL-1 and thus utilise a distinct cell surface receptor. Recent studies have shown that members of the TLR family, which utilise the IL-1R signalling pathway, are important mediators of LPS signalling (Yang *et al.,* 1998b; 1999; Kirchshning *et al.,* 1998; Chow *et al.,* 1999). However, further studies are needed to determine the cell surface receptor that transduces the effects of LPS in the up-regulation of the rat BKB_1 receptor gene. Molecular determination of the inducing effects of these mediators has strengthened the role of these mediators as inducing agents of BKB_1 receptor expression *in vivo.* It would therefore, be interesting to examine the extent to which these mediators exert synergistic effects. One could postulate that the complex network of interactions between these mediators *in vivo* may contribute to the maintenance of elevated levels of BKB_1 receptors observed at the sites of tissue injury.

Mechanisms of BKB_1 receptor gene regulation

Maximal levels of IL-1β and LPS-induced BKB_1 receptor mRNA levels were shown to be attained through increased transcription in a process independent of new protein synthesis. This increased transcription generates multiple transcripts by read-through of the RNA polymerase at the 3' end of the gene. Given that these transcripts exhibit equivalent profiles of expression and mRNA half-lives, their functional significance, if any, remains unclear. Evidence has been presented showing the involvement of NF-κB in the IL-1β and LPS-mediated transcriptional up-regulation of the BKB_1 receptor gene.

In addition, IL-1β and LPS-mediated activation of PI 3-kinase are also involved in BKB_1 receptor up-regulation. Given these roles for NF-κB and PI 3-kinase it would be interesting to determine if and how these pathways converge. Evidence exists for the involvement of PI 3-kinase in both the IκB degradation pathway (Ozes *et al.,* 1999; Romashkova and Makarov, 1999) and the parallel pathway leading to phosphorylation of NF-κB (Sizemore *et al.,* 1999). In addition to the NF-κB-dependent pathway this study has provided evidence for an NF-κB-independent pathway for IL-1β and LPS-mediated transcriptional up-regulation of the BKB_1 receptor gene. Further investigations are required to determine the components of this pathway although one potential candidate is activated CREB. Studies presented in this thesis have shown that the addition of dbcAMP up-regulates BKB_1 receptor expression via an NF-κB-independent pathway. Additional studies are required to further delineate the signal transduction pathways involved in both the NF-κB-dependent and independent mechanisms of BKB_1 receptor induction. The availibility of a plethora of tools are available to examine these pathways, including specific inhibitors and activators, dominant negative and constitutively active mutants, *in vitro* kinase assays and phosphorylation-specific antibodies, will allow these pathways to be closely examined.

Preliminary studies have provided evidence for the existence of cell type-specific regulatory cis elements in the BKB_1 receptor gene. However, in contrast to studies by Ni *et al.,* (1998b), the rat BKB_1 receptor reporter gene expression used in this thesis was not induced following the addition of IL-1β or LPS. The reasons for these differences are unclear, although the transiently transfected DNA in these studies may not adequately model the chromatin configuration necessary for BKB_1 receptor induction.

Examination of stable, replicating reporter constructs would allow these issues to be resolved. Comparitive analysis of these stable replicating templates in BKB_1-expressing and non-expressing cell lines combined with mutagenesis studies would be useful for further characterisation of the elements involved in BKB_1 receptor regulation. The identification of numerous putative regulatory cis elements in the 5' flanking region of the rat BKB_1 receptor gene suggests that a complex transcription factor network may be involved in the receptor gene regulation. Interestingly, the transcription of a number of genes can be increased via the synergistic activation of transcription factors. In particular, NF-κB has been shown to activate transcription synergistically with Sp1 (Perkins *et al.,* 1994), the C/EBP families (Matsusaka *et al.,* 1993; Kunsch *et al.,* 1994; LeClair *et al.,* 1992) and AP-1 (Stein *et al.,* 1993). Such interactions have been shown to magnify IL-1β and LPS-meditated responses (Matsusaka *et al.,* 1993; Klampfer *et al.,* 1994; Ray *et al.,* 1995; Collins *et al.,* 1995). Therefore, it would be interesting to determine whether a similar synergistic mechanism exists to increase BKB_1 receptor expression. *In vitro* studies presented in this thesis have shown that the CRE site can bind CREB and the NF-κB sites exhibit weak nuclear protein binding affinity. However it is possible that additional 5' flanking sequences are required to facilitate binding. Analysis of DNase I protection mapping could be used as an alternative approach to determine which regions of the BKB_1 receptor gene bind nuclear proteins.

The inability to induce reporter gene expression has led to a proposed role for chromatin re-modelling in BKB_1 receptor transcriptonal regulation. Two important chromatin re-modelling systems are involved in the transcription process. One system includes several members of the evolutionarily conserved SWI/SNF family with ATP-dependent nucleosome destabilising activity (reviewed in Tsukiyama and Wu, 1997); the other

system involves post-translational modifications of chromatin components, in particular histone acetylation (reviewed in Berger, 1999). For a given gene it is thought that the binding of transcriptional activators recruits large co-activator complexes which function in part as chromatin re-modelling factors. Many of the individual proteins within these co-activator complexes are shared between the different complexes. Recent studies suggest that the role of these co-activators are dependent upon alternative interaction interfaces in the co-activator configuration (Korzus et al., 1998; Perissi *et al.,* 1999). Particularly interesting, is the multi-functional co-activator CBP/p300. This was originally identified as a protein that interacts with phosphorylated CREB (Chrivia *et al.,* 1993; Kwok *et al.,* 1994), however, more recent studies have shown that these co-factors make contact with and connect the functions of many transcription factors, including NF-κB (Gerristen *et al.,* 1997; Perkins *et al.,* 1997; Zhong *et al.,* 1998). Thus, CBP/p300 is an attractive target to co-ordinate the findings from these studies. This co-activator acts as an integrator of multiple signal transduction pathways in the nucleus and functions as a "molecular scaffold" in the assembly of multiprotein complexes (Kamei *et al.,* 1996; Perissi *et al.,* 1999). In addition, competition for limiting levels of CBP/p300 between signal transduction pathways is thought to contribute to the control of specific gene expression patterns (Kamei *et al.,* 1996; Zhang *et al.,* 1996; Wadgaonkar *et al.,* 1999). Furthermore, regulation of transcription by CBP/p300 has been demonstrated by a number of mechanisms including its intrinsic HAT activity (Balbas *et al.,* 1998; Perissi *et al.,* 1999). Experiments using anti-CBP antibodies could be used to determine whether this co-activator plays a crucial role in co-ordinating the multiple transduction pathways activated by IL-1β or LPS in the inducible BKB_1 receptor expression.

In conclusion, it is hoped that the work presented in this thesis will stimulate and aid further experimentation into the regulation of BKB_1 receptor gene expression. Further studies are required to dissect the signal transduction pathways that are involved in the up-regulation and the convergence of these pathways on specific transcription factors. Specifically and importantly, the role of chromatin-remodelling and the analysis of co-activator complexes will need to be investigated. Knowledge of the molecular mechanisms of the regulation and induction of the BKB_1 receptor gene will lead to a better understanding of the role of the BKB_1 receptor in healthy and injured tissues. Moreover, this research may be useful in understanding the mechanisms of regulation of other inducible genes at the site of tissue injury. The determination of these mechanisms could allow the development of novel therapeutic tools for controlling BKB_1 receptor gene expression.

AbdAlla, S., Godovac-Zimmermann, J., Braun, A. and Roscher, A.A. (1996) Structure of the bradykinin B_2 receptors' amino terminus. *Biochemistry* **35,** 7514-7519.

Abelous, J.E. and Bardier, E. (1909) Les substances hypotensives de l'urine humaine normale. *C.R. Soc. Biol.* **66**, 511-512.

Ahluwalia, A. and Perretti, M. (1996) Involvement of bradykinin B_1 receptors in the polymorphonuclear leukocyte accumulation induced by IL-1β in vivo in the mouse. *J. Immunol.* **156,** 269-274.

Alfie, M.E., Alim, S., Mehta, D., Shesley, E.G. and Carretero, O.A. (1999) An enhanced effect of arginine vasopressin in bradykinin B_2 receptor null mutant mice. *Hypertension* **33,** 1436-1440.

Alfie, M.E., Yang, X., Hess, F. and Carretero, O.A. (1996) Salt-sensitive hypertension in bradykinin B_2 receptor knockout mice. *Biochem. Biophys. Res.Comm.* **224,** 625-630.

Allogho, S.N., Gobeil, F., Pheng, L.H., Nguyen-Le, X.K., Neugebauer, W. and Regoli, D. (1995) Kinin B_1 and B_2 receptors in the mouse. *Can. J. Physiol. Pharmacol.* **73,** 1759-1764.

Andrisani, O.M. (1999) CREB-mediated transcriptional control. *Crit. Rev. Euk. Gene Exp.* **9,** 19-32.

Anrather, J., Csizmadia, V., Soares, M.P. and Winkler, H. (1999) Regulation of NF-κB RelA phosphorylation and transcriptional activity by $p21^{ras}$ and protein kinase Cζ in primary endothelial cells. *J. Biol. Chem.* **274,** 13594-13603.

Aramori, I., Zenkoh, J., Morikawa, N., O'Donnell, N., Asano, M., Nakamura, K., Iwami, M., Kojo, H. and Notsu, Y. (1997) Novel subtype-selective nonpeptide bradykinin receptor antagonists FR167344 and FR173657. *Mol. Pharmacol.* **51,** 171-176.

Archer, T.K., Lefebvre, P., Wolford, R.G. and Hager, G.L. (1992) Transcription factor loading onto the MMTV promoter: A bimodal mechanism for promoter activation. *Science* **255,** 1573-1576.

Asakura, S., Hurley, R. W., Skorstengaard, K., Ohkubo, I. and Mosher, D. F. (1992) Inhibition of cell adhesion by molecular weight kininogen. *J. Cell Biol.* **116,** 465-476.

Audet, R., Petitclerc, E., Drapeau, G., Rioux, F. and Marceau, F. (1994) Further analysis of the upregulation of bradykinin B_1 receptors in isolated rabbit aorta by using metabolic inhibitors. *Eur. J. Pharmacol.* **271,** 551-555.

Audet, R., Rioux, F., Drapeau, G. and Marceau, F. (1997) Cardiovascular effects of Sar-[D-Phe^8]des-Arg^9-bradykinin, a metabolically protected agonist of the B_1 receptor for kinins, in the anesthetized rabbit pretreated with a sublethal dose of bacterial lipopolysaccharide. *J. Pharmacol. Exp. Ther.* **280,** 6-15.

Austin, K.E., Faussner, A., Robinson, H.E., Chakravarty, S., Kyle, D.J., Bathon, J.M. and Proud, D. (1997) Stable expression of the human kinin B_1 receptor in Chinese hamster ovary cells. *J. Biol. Chem.* **272,** 11420-11425.

Babbedge, R., Dray, A. and Urban, L. (1995) Bradykinin depolarises the rat isolated superior cervical ganglion via B_2 receptor activation. *Neurosci. Lett.* **193,** 161-164.

Bachvarov, D.R., Hess, J.F., Menke, J.G., Larrivee, J-F. and Marceau, F. (1996) Structure and genomic organization of the human B_1 receptor gene for kinins. *Genomics* **33**, 374-381.

Bachvarov, D.R., Landry, M., Houle, S., Pare, P. and Marceau, F. (1998b) Altered frequency of a polymorphic allele of the kinin B_1 receptor gene in inflammatory bowel disease. *Gasteroenterology* **115,** 1045-1048.

Bachvarov, D.R., Landry, M., Pelletier, IL-1β ., Chevrette, M., Betard, C., Houde, I., Bergeron, J., Lebel, M. and Marceau, F. (1998a) Characterization of two polymorphic sites in the human kinin B_1 receptor gene: Altered frequency of an allele in patients with a history of end stage renal failure. *J. Am. Soc. Nephrol.* **9,** 598-604.

Bader, M., Aranjo, R.C., Oliveira, S.M., Kettritz, R., Walther, T. and Pesquero, J.B. (1997) Phenotypic characterization of mice lacking the kinin B_1 receptor [abstract]. *Hypertension* **30,** 998.

Baeuerle, P.A. and Baltimore, D. (1996) NF-κB: ten years after. *Cell* **87,** 13-20.

Baldwin, A.S. (1996) The NF-κB and IκB protein: new discoveries and insights. *Annu. Rev. Immunol.* **14,** 649-681.

Bankers-Fulbright, J.L., Kalli, K.R. and McKean, D.J. (1996) Interleukin-1 signal transduction. *Life Sci.* **59,** 61-83.

Barrios-Rodiles, M., Tiraloche, G. and Chadee, K. (1999) Lipopolysaccharide modulates cyclooxygenase-2 transcriptionally and posttranscriptionally in human macrophages independently from endogenous IL-1β and TNF-α. *J. Immunol.* **163,** 963-969.

Bascands, J-L, Pecher, C., Rouaud, S., Emond, C., Tack, J.L., Bastie, M.J., Burch, R., Regoli, D. and Girolami, J.P. (1993) Evidence for existence of two distinct bradykinin receptors on rat mesangial cells. *Am. J. Physiol. Renal Fluid Electrolyte Physiol.* **264/3** 33-3 (F548-F556).

Bastian, S., Lollier B., Paquet, J.L. and Pruneau, D. (1997) Stable expression of human kinin B_1 receptor in 293 cells: Pharmacological and functional characterisation. *Br. J. Pharmacol.* **122,** 393-399.

Bastian, S. Paquet, J., Robert, C., Cremers, B., Loillier, B., Larrivee, J., Bachvarov, D.R., Marceau, F. and Pruneau, D. (1998) Interleukin 8 (IL-8) induces the expression of kinin BKB_1 receptor in human lung fibroblasts. *Biochem. Biophys. Res. Commun.* **253,** 750-755.

Bathon, J.M., Manning, D.C., Goldman, D.W., Towns, M.C. and Proud, D. (1992) Characterization of kinin receptors on human synovial cells and upregulation of receptor number by interleukin-1. *J. Pharmacol. Exp. Ther.* **260,** 384-392.

Batt, D.B., Luo, Y. and Carmichael, G.G. (1994) Polyadenylation and transcription termination in gene constructs containing multiple tandem polyadenylation signals. *Nucleic.Acid.Res.*. **22**, 2811-2816.

Baumgarten, C.R., Lehmkuhl, B., Henning, R., Brunnee, T., Dorow, P., Schilling, W. and Kunkel, G. (1992) Bradykinin and other inflammatory mediators in BAL-fluid from patients with active pulmonary inflammation. *Agents Actions Suppl.* **3,** 475-481.

Becker-Andre, M., Hooft van Huijsduijnen, R., Losberger, C., Whelan, J. and DeLamarter, J.F. (1992) Murine endothelial leukocyte-adhesion molecule 1 is a close structural and functional homologue of the human protein. *Eur. J. Biochem.* **206,** 401-411.

Bedi, G.S., Balwierczak, J. and Back, N. (1983) Rodent kinin-forming enzyme systems I. Purification and characterisation of plasma kininogen. *Biochem.Pharmacol.* **32,** 2061-2069.

Belichard, P., Loillier, B., Paquet, J.L., Luccarini, J.M. and Pruneau, D. (1996) Haemodynamic and cardiac effects of kinin B_1 and B_2 receptor stimulation in conscious instrumented dogs. *Br. J. Pharmacol.* **117,** 1565-1571.

Berger, S.L. (1999) Gene activation by histone and factor acetyltransferases. *Curr. Opin. Cell Biol.* **11,** 336-341.

Bergmann, M., Hart, L., Lindsay, M., Barnes, P.J. and Newton, R. (1998) IκBα degradation and nuclear factor-κB DNA binding are insufficient for interleukin-1β and tumor necrosis factor-α-induced κB-dependent transcription. *J. Biol. Chem.* **273,** 6607-6610.

Beyaert, R., Cuenda, C., Vanden Berghe, W., Plaisance, S., Lee, J.C., Haegemann, G., Cohen, P. and Fiers, W. (1996) The p38/ERK mitogen-activated protein kinase regulates regulates interleukin-6-synthesis in response to tumor necrosis factor. *EMBO J.* **15,** 1914-1923.

Bhoola, K.D., Figueroa, C.D. and Worthy, K. (1992) Bioregulation of kinins: Kallikreins, kininogens and kininases. *Pharmacol. Rev.* **44,** 1-80.

Bhoola, R., Ramsaroop, R., Naidoo, S., Muller, E.W. and Bhoola, K.D. (1997) Kinin receptor status in normal and inflammed gastric mucosa. *Immunopharmacology* **36,** 161-165.

Bird, T.A., Schooley, K., Dower, S.K., Hagen, H. and Virca, G.D. (1997) Activation of nuclear transcription factor NF-κB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. *J. Biol. Chem.* **272,** 32606-32612.

Bkaily, G., Jaalouk, D., Jacques, D., Economos, D., Hassan, G., Simaan, M., Regoli, D. and Pothier, P. (1997) Bradykinin activates R-, T- and L-type Ca^{2+} channels and induces a sustained increase of nuclear Ca^{2+} in aortic vascular smooth muscle cells. *Can. J. Physiol. Pharmacol.* **75,** 652-660.

Blaukat, A., Pizard, A., Rajerison, R.M., Alhenc-Gelas, F., Muller-Esterl, W. and Dikic, I. (1999) Activation of mitogen-activated protein kinase by the bradykinin B_2 receptor is independent of receptor phosphorylation and phosphorylation-triggered internalization. *FEBS Letts.* **451,** 337-341.

Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R. and Wymann, M.P. (1998) Bifurcation of lipid and protein kinase signals of PI3Kγ to the protein kinases PKB and MAPK. *Science* **282,** 293-296.

Bonizzi, G., Piette, J., Schoonbroodt, S., Merville, M-P., and Bours, V. (1999) Role of the protein kinase C λ/ι isoform in nuclear factor-kB activation in interleukin-1β or tumor necrosis factor-α: Cell type specificities. *Biochem. Pharmacol.* **57,** 713-720.

Borkowski, J.A., Ransom, R.W., Seabrook, G.R., Trumbauer, M., Chen, H., Hill, R.G., Strader, C.D. and Hess, J.F. (1995) Targeted disruption of a B_2 bradykinin receptor gene in mice eliminates bradykinin action in smooth muscle and neurons. *J. Biol. Chem.* **270,** 13706-13710.

Bouchard, J.F., Chouinard, J. and Lamontagne, D. (1998) Role of kinins in the endothelial protective effect of ischaemic preconditioning. *Br. J. Pharmacol.* **123,** 413-420.

Bouthillier, J., Deblois, D. and Marceau, F. (1987) Studies on the induction of pharmacological responses to des-Arg^9-bradykinin in vitro and in vivo. *Br. J. Pharmacol.* **92,** 257-264.

Brockman, J.A., Scherer, D.C., McKinsey, T.A., Hall, S.M., Qi, X., Lee, W.Y. and Ballard, D.W. (1995) Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. *Mol. Cell. Biol.* **15,** 2809-2818.

Bulla, G.A., DeSimone, V., Cortese, R. and Fournier, R.E.K. (1992) Extinction of a1-antitrypsin gene expression in somatic cell hybrids: evidence for multiple controls. *Genes Dev.* **6,** 316-327.

Burch, R.M. and DeHaas, C. (1990) A bradykinin antagonist inhibits carrageenan edema in rats. *Naunyn-Schmiedeberg Arch. Pharmacol.* **342,** 189-193.

Burch, R.M. and Kyle, D.J. (1992) Recent developments in the understanding of bradykinin receptors. *Life Sciences* **50,** 829-838.

Burkard, M., ZU.S.A.ck, J.S., Jones, S., Francis, M., Whalley, E.T., Stewart, J.M. and Gera, L. (1996) Comparitive profile of novel potent bradykinin antagonists at human B_1 and B_2 receptors. *Immunopharmacology* **33,** 186-190.

Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J-L., Di Marco, F., French, L. and Tschopp, J. (1998) MyD88, an adapter protein involved in interleukin-1 signaling. *J. Biol. Chem.* **273,** 12203-12209.

Busser, B.W., Hammond, T.G., Bjorling, D.E. and Saban, R. (1996) Lipopolysaccharide upregulates bradykinin 1 receptors in the isolated mouse bladder. *J. Urol.* **160,** 2267-2273.

Butt, S.K., Dawson, L.G. and Hall, J.M. (1995) Bradykinin BKB_1 receptors in the rabbit urinary bladder: Induction of responses, smooth muscle contraction and phosphatidylinositol hydrolysis. *Br. J. Pharmacol.* **114,** 612-617.

Campos, A.H. and Calixto, J.B. (1994) Mechanisms involved in the contractile responses of kinins in rat portal vein rings: Mediation by B_1 and B2 receptors. *J. Pharmacol. Exp. Ther.* **268/2,** 902-909.

Campos, M.M. and Calixto, J.B. (1995) Involvement of B_1 and B_2 receptors in bradykinin-induced rat paw oedema. *Br. J. Pharmacol.* **114,** 1005-1013.

Campos, M.M. Henriques, M.G.M.O. and Calixto, J.B. (1997) The role of B_1 and B_2 receptors in oedema formation after long-term treatment with *Mycobacterium bovis* bacillus calmette-guerin (BCG) *Br. J. Pharmacol.* **120,** 502-508.

Campos, M.M., Mata, L.V. and Calixto, J.B. (1995) Expression of BKB_1 kinin receptors mediating paw oedema and formalin-induced nociception. Modulation by glucorticoids. *Can. J. Physiol. Pharmacol.* **73,** 812-819.

Campos, M.M., Souza, G.E.P. and Calixto, J.B. (1996) Upregulation of B_1 receptor mediating des-Arg^9-BK-induced rat paw oedema by systemic treatment with bacterial endotoxin. *Br. J. Pharmacol.* 117, 793-798.

Cannon, P., Kim, S-H., Ulich, C. and Kim, S. (1994) Analysis of Tat function in human immunodeficiency virus type 1-infected low-level-expression cell lines U1 and ACH-2. *J. Virol.* **68,** 1993-1997.

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. and Goeddel, D.V. (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* **383,** 443-446.

Capeyrou, R., Riond, J., Corbani, M., Lepage, J-F., Bertin, B. and Emorine, L.J. (1997) Agonist-induced signaling and trafficking of the μ-opioid receptor: role of serine and threonine residues in the third cytoplasmic loop and C-terminal domain. *FEBS Lett.* **415,** 200-205.

Carl, V.S., Moore, E.E., Moore, F.A. and Whalley, E.T. (1996) Involvement of bradykinin B_1 and B_2 receptors in human PMN elastase release and increase in endothelial cell monolayer permeability. *Immunopharmacology* **33,** 325-329.

Carter, A.B., Monick, M.M. and Hunninghake, G.W. (1999) Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am. J. Respir. Cell. Mol. Biol.* **20,** 751-758.

Carter, D.B., Deibel, M.R.J., Dunn, C.J., Tomich, C.S.C., Laborde, A.L., Slightom, J.L., Berger, A.E., Bienkowski, M.J., Sun, F.F., McEwan, R.N., Harris, P.K.W., Yem, A.W., Waszak, G.A., Chosay, J.G., Sieu, L.C., Hardee, M.M., Zurcher-Neely, H.A., Reardon, I.M., Heinrickson, R.L., Truesdell, S.E., Shelly, J.A., Eessalu, T.E., Taylor, B, M. and Tracey, D.E. (1990) Purification, cloning, expression and biological characterisation of an interleukin-1 receptor antagonist protein. *Nature* **344,** 633-638.

Cereghini, S. and Yaniv, M. (1984) Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. *EMBO J.* **3,** 1243-1253.

Chahine, R., Adam, A., Yamaguchi, N., Gaspo, R., Regoli, D. and Nadeau, R. (1993) Protective effects of bradykinin on the ischaemic heart: Implications of the B_1 receptor. *Br. J. Pharmacol.* **108,** 318-322.

Chai, K.X., Ni, A., Wang, D., Ward, D.C., Chao, J. and Chao, L. (1996) Genomic DNA sequence, expression, and chromosomal localization of the human B1 bradykinin receptor gene BDKRB1. *Genomics* **31,** 51-57.

Chandra, G., Cogswell, J.P., Miller, L.R., Godlevski, M.M., Stinnett, S.W., Noel, S.L., Kadwell, S.H., Kost, T.A. and Gray, J.G. (1995) Cyclic AMP signalling pathways are important in IL-1β transcriptional regulation. *J. Immunol.* **155,** 4535-4543.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162,** 156-159.

Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J. and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* **274,** 10689-10692.

Christiansen, S.C., Proud, D. and Cochrane, C.G. (1987) Detection of tissue kallikrein in the bronchoalveolar lavage fluid of asthmatic subjects. *J. Clin. Invest.* **1,** 188-197.

Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) Phosphorylated CREB binds specifically to nuclear protein CBP. *Nature* **365,** 855-859.

Chu, W., Presky, D.H., Swerlick, R.A. and Burns, D.K. (1994) Alternatively processed human E-selectin transcripts linked to chronic expression of E-selectin in vivo. *J. Immunol.* **153,** 4179-4189.

Churchill, L. and Ward, P.E. (1986) Relaxation of isolated mesenteric arteries by des-Arg^9-bradykinin stimulation of B_1 receptors. *Eur. J. Pharmacol.* **130,** 11-18.

Coelho, M.M., Oliveira, C.R., Pajolla, G.P., Calixto, J.B. and Pela, I.R. (1997) Central involvement of kinin B_1 and B_2 receptors in the febrile response induced by endotoxin in rats. *Br. J. Pharmacol.* **121,** 296-302.

Collins, T., Read, M.A., Neish, A.S., Whitley, M.Z., Thanos, D. and Maniatas, T. (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J.* **9,** 899-909.

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Sims, J.E. and Mantovani, A. (1993) Interleukin 1 type II receptor: a decoy target for IL1 that is regulated by IL4. *Science* **261,** 472-475.

Correa, C.R. and Calixto, J.B. (1993) Evidence for participation of B_1 and B_2 kinin receptors in formalin-induced nociceptive response in the mouse. *Br. J. Pharmacol.* **110,** 193-198.

Couture, R., Mizrahi, J., Regoli, D. and Devroede, G. (1981) Peptides and the human colon: An in vitro pharmacological study. *Can. J. Physiol. Pharmacol.* **59,** 957-964.

Croft, D., McIntyre, P., Wibulswas, A. and Kramer, I. (1999) Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-α in combination with either IL-4 or IL-13 through increased mRNA stability. *Am. J. Pathol.* **154,** 1149-1158.

Cruwys, S.C., Garrett, N.E., Perkins, M.N., Blake, D.R. and Kidd, B.L. (1994) The role of bradykinin B_1 receptors in the maintenance of intra-articular plasma extravasation in chronic antigen-induced arthritis. *Br. J. Pharmacol.* **113,** 940-944.

Curnow, K.M., Pascoe, L. and White, P.C. (1992) Genetic analysis of the human type-1 angiotensin II receptor. *Mol. Endocrinol.* **6,** 1113-1118.

Damas, J. and Adam, A. (1985) The relationship between kininogens and kallikreins in Brown Norway deficient rat plasma. *Mol. Physiol.* **8,** 307-316.

Damas, J., Bourdon, V., Remacle-Volon, G. and Adam, A. (1990) Kinins and peritoneal exudates induced by carrageenin and zymosan in rats. *Br. J. Pharmacol.* **107,** 660-664.

Davis A.J. and Perkins, M.N. (1994a) Induction of B1 receptors in vivo in a model of persistant inflammatory mechanical hyperalgesia in the rat. *Neuropharmacology* **33,** 127-133.

Davis A.J. and Perkins, M.N. (1994b) The involvement of bradykinin B_1 and B_2 receptor mechanisms in cytokine-induced mechanical hyperalgesia in the rat. *Br. J. Pharmacol.* **113,** 63-68.

Davis A.J. and Perkins, M.N. (1996) Substance P and capsaicin-induced mechanical hyperalgesia in the rat knee joint; the involvement of B1 and B2 receptors. *Br. J. Pharmacol.* **118,** 2206-2212.

Davis A.J. and Perkins, M.N. (1997) Des-Arg^9-BK-induced mechanical hyperalgesia and analgesia in the rat: Involvement of IL-1, prostaglandins and peripheral opioids. *Br. J. Pharmacol.* **120,** 74P.

Davis, C.L., Naeem, S., Phagoo, S.B., Campbell, E.A., Urban, L. and Burgess, G.M. (1996) B_1 bradykinin receptors and sensory neurones. *Br. J. Pharmacol.* **118,** 1469-1476.

Deblois, D., Bouthillier, J. and Marceau, F. (1988) Effect of glucorticoids, monokines and growth factors on the spontaneously developing responses of the rabbit aorta to des-Arg^9-bradykinin. *Br. J. Pharmacol.* **93,** 969-977.

Deblois, D., Bouthillier, J. and Marceau, F. (1989) Pharmacological modulation of the up-regulated responses to des-Arg^9-bradykinin in vivo and in vitro. *Immunopharmacology* **17,** 187-198.

Deblois, D., Bouthillier, J. and Marceau, F. (1991) Pulse exposure to protein synthesis inhibitors enhances tissue response to des-Arg^9-bradykinin: Possible role of interleukin-1. *Br. J. Pharmacol.* **103,** 1057-1066.

Deblois, D. and Marceau, F. (1987) The ability of des-Arg^9-bradykinin to relax rabbit isolated mesenteric arteries is acquired during in vitro incubation. *Eur. J. Pharmacol.* **142,** 141-144.

Decarie, A., Adam, A. and Couture, R. (1996) Effect of captopril and icatibant on bradykinin (BK) and des[Arg^9]BK in carrageenan-induced edema. *Peptides* **17,** 1009-1015.

Decarie, A., Drapeau, G., Closset, J., Couture R. and Adam, A. (1994) Development of digoxigenin-labeled peptide: Application to chemiluminoenzyme immunoassay of bradykinin in inflamed tissues. *Peptides* **15,** 511-518.

DeCesare, D., Fimia, G.M. and Sassone-Corsi, P. (1999) Signaling routes to CREM and CREB: plasticity in transcriptional activation. *Trends Biochem. Sci.* **24,** 281-285.

DeCesare, D., Jacquot, S., Hanauer, A. and Sassone-Corsi, P. (1998) Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of *c-fos* gene. *Biochemistry* **95**, 12202-12207.

Delhase, M., Hayakawa, M., Chen, Y. and Karin, M. (1999) Positive and negative regulation of the IκB kinase activity through IKKβ subunit phosphorylation. *Science* **284,** 309-313.

Deperthes, D., Marceau, F., Frenette, G., Lazure,C., Tremblay, R.R. and Dube, J.Y. (1997) Human kallikrein hK2 has low kininogenase activity while prostate-specific anitgen (hK3) has none. *Biochim. Biophys. Acta.* **1343,** 102-106.

DeWeerd, W.F.C. and Leeb-Lundberg, L.M.F. (1997) Bradykinin sequesters B_2 bradykinin receptors and the receptor-coupled G alpha subunits G alpha (q) and G alpha (i) in caveolae in DDT-1 and MF-2 smooth muscle cells. *J. Biol. Chem.* **272** 17858-17866.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E. and Karin, M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388,** 548-554.

Dinarello,C.A. (1984) Interleukin-1. *Ref. Inf. Dis.* **6,** 51-95.

Dinarello, C.A. (1992) Role of interleukin 1 in systemic responses to LPS. In *Bacterial endotoxic lipopolysaccharides* (J.L. Ryan, D.C. Morrison, eds.), Boca Raton, FL, CRC Press, 105-144.

Dinarello, C.A. (1994) The interleukin-1family: 10 years of discovery. *FASEB J.* **8,** 1314-1325.

Dinarello, C.A. and Kreuger, J.M. (1986) Induction of interleukin 1 by synthetic and naturally occuring muramyl peptides. *Fed. Proc.* **45,** 1545-2548.

Dixon, B. S. (1994) Cyclic AMP selectively enhances bradykinin receptor synthesis and expression in cultured arterial smooth muscle: Inhibition of angiotensin II and vasopressin response. *J. Clin. Invest.* **93,** 2535-2544.

Dixon, B. S. and Denis., M. J. (1997) Regulation of mitogenesis by kinins in arterial smooth muscle cells. *Am. J. Physiol.* **273 (Pt 1)**: C7-20.

Dixon, B.S., Sharma, R.V. and Dennis, M.J. (1996) The bradykinin B_2 receptor is a delayed early response gene for platelet-derived growth factor in arterial smooth muscle cells. *J. Biol. Chem.* **271,** 13324-13332.

Dolovich, J., Back, N. and Arbesman, C.E. (1970) Kinin-like activity in nasal secretions of allergic patients. *Int. Arch. Allergy* **38,** 337-344.

Donaldson, A., Daphna-Iken, D., Tetsuka, T. and Morrison, A.R. (1996) Interleukin-1β activates PI 3-kinase in renal mesangial cells. *Biochem. Biophys. Res. Comm.* **277,** 289-293.

Drapeau, G., Audet, R., Levesque, L., Godin D. and Marceau, F. (1993) Development and in vivo evaluation of metabolically resistant antagonists of BKB_1 receptor for kinins. *J. Pharmacol. Exp. Ther.* **266,** 192-199.

Drapeau, G., Deblois, D. and Marceau, F. (1991) Hypotensive effects of Lys-des-Arg9-bradykinin and metabolically protected agonists of B_1 receptors for kinins. *J. Pharmacol. Exp. Ther.* **259,** 997-1003.

Dray, A., Bettaney, J., Forster, P. and Perkins, M.N. (1988) Activation of a bradykinin receptor in peripheral nerve and spinal cord in the neonatal rat in vitro. *Br. J. Pharmacol.* **95,** 1008-1010.

Dray, A., Patel, I.A., Perkins, M.N. and Rueff, A. (1992) Bradykinin-induced activation of nociceptors: receptor and mechanistic studies on the neonatal rat spinal cord-tail preparation *in vitro. Br. J. Pharmacol.* **107,** 1129-1134.

Dray, A. and Perkins. M.N (1993) Bradykinin and inflammatory pain. *Trends Neurosci.* **16,** 99-104.

Drummond. G.R. and Cocks, T.M. (1995) Endothelium-dependent relaxation mediated by inducible B_1 and constitutive B_2 kinin receptors in the bovine isolated coronary artery. *Br. J. Pharmacol.* **116,** 2473-2481.

Du, K. and Montminy, M. (1998) CREB is a regulatory target for the protein kinase Akt/PKB. *J.Biol. Chem.* **273,** 32377-32379.

Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994) Molecular cloning and functional analysis of the adenovirus E1A-associated 300 kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.* **8,** 869-884.

Edmonson, D.G., Smith, M.M. and Roth S.Y. (1996) Repression domain of the yeast global repressor Tup1 directly interacts with histones H3 and H4. *Genes Dev.* **10,** 1247-1259.

Edwalds-Gilbert, G., Prescott, J. and Falck-Pedersen, E. (1993) 3' RNA processing efficiency plays a primary role in generating termination-competent RNA polymerase II elongation complexes. *Mol. Cell. Biol.* **13,** 3472-3480.

Edwalds-Gilbert, G., Veraldi, K.L. and Milcarek, C. (1997) Alternative poly(A) site selection in complex transcription units: means to an ends? *Nucleic Acids Res.* **25,** 2547-2561.

Eggerickx, D., Raspe, E., Bertrand, D., Vassart, G. and Parmentier, M. (1992) Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B2 receptor gene. *Biochem. Biophys. Res. Commun.* **187**, 1306-1313.

Eisenberg, S.P., Evans, R.J., Arend, W.P., Verderber, E., Brewer, M.T., Hannum, C.H. and Thompson, R.C. (1990) Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. *Nature* **343,** 341-346.

Elbein, K.B. (1981) The tunicamycins: Useful tools for studies on glycoproteins. *Trends Biochem. Sci.* **6,** 219-221.

Elliot, D.F., Horton, E.W., Lewis, G.P. (1960) Actions of pure bradykinin. *J. Physiol.* **153,** 473-480.

Emorine, L.J., Marullo, S., Delavier-Klutchko, C., Kaveri, S.V., Durieu-Trautmann, O. and Strosberg, A.D. (1987) Structure of the gene for the human β_2-adrenergic receptor: Expression and promoter characterisation. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 6995-6999.

Enjyoji, X., Kato, H., Hayashi, I., Oh ishi, S.and Iwanaga, S. (1988) Purification and characterization of rat T-kininogens isolated from plasma of adjuvant-treated rats. Identification of three kinds of T-kininogens. *J. Biol. Chem.* **263,** 973-979.

Faggioli, L., Costanzo, C., Merola, M., Furia, A. and Palmieri, M. (1997) Protein synthesis inhibitors cycloheximide and anisomycin induce interleukin-6 gene expression and activate transcription factor NF-kappaB. *Biochem. Biophys. Res. Commun.* **233,** 507-513.

Farmer, S.G., Burch, R.M., Meeker, S.A. and Wilkins, D.E. (1989) Evidence for a pulmonary B3 bradykinin receptor. *Mol. Pharmacol.* **36,** 1-8.

Farmer, S.G., McMillan, B.A., Meeker, S.N. and Burch, R.M. (1991) Induction of vascular smooth muscle bradykinin B_1 receptors in vivo during antigen arthritis. *Agents Actions* **34,** 191-193.

Fathy, D.B., Mathis, S.A., Leeb, T. and Leeb-Lundberg, L.M.F. (1997) The third transmembrane domains of the human B1 and B2 bradykinin receptor subtypes are involved in discriminating between subtype-selective ligands [abstract]. *FASEB J.* **11,** A1328.

Faussner, A., Proud, D., Towns, M. and Bathon, J. (1998) Influence of the cytosolic carboxyl termini of human B1 and B2 kinin receptors on receptor sequestration, ligand internalization, and signal transduction. *J. Biol. Chem.* **273,** 2617-2623.

Fenoy, F.J. and Roman, R.J. (1992) Effect of kinin receptor antagonists on renal hemodynamic and natriuretic responses to volume expansion. *Am. J. Physiol.* **263,** R1136-R1140.

Feres, T., Paiva, A.C.M. and Paiva, T.B. (1992) BK_1 and BK_2 bradykinin receptors in the rat duodenum smooth muscle. *Br. J. Pharmacol.* **107,** 991-995.

Forrester, W.C., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G. and Groudine, M. (1987) Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. *Nucleic Acids Res.* **15,** 10159-10177.

Foucart, S., Grondin, L., Couture, R. and Nadeau, R. (1997) Modulation of noradrenaline release by B_1 and B_2 kinin receptors during metabolic anoxia in the rat isolated atria. *Can. J. Physiol. Pharmacol.* **75,** 639-645.

Frohman, M.A.M., Dush, M.K. and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 8998-9002.

Furuto-Kato, S., Matsumo, A., Kitamura, N., and Nakanishi, S. (1985) Primary structures of the mRNAs encoding the rat precursors for bradykinin and T-kinin. Structural relationship of kininogens with major acute phase protein and a1-cysteine proteinase inhibitor. *J. Biol. Chem.* **260,** 12054-12059.

Galizzi, J.P., Bodinier, M.C., Chapelain, B, Ly, S.M., Coussy, L., Gireaud, S., Neliat, G. and Jean, T. (1994) Up-regulation of [^{3}H]-des-Arg^{10}-kallidin binding to the bradykinin B_1 receptor by interleukin-1β in isolated smooth muscle cells: Correlation with B_1 agonist-induced PGI_2 production. *Br. J. Pharmacol.* **113,** 389-394.

Gay, N. and Keith, F. (1991) *Drosophila* Toll and IL-1 receptor. *Nature* **351,** 355-356.

Gera, L., Stewart, J.M., Whalley, E.T., Burkard, M. and ZU.S.A.ck, J.S. (1996) New bradykinin antagonists having high potency at B_1 receptors. *Immunopharmacology* **33,** 183-185.

Gerristen, M.E., Williams, A.J., Neish, A.S., Moore, S., Shi, Y. and Collins, T. (1997) CREB-binding protein/p300 are transcriptional co-activators of p65. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2927-2932.

Giles, R.H., Peters, D.J.M. and Breuning, M.H. (1998) Conjunction dysfunction: CBP/p300 in human disease. *Trends Genetics* **14,** 178-184.

Gobeil, F., Neugebauer, W., Nguyen-Le, X. K., Allogho, S. N., Pheng, L.H., Blouin, D., Whalley, E. T. and Regoli, D. (1997) Pharmacological profiles of the human and rabbit B_1 receptors. *Can. J. Physiol. Pharmacol.* **75,** 591-595.

Goldman, P.S., Tran, V.K. and Goodman.R.H. (1997) The multi-functional role of the co-activator CBP and transcriptional regulation. *Recent Prog. Horm. Res.* **52,** 103-120.

Goldstein, R.H. and Wall, M. (1984) Activation of protein formation and cell division by bradykinin and des-Arg^9-bradykinin. *J. Biol. Chem.* **259,** 9263-9268.

Gonzalez, G.A. and Montiminy, M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59,** 675-680.

Greenberg and Ziff, E.B. (1984) Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature* **311,** 433-438.

Greenfeeder, S.A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R.A. and Ju, G. (1995) Molecular cloning and characterisation of a second subunit of the interleukin 1 receptor complex. *J. Biol. Chem.* **270,** 13757-13765.

Greger, I.H. and Proudfoot, N.J. (1998) Poly(A) signals control both transcriptional termination and initiation between the tandem GAL10 and GAL7 genes of Saccharomyces cerevisiase. *EMBO J.* **17,** 4771-4779.

Guesdon, F., Knight, C.G., Rawlinson, L.M., Saklatvala, J. (1997) Dual specificity of the IL-1 and TNF-activated β casein kinase. *J. Biol. Chem.* **272,** 30017-30024.

Haberman, E. (1963) Uber pH-bedingte modifikationen des kininliefernden a-globulin (kininogen) aus rinderserum und das molekulargewicht von kininogen. *Biochem. Z.* **337,** 4440.

Hagiwara, M.. Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R. and Montminy, M.R. (1993) Coupling of hormonal stimulation and transcription via the cyclic AMP responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol. Cell. Biol.* **13,** 4852-4859.

Haley, J.E., Dickenson, A.H. and Schachter, M. (1989) Electrophysiological evidence for a role of bradykinin in chemical nociception in the rat. *Neurosci. Lett.* **97,** 198-202.

Hall, A.J., Vos, H.L. and Bertina, R.M. (1999) Lipopolysaccharide induction of tissue factor in THP-1 cells involves jun protein phosphorylation and nuclear factor κB nuclear translocation. *J. Biol. Chem.* **274,** 376-383.

Hall, J. M. (1992) Bradykinin receptors: Pharmacological properties and biological roles. *Pharmac. Ther.* **56,** 131-190.

Han, J., Lee, J-D., Bibbs, L. and Ulevitch, R.J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265,** 808-811.

Hargreaves, K.M., Troullos, E.S., Dionne, R.A., Schmidt, E.A., Schafer, S.C. and Joris, J.L. (1988) Bradykinin is increased during acute and chronic inflammation: therapeutic implications. *Clin. Pharmacol. Ther.* **44,** 613-621.

Heapy, C.G., Shaw, J.S. and Farmer, S.C. (1993) Differential sensitivity of antinociceptive assays to the bradykinin antagonist Hoe 140. *Br. J. Pharmacol.* **108,** 209-213.

Helms, J.B and Rothman, J.E. (1992) Inhibition by brefeldin A of a golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. *Nature* **360,** 352-354.

Hess, J.F., Borkowski, J.A., MacNeil, T., Stonesifer, G.Y., Fraher, J. and Strader, C.D. (1994) Differential pharmacology of cloned human and mouse B_2 bradykinin receptors. *Mol. Pharmacol.* **45,** 1-8.

Hess, J.F., Borkowski, J.A., Young, G.S., Strader, C.D. and Ransom, R.W. (1992) Cloning and pharmacological characterization of a human bradykinin (bradykinin -2) receptor. *Biochem. Biophys. Res. Commun* **184,** 260-268.

Hess, J.F., Derrick, A.W., MacNeil, T. and Borkowski, J.A. (1996) The agonist selectivity of a mouse B_1 -bradykinin receptor differs from human and rabbit B_1 receptors. *Immunopharmacology* **33,** 1-8.

Higashiyama, S., Ishiguro, H., Ohkulso, I., Fujimoto, S., Matsuda, T. and Saski, M. (1986) Kinin release from kininogens by calpains. *Life Sci.* **39,** 1639-1644.

Hirose,Y. and Manley, J.L. (1998) RNA polymerase II is an essential mRNA polyadenylation factor. *Nature* **395,** 93-96.

Hock, F.J., Wirth, K.J., Albus, U., Linz, W., Gerhards, H.J., Wiemer, G., Henke, S., Breipohl, G., Konig, W., Knolle, J. and Scholkens, B.A. (1991) Hoe 140 a new potent and long acting bradykinin antagonist: In vitro studies. *Br. J. Pharmacol.* **102,** 769-773.

Hu, D. and Fan, T.D. (1993) [Leu^8]des-Arg^9-bradykinin inhibits the angiogenic effect of bradykinin and interleukin-1 in rats. *Br. J. Pharmacol.* **109,** 14-17.

Huang, T-J., Haddad, E-B., Fox, A.J., Salmon, M., Jones, C., Burgess, G., Chung, K.F. (1999) Contribution of bradykinin BKB_1 and BKB_2 receptors in the allergen-induced bronchial hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* (In press).

Ishida, H., Scicli, A.G. and Carretero, O.A., (1989a) Contributions of various rat plasma peptidases to kinin hydrolysis. *J. Pharmacol. Exp.Ther.* **251,** 817-820.
Ishida, H., Nagata, N. and Koshihara, Y. (1989b) Role of angiotensin converting enzyme and other peptidases in in vivo metabolism of kinins. *Hypertension* **14**, 322-327.

Issandou, M. and Darbon, J.M. (1991) Des-Arg^9 bradykinin modulates DNA synthesis, phospholipase C, and protein kinase C in cultured mesangial cells. *J. Biol. Chem.* **266,** 21037-21043.

Jackson, R.J. and Standart, N. (1990) Do the poly(A) tail and 3' untranslated region control mRNA translation. *Cell* **62,** 15-24.

Jacobsen, S. (1966) Separation of two different substrates for plasma kinin-forming enzymes. *Nature* **210,** 98-99.

Jaffa, A.A., Miller, B.S., Rosenzweig, S.A., Naidu, P.S., Velarde, V. and Mayfield, R.K. (1997) Bradykinin induces tubulin phosphorylation and nuclear translocation of MAP kinase in mesengial cells. *Am. J. Physiol.* **273,** F916-924.

Jeong, S. and Stein, A. (1994) Micrococcal nuclease digestion of nuclei reveals extended nucleosome ladders having anomalous DNA lengths for chromatin assembled on non-replicating plasmids in transfected cells. *Nucleic Acids Res.* **22,** 370-375.

Jiang, Y. and Schmaier, A.H. (1992) Kininogens' domain 3 contains heavy chain cell binding region. *Agents Actions* **36**, 34-39.

Jones, C., Phillips, E., Davis, C., Arbuckle, J., Yaqoob, M., Burgess, G., Docherty, R. J., Webb, M., Bevan, S. J. and McIntyre, P. (1999) Molecular characterisation of cloned bradykinin B_1 receptors from rat and human. *Eur. J. Pharmacol.* **374,** 423-433.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, T., Gloss, B., Lin, S-C., Heyman, R.A., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85,** 403-414.

Kammerer, S., Braun, A., Arnold, N. and Roscher, A.A. (1995) The human bradykinin B_2 receptor gene: Full length cDNA, genomic organization and identification of the regulatory region. *Biochem. Biophys. Res. Commun.* **211,** 226-233.

Kane, L.P., Shapiro, V.S., Stokoe, D. and Weiss, A. (1999) Induction of NF-κB by the Akt/PKB kinase. *Curr. Biol.* **9,** 601-604.

Kaplan, A.P. (1978) Initiation of the intrinsic coagulation and fibrinolytic pathways in man: The role of surfaces, hageman factor, prekallikreins, high molecular weight kininogen and factor XI. *Prog. Hemost. Thromb.* **4,** 127-175.

Kellermann, J., Lottspeich, F., Geiger, R. and Deutzmann, R. (1988) Human urinary kallikrein-amino acid sequence and carbohydrate attachment sites. *Protein Seq. Data. Anal.* **1,** 177-182.

Khasar, S.G., Miao, F.J.P. and Levine, J.D. (1995) Inflammation modulates the contribution of receptor-subtypes to bradykinin-induced hyperalgesia in the rat. *Neuroscience* **69,** 685-690.

Kim, J.Y.H., Gonzalez-Scarano, F., Zeicher, S.L. and Alwine, J.C. (1993) Replication of type I human immunodeficiency viruses containing linker substitution mutations in the -201 to -130 long terminal repeat. *J. Virol.* **67,** 1658-1662.

Kirschning, C.J., Wesche, H., Merrill Ayres, T. and Rothe, M. (1998) Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* **188,** 2091-2097.

Kitabayashi, I., Kawakami, Z., Chiu, R., Ozawa, K., Matsuoka, T., Toyoshima, S., Umesono, K., Evans, R.M., Gachelin, G. and Yokoyama, K. (1992) Transcriptional regulation of the c-jun gene by retinoic acid and E1A during differentiation of F9 cells. *EMBO J.* **11,** 167-175.

Kitamura, N., Takagaki, Y., Furuto, S., Tanaka, T., Nawa, H. and Nakanishi, S. (1983) A single gene for bovine high molecular weight and low molecular weight kininogens. *Nature* **305,** 545-549.

Klampfer, L., Lee, T.H., Hsu, W., Vilcek, J. and Chenkiang, S. (1994) NF-IL6 and AP-1 co-operatively modulate the activation of the TSG-6 gene by tumor necrosis factor alpha and interleukin-1. *Mol. Cell. Biol.* **14,** 6561-6569.

Kobilka, B.K., Frielle, T., Dohlman, H.G., Bolanowski, M.A., Dixon, R.A.F., Keller, P., Caron, M.G. and Lefkowitz, R.F. (1987a) Delineation of the intronless structure of the genes for the human and hamster beta 2-adrenergic receptor and their putative promoter regions. *J. Biol. Chem.* **262,** 7321-7327.

Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, M.G., Lefkowitz, R.J. and Regan, J.W. (1987b) Cloning, sequencing and expression of the gene encoding for the human platelet α_2-adrenergic receptor. *Science* **238,** 650-656.

Kolesnick, R. and Golde, D.W. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77,** 325-328.

Korzus, E., Torchia, J., Rose, D.W., Xu, L., Kurokawa, R., McInerney, E.M., Mullen, T-M., Glass, C.K., Rosenfeld, M.G. (1998) Transcription-factor-specific requirements for coactivators and their acetyltransferase functions. *Science* **279,** 703-707.

Krushel, L.A., Cunningham, B.A., Edelman, G.M. and Crossin, K.L. (1999) NF-κB activity is induced by neural cell adhesion molecule binding to neurons and astrocytes. *J. Biol. Chem.* **274,** 2432-2439.

Kunsch, C., Lang, R.K., Rosen, C.A. and Shannon, M.F. (1994) Synergistic transcriptional activation of IL-8 gene by NF-κB p65 (RelA) and NF-IL-6. *J. Immunol.* **153,** 153-164.

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370,** 223-226.

Kyriakis, J.M. and Avruch, J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* **271,** 24313-24316.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmed, M.F., Avurch, J. and Woodgett, J.R. (1994) The stress-activated protein kinase sub-family of c-jun kinases. *Nature* **369,** 156-160.

Langford, K., Frenzel, K., Martin, B.M. and Bernstein, K.E. (1992) The genomic organization of the rat AT_1 angiotensin receptor. *Biochem. Biophys. Res. Commun.* **183,** 1025-1032.

Larrivee, J.F., Bachvarov, D.R., Houle, F., Landry, J., Huot, J. and Marceau, F. (1998) Role of the mitogen-activated protein kinases in the expression of the kinin B_1 receptors induced by tissue injury. *J. Immunol.* **160,** 1419-1426.

Latchman, D. (1995) Tissue-specific expression of proteins and messenger RNAs. In: Gene regulation: a eukaryotic perspective, 1-9. (ed. Latchman, D.), London, Chapman and Hall.

LeClair, K.P., Blanar, M.A. and Sharp, P.A. (1992) The p50 subunit of NF-kappa B associates with the NF-IL6 transcription factor. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 8145-8149.

Leeb, T, Mathis S.A. and Leeb-Lundberg, L.M. F. (1997) The sixth transmembrane domains of human B_1 and B2 bradykinin receptors are structurally compatible and involved in discriminating between subtype-selective agonists. *J. Biol. Chem.* **272,** 311-317.

Leff, S.E., Rosenfeld, M.G. and Evans, R.M. (1986) Complex transcriptional units: Diversity in gene expression by alternative RNA processing. *Annu. Rev. Biochem.* **55,** 1091-1117.

Lemaitre, B., Nicolas, E., Michaut, L., Reichart, J-M. and Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86,** 973-983.

Lerner, U.H. and Modeer, T. (1991) Bradykinin B1 and B2 receptor agonists synergistically potentiate interleukin-1 induced prostaglandin biosynthesis in human gingival fibroblasts. *Biosci. Rep.* **12,** 263-271.

Levesque, L., Drapeau, G., Grose, J.H., Rioux, F. and Marceau, F. (1993) Vascular mode of action of kinin B_1 receptors and development of a cellular model for the investigation of these receptors. *Br. J. Pharmacol.* **109,** 1254-1262.

Levesque, L., Harvey, N., Rioux, F., Drapeau, G. and Marceau, F. (1995b) Development of a binding assay for the B_1 receptors for kinins. *Immunopharmacology* **29,** 141-147.

Levesque, L., Larrivee, J.F., Bachvarov, D.R., Rioux, F., Drapeau, G. and Marceau, F. (1995a) Regulation of kinin induced contraction and DNA synthesis by inflammatory cytokines in the smooth muscle of the rabbit aorta. *Br. J. Pharmacol.* **116,** 1673-1679.

Libby, P., Ordovas, J.M., Birinyi, L.K., Auger, K.R. and Dinarello, C. (1986) Inducible interleukin-1 gene expression in adult human vascular smooth muscle cells. *J. Clin. Invest.* **78,** 1432-1438.

Linz, W., Wiemer, G., Gohlke, P., Unger, T. and Scholkens, B.A. (1995) Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol. Rev.* **47,** 25-49.

Lortie, M., Regoli, D., Rhaleb, N.E. and Plante, G.E. (1992) The role of B_1- and B_2 - kinin receptor in the renal tubular and hemodynamic response to bradykinin. *Am. J. Physiol.* **262,** R72-R76.

Lu, D.L. and Menon, K.M.J. (1996) 3' untranslated region-mediated regulation of luteinizing hormone/human chorionic gonadotropin receptor expression. *Biochem.* **35,** 12347-12353.

Lukiw, W.J. and Bazan, N.G. (1998) Strong nuclear factor-κB-DNA binding parallels cyclooxygenase-2 gene transcription in aging and in sporadic alzheimer's disease superior temporal lobe neocortex. *J. Neuro. Res.* **53,** 583-592.

Lung, C., Jagels, M.A., Daffern, P.J., Tan, E.M. and Zuraw, B.L. (1998) Induction of human B2 bradykinin receptor mRNA and membrane receptors by IFNγ. *Immunopharmacology* **39,** 243-253.

Ma, J-X., Wang, D-Z., Chao, L. and Chao, J. (1994b) Cloning and sequence analysis and expression of the gene encoding the mouse bradykinin B_2 receptor. *Gene* **149,** 283-288.

Ma, J-X., Wang, D-Z., Ward, D.C., Chen, L., Dessai, T., Chao, J. and Chao, L. (1994a) Structure and chromosomal localization of the gene (BDKRB2) encoding human bradykinin B_2 receptor. *Genomics* **23**, 362-369.

MacNeil, T., Bierilo, K.K., Menke, J.G. and Hess, J.F. (1995) Cloning and pharmacological characterization of a rabbit bradykinin B_1 receptor. *Biochim Biophys Acta.* **1264,** 223-228.

MacNeil, T., Feighner, S., Hreniuk, D.L., Hess, J.F. and Van der Ploeg, L.H.T. (1997) Partial agonists and full antagonists at the human and murine bradykinin B_1 receptors. *Can. J. Physiol. Pharmacol.* **75,** 735-740.

Madeddu, P., Emanueli, C., Varoni, M.V., Demontis, M.P., Anania, V., Glorioso, N. and Chao, J. (1997b) Regulation of bradykinin B_2- receptor expression by estrogen. *Br. J. Pharmacol.* **121,** 1763-1769.

Madeddu, P., Varoni, M.V., Emanueli, C., Paci. M.V., Fulgheri, P.D., Sarzani, R. and Glorioso, N. (1997a) Enhanced vasopressor response to angiotensin II in mice with disruption of bradykinin B2-receptor gene [abstract]. *Hypertension* **30,** 494.

Majima, M. and Katori, M. (1995) Approaches to the development of novel antihypertensive drugs: crucial role of the renal kallikrein-kinin system. *Trends Pharmacol.* **16,** 239-246.

Malinin, N.L., Boldin, M.P., Kovalenko, A.V. and Wallach, D. (1997) MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature* **385,** 540-544.

Mandle, R. J., Colman, R.W. and Kaplan, A.P. (1976) Identification of prekallikrein and HMW-kininogen as a circulating complex in human plasma. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4179- 4183.

Mann, K.P., Weiss, E.A. and Nevins, J.R. (1993) Alternative poly (A) site utilization during adendovirus infection coincides with a decrease in the activity of a poly (A) site processing factor. *Mol. Cell. Biol.* **13,** 2411-2419.

Marceau, F. (1997b) Is there a role for bradykinin in the therapeutic and side effects of angiotensin-converting enzyme inhibitors? *Can. J. Cardiol.* **13,** 187-194.

Marceau, F., Barabe, J., St-Pierre, S. and Regoli, D. (1980) Kinin receptors in experimental inflammation. *Can. J. Physiol. Pharmacol.* **58,** 536-542.

Marceau, F., Gendreau, M., Barabe, J., St-Pierre, S. and Regoli, D. (1981) The degradation of bradykinin (BK) and of des-Arg^9-BK in plasma. *Can. J. Physiol. Pharmacol.* **59,** 131-138.

Marceau, F., Hess J. H. and Bachvarov, D. R. (1998) The B_1 receptor for kinins. *Pharmacol. Rev.* **50,** 357-386.

Marceau, F., Larrivee, J.F., Saint-Jacques, E. and Bachvarov, D.R. (1997a) The kinin B_1 receptor: An inducible G protein coupled receptor. *Can. J. Physiol. Pharmacol.* **75,** 725-730.

Marceau, F., Lussier, A. and St-Pierre, S. (1984) Selective induction of cardiovascular responses to des-Arg^9-bradykinin by bacterial endotoxin. *Pharmacology* **29,** 70-74.

Marin-Castano, M.E., Schanstra, J.P., Praddaude, F., Pesquero, J.B., Ader, J., Girolami, J. and Bascands, J. (1998) Differential induction of functional B1-bradykinin receptors along the rat nephrom in endotoxin induced inflammation. *Kidney Int.* **54,** 1888-1898.

Marmiroli, S., Bavelloni, A., Faenza, I., Sirri, A., Ognibene, A., Cenni, V., Tsukada, J., Koyama, Y., Ruzzene, M., Ferri, A., Auron, P.E., Toker, A. and Maraldi, N.M. (1998) Phosphatidylinositol 3-kinase in recruited to a specific site in the activated IL-1 receptor 1. *FEBS Letts.* **438,** 49-54.

Marsh, K.A. and Hill, S.J. (1994) Des-Arg^9-bradykinin-induced increases in intracellular calcium ion concentration in single bovine tracheal smooth muscle cells. *Br. J. Pharmacol.* **112,** 934-938.

Martin, M.U., Wesche, H., Lang, D. and Knop, J. (1997) Effects of overexpression of IRAK on NF-κB-mediated IL2 production and stress activated protein kinase activity. *Cytokine* **9,** 920 (Abstract 121).

Martinez-Balbas, M.A., Bannister, A.J., Martin, K., Haus-Seuffert, P., Meisterernst M. and Kouzarides, T. (1998) The acetyltransferase activity of CBP stimulates transcription. *EMBO J.* **17,** 2886-2893.

Mathis, S.A., Criscimagna, N.L. and Leeb-Lundberg, L.M.F. (1996) B1 and B2 kinin receptors mediate distinct patterns of intracellular Ca^{2+} signaling in single cultured vascular smooth muscle cells. *Mol. Pharmacol* **50,** 128-139.

MatsU.S.A.ka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima K., Kishimoto, T. and Akira, S. (1993) Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10193-10197.

McAllister, B.S., Leeb-Lundberg, F. and Olson, M.S. (1993) Bradykinin inhibition of EGF-and PDGF-induced DNA synthesis in human fibroblasts. *Am. J. Physiol.* **265,** C477-C484.

McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S.D., Wickens, M. and Bentley, D.L. (1997) The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. *Nature* **385,** 357-381.

McEachern, A., Shelton, E., Bhakta, S., Obernolte, R., Bach, C., Zuppan, P., Fujisaki, J., Aldrich, R. and Jarnagin, K. (1991) Expression cloning of a rat B_2 bradykinin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7724-7728.

McIntyre, P., Phillips, E., Skidmore, E., Brown, M. and Webb, M. (1993) Cloned murine bradykinin receptor exhibits a mixed B_1 and B_2 pharmacological sensitivity. *Mol. Pharmacol.* **44,** 346-355.

Medzhitov, R., Preston-Hurlburt, P. and Janeway Jr., C.A. (1997) A human homologue of the *Drosophila* toll protein signals activation of adaptive immunity. *Nature* **388,** 394-397.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadien, A., Chen, C., Ghosh, S. and Janeway, Jr. C.A. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell* **2,** 253-258.

Meini, S., Lecci, A. and Maggi, C.A. (1996) The longitudinal muscle of rat ileum as a sensitive monoreceptor assay for bradykinin B_1 receptors. *Br. J. Pharmacol.* **117**, 1619-1624.

Meloni, F. J., Gustafson, E. J. and Schmaier, A. H. (1992) High molecular weight kininogen binds to platelets by its heavy and light chains and when bound has altered susceptibility to kallikrein cleavage. *Blood* **79**, 1233-1244.

Menke, G.J., Borkowski, J.A., Bierilo, K.K., MacNeil, T., Derrick, A.W., Schneck, K.A., Ransom, R.W., Strader, C.D., Linemayer, D.L. and Hess, J.F. (1994) Expression cloning of a human B_1 bradykinin receptor. *J. Biol. Chem.* **269**, 21583-21586.

Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J.W., Young, D.B., Barbosa, M., Mann, M., Manning, A. and Rao, A. (1997) IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. *Science* **278,** 860-866.

Miyamoto, S., Chiorini, J.A., Urcelay, E. and Safer, B. (1996) Regulation of gene expression for translation initiation factor eIF-2α: importance of the 3' untranslated region. *Biochem J.* **315,** 791-798.

Montminy, M. (1997) Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem.* **66,** 807-22.

Morisato, D. and Anderson, K.V. (1995) Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. *Annu. Rev. Genet.* **29,** 371-99.

Muroi, M. and Suzuki, T. (1993) Role of protein kinase A in LPS-induced activation of NF-kappaB proteins in the mouse macrophage-like cell line. *Cell. Signal.* **5,** 289-298.

Murtagh, J.J., Eddy, R., Shows, R.B., Moss, J. and Vaughan, M. (1991) Different isoforms of Go alpha mRNA arise by alternative splicing of transcripts from a single gene on human chromosome 16. *Mol. Cell. Biol.* **11,** 1146-1155.

Muzio, M., Natoli, G., Saccani, S., Levrero, M. and Mantovani, A. (1998) The human toll signalling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF 6*)* *J. Exp. Med.* **187,** 2097-2101.

Muzio, M., Ni, J., Feng, P. and Dixit, V.M. (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278,** 1612-1615.

Nakajima, M., Mukoyama, M., Pratt, R.E., Horiuchi, M and Dzau, V.J. (1993) Cloning of cDNA and analysis of the gene for mouse angiotensin II type 2 receptor. *Biochem. Biophys. Res. Commun.* **197,** 393-399.

Nakhostine, N., Ribuot, C., Lamontagne, D., Nadeau, R. and Couture, R. (1993) Mediation by B_1 and B_2 receptors of vasodepressor responses to intravenously administered kinins in anaesthetized dogs. *Br. J. Pharmacol.* **110,** 71-76.

Naraba, H., Ueno, A., Kosugi, Y., Yoshimrua, M., Murakami, M., Kudo, I. and Ohishi, S. (1998) Agonist stimulation of B_1 and B_2 kinin receptors causes activation of the MAP kinase signalling pathway, resulting in the translocation of AP-1 in HEK 293 cells. *FEBS. Letts.* **435,** 96-100.

Nasuhara, Y., Adcock, I.M., Catley, M., Barnes, P.J. and Newton, R. (1999) Differential IκB kinase activation and IκBα degradation by interleukin-1β and tumor necrosis factor-α in human U937 monocytic cells. *J. Biol. Chem.* **274,** 19965-19972.

Naumann, M. and Scheidereit, C. (1994) Activation of NF-κB in vivo is regulated by multiple phosphorylations. *EMBO J.* **13,** 4597-4607.

Newton, R., Adcock, I.M. and Barnes, P.J. (1996) Superinduction of NF-κB by actinomycin D and cycloheximide in epithelial cells. *Biochem. Biophys. Res. Commun.* **218,** 518-523.

Newton, R., Kuitert, L.M.E., Bergmann, M., Adcock, I.M. and Barnes, P.J. (1997b) Evidence for involvement of NF-κB in the transcriptional control of COX-2 gene expression by IL-1β. *Biochem. Biophys. Res. Commun.* **237,** 28-32.

Newton, R., Seybold, J., Liu, S.F. and Barnes, P.J. (1997a) Alternative COX-2 transcripts are differentially regulated: implications for post-transcriptional control. *Biochem. Biophys. Res. Commun.* **234,** 85-89.

Newton, R., Stevens, D.A., Hart, L.A., Lindsay, M., Adcock, I.M. and Barnes. P.J. (1997c) Superinduction of COX-2 mRNA by cycloheximide and interleukin-1beta involves increased transcription and correlates with increased NF-kappaB and JNK activation. *FEBS Letts.* **418,** 135-138.

Ni, A., Chai, K. X., Chao, L. and Chao, J. (1998b) Molecular cloning and expression of rat bradykinin B_1 receptor. *Biochem. Biophys. Acta* **1442,** 177-185.

Ni, A., Chao, L. and Chao, J. (1998a) Transcription factor nuclear factor kappaB regulates the inducible expression of the human B1 receptor gene in inflammation. *J. Biol. Chem.* **273,** 2784-2791.

Nicolau, M., Feltrin, M.R. and Regoli, D. (1996) Induction of bradykinin B_1 hypotensive receptors in rats by lipopolysaccharide. *Can. J. Physiol. Pharmacol.* **74,** 337-340.

Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J-I., Cao, Z. and Matsumoto, K. (1999) The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **398,** 252-256.

Nwator, I.A.A. and Whalley, E.T. (1989) Angiotensin converting enzyme inhibitors and expression of des-Arg^9-BK (kinin B_1) receptors in vivo. *Eur. J. Pharmacol.* **160,** 125-132.

O'Connell, M.A., Bennett, B.L., Mercurio, F., Manning, A.M. and Mackman, N. (1998) Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. *J. Biol. Chem.* **273,** 30410-30414.

Odya, C. E., Wilgis, F.P., Walker, J.F. and Oparil S., (1983) Immunoreactive bradykinin and [desArg^9]-bradykinin in low-renin essential hypertension before and after treatment with enalapril (MK-421). *J. Lab. Clin. Med.* **102,** 714-721.

Okamoto, H. and Greenbaum, L.M. (1983) Isolation and structure of T-kinin. *Biochem. Biophys. Res. Commun.* **112,** 701-708.

O'Neill, L.A.J. (1995) Towards an understanding of the signal transduction pathways of interleukin 1. *Biochim. Biophys. Acta* **1266,** 31-44.

O'Neill, L.A.J. and Greene, C. (1998) Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J. Leukoc. Biol.* **63,** 650-657.

Ozes, O. N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401,** 82-85.

Pan, Z.K., Christiansen, S.C., Ptasznik, A. and Zuraw, B.L. (1999) Requirement of phosphatidylinositol 3-kinase activity for bradykinin stimulation of NF-κB activation in cultured human epithelial cells. *J. Biol. Chem.* **274,** 9918-9922.

Pan, Z.K., Ye, R.D., Christiansen, S.C., Jagels, M.A., Bokoch, G.M. and Zuraw, B.L. (1998) Role of the Rho GTPase in bradykinin-stimulated nuclear factor-κB activation and IL-1β gene expression in cultured human epithelial cells. *J. Immunol.* **160,** 3038-3045.

Pan, Z.K., Zuraw, B.L., Lung, C., Prossnitz, E.R., Browning, D.D. and Ye, R.D. (1996). Bradykinin stimulates NF-κB activation and interleukin 1β gene expression in cultured human fibroblasts. *J. Clin. Invest.* **98,** 2042-2049.

Parker, D., Ferreri, K., Nakajima, T., LaMorte, V.J., Evans, R., Koerber, S.C., Hoeger, C. and Montiminy, M.R. (1996) Phosphorylation of CREB at Ser133 induces complex formation with CBP via a direct mechanism. *Mol. Cell. Biol.* **16,** 694-703.

Patel, K.V. and Schrey, M.P. (1992) Inhibition of DNA synthesis and growth in human breast stromal cells by bradykinin: evidence for independent roles of B_1 and B_2 receptor in the respective control of cell growth and phospholipid hydrolysis. *Cancer Res.* **52,** 334-340.

Paul, A., Wilson, S., Belham, C.M., Robinson, C.J.M., Scott, P.H., Gould, G.W. and Plevin, R. (1997) Stress-activated protein kinases: Activation, regulation and function. *Cell. Signal.* **9,** 403-410.

Pepitoni, S., Wood, I.C. and Buckley, N.J. (1997) Structure of the m1 muscarinic acetycholine receptor gene and its promoter. *J. Biol. Chem.* **272,** 17112-17117.

Perissi, V., Dasen, J.S., Kurokawa, R., Wang, Z., Korzus, E., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1999) Factor-specific modulation of CREB-binding protein acetyltransferase activity. *Biochemistry* **96,** 3652-3657.

Perkins, M.N., Campbell, E. and Dray, A. (1993a) Antinociceptive activity of the bradykinin B_1 and B_2 receptor antagonists, des-Arg^9,[Leu^8]-BK and Hoe 140, in two models of persistant hyperalgesia in the rat. *Pain* **53,** 191-197.

Perkins, M.N. and Kelly, D. (1993) Induction of bradykinin B_1 receptors in vivo in a model of ultra-violet irradiation-induced thermal hyperalgesia in the rat. *Br. J. Pharmacol.* **110,** 1441-1444.

Perkins, M.N. and Kelly, D. (1994) Interleukin-1β induced-desArg^{9}bradykinin-mediated thermal hyperalgesia in the rat. *Neuropharmacology* **33,** 657-660.

Perkins, N.D., Edwards, N.L., Duckett, C.S., Agranoff, A.B., Schmid, R.M. and Nabel, G.J. (1993b) A cooperative interaction between NF-κB and Sp1 is required for HIV-1 enhancer activation. *EMBO J.* **12,** 3551-3558.

Perkins, N.D., Felzien, L.K., Betts, J.C., Leung, K., Beach, D.H. and Nabel, G.J. (1997) Regulation of NF-κB by cyclin-dependent kinases associated with the p300 co-activator. *Science* **275,** 523-527.

Pesquero, J.B., Lindsey, C.J., Zeh, K., Paiva, A.C.M., Ganten, D. and Bader, M. (1994) Molecular structure and expression of rat bradykinin B_2 receptor gene: evidence for alternative splicing. *J. Biol. Chem.* **269**, 26920-26925.

Pesquero, J.B., Pesquero, J.L., Oliveria, S.M., Roscher, A.A., Metzger, R., Ganten, D. and Bader, M. (1996) Molecular cloning and functional characterization of a mouse bradykinin B_1 receptor gene. *Biochem. Biophys. Res. Comm.* **220,** 219-225.

Petitclerc, E., Abel, S., Deblois, D., Poubelle, P.E. and Marceau, F. (1992) Effects of interleukin-1 receptor antagonist on three types of responses to interleukin-1 in rabbit isolated blood vessels. *J. Cardiovasc. Pharmacol.* **19,** 821-829.

Phagoo, S.B., Yaqoob, M., McIntyre, P., Jones, C. and Burgess, G. (1997) Cytokines increase B1 bradykinin receptor mRNA and protein levels in human lung fibroblasts [Abstract]. *Biochem. Soc. Trans.* **25,** 43S.

Phagoo, S.B., Poole, S. and Leeb-Lundberg, L.M.F. (1999) Autoregulation of bradykinin receptors: Agonists in the presence of interleukin-1β shift the repertoire of receptor subtypes from B2 to B1 in human lung fibroblasts. *Mol. Pharmacol.* **56,** 325-333.

Phillips, E., Conder, M., Bevan, S., McIntyre, P. and Webb, M. (1992) Expression of functional bradykinin receptors in *Xenopus* oocytes. *J. Neurochem.* **58,** 243-249.

Popoff, M.R., Chaves-Olarte, E., Lemichez, E., Von Eichel-Streiber, C., Thelestam, M., Chardin, P., Cussac, D., Antonny, B., Chavrier, P., Flatau, G., Giry, M., De Gunzburg, J. and Boquet, P. (1996) Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *J. Biol. Chem.* **271,** 10217-10224.

Prado, G.N., Taylor, L. and Polgar, P. (1997) Effects of intracellular tyrosine residue mutation and carboxyl terminus truncation on signal transduction and internalization of the rat bradykinin B2 receptor. *J. Biol. Chem.* **272,** 14638-14642.

Prestridge, D.S. (1991) SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. *Comput. Appl. Biosci.* **7,** 203-206.

Proud, D. and Kaplan, A.P. (1988) Kinin formation: Mechanisms and role of inflammatory disorders. *Ann. Rev. Immunol.* **6,** 49-83.

Proud, D., Siekerski, E.S. and Bailey, G. (1987a) Human lung mast cell tryptase is a kininogenase. *Fed. Proc.* **46,** 932.

Proud, D., Togias, A., Naclerio, R.M., Crush, S. A., Norman, P.S. and Lichtenstein, L.M., (1983) Kinins are generated in vivo following nasal airway challenge of allergic individuals with allergen. *J. Clin. Invest.* **72,** 1678-1685.

Pruneau, D. and Belichard, P. (1993) Induction of bradykinin B_1 receptor-mediated relaxation in the isolated rabbit carotid artery. *Eur. J. Pharmacol.* **239,** 63-67.

Pruneau, D., Luccarini, J.M., Robert, C. and Belichard, P. (1994) Induction of kinin B_1 receptor-dependent vasoconstriction following balloon catheter injury to the rabbit carotid artery. *Br. J. Pharmacol.* **111,** 1029-1034.

Raidoo, D.M. and Bhoola, K.D. (1997) Kinin receptors on human neurones. *J. Neuroimmunology* **77,** 39-44.

Rameh, L.E. and Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* **274,** 8347-8350.

Rang, H.P., Bevan, S. and Dray, A. (1991) Chemical activation of nociceptive peripheral neurones. *Br. Med. Bull.* **47,** 534-548.

Ray, A., Hannink, M. and Ray, B.K. (1995) Concerted participation of NF-κB and C/EBP heteromer in lipopolysaccharide induction of serum amyloid A gene expression in the liver. *J. Biol. Chem.* **270,** 7365-7374.

Raymond, P., Drapeau, G., Raut, R., Audet, R., Marceau, F., Ong, H. and Adam, A. (1995) Quantification of des-Arg^9-bradykinin using a chemiluminescence enzyme immunoassay: Application to its kinetic during plasma activation. *J. Immunol. Methods* **180,** 247-257.

Reddy, S.A.G., Huang, J.H. and Liao, W.S.L. (1997) Phosphatidylinositol 3-kinase in interleukin 1 signaling. *J. Biol. Chem.* **272,** 29167-29173.

Reeves, R., Gorman, C.M. and Howard, B. (1985) Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. *Nucleic Acids Res.* **13,** 3599-3615.

Regoli, D. and Barabe, J. (1980) Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.* **32,** 1-46.

Regoli, D., Barabe, J. and Park, W.K. (1977) Receptors for bradykinin in rabbit aorta. *Can. J. Physiol. Pharmacol.* **55,** 855-867.

Regoli, D., Gobeil, F., Nguyen, Q.T., Jukic, E., Seoane, P.R., Salvino, J.M. and Sawutz, D.G. (1994) Bradykinin receptor types and B2 subtypes. *Life. Sci.,* **55,** 735-749.

Regoli, D., Marceau, F. and Barabe, J. (1978) De novo formation of vascular receptors for bradykinin. *Can J. Physiol. Pharmacol.* **56,** 674-677.

Regoli, D., Marceau, F. and Lavigne, J. (1981) Induction of B_1-receptors for kinins in the rabbit by a bacterial lipopolysaccharide. *Eur. J. Pharmacol.* **71,** 105-115.

Rhaleb, N.E, Gobeil, F. and Regoli, D. (1992) Non-selectivity of new bradykinin antagonists for B_1 receptors. *Life Sci.* **51,** PL125-PL129.

Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T. and Hia, T. (1994) Induction of cyclooxygenase-2 by interleukin-1α. *J. Biol. Chem.* **269,** 11769-11775.

Roch E Silva, M., Beraldo, W. T. and Rosenfeld, G. (1949) Bradykinin hypotensive and smooth muscle stimulating factor released from plasma globulins by snake venoms and by trypsin. *Am. J. Physiol.* **156,** 261-273.

Rock, F.L., Hardiman, J.C., Timans, R., Kastelein, R. and Bazan, J.F. (1998) A family of human receptors structurally related to *Drospophila* Toll. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 588-593.

Romashkova, J.A. and Makarov, S.S. (1999) NF-κB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401,** 86-90.

Roslan, R., Campbell, E.A. and Dray, A. (1995) The induction of bradykinin B_1 reeptor in non-inflamed and inflamed rat urinary bladder. *Br. J. Pharmacol.* **114,** 228P.

Rueff, A., Dawson, A.J. and Mendell, L.M. (1996) Characteristics of nerve growth factor induced hyperalgesia in adult rats: Dependence on enhanced bradykinin-1 receptor activity but not neurokinin-1 receptor activation. *Pain* **66,** 359-372.

Rupniak, N.M.J., Boyce, S., Webb, J.K., Williams, A.R., Carlson, E.J., Hill, R.G., Borkowski, J.A. and Hess, J.F. (1997) Effects of the bradykinin B_1 receptor antagonist des-Arg^9[Leu^8]bradykinin and genetic disruption of the B_2 receptor on nociception in rats and mice. *Pain* **71,** 89-97.

Ryan, J.W., Denslow, N.D., Greenwald, J.A. and Rogoff, M.A. (1994) Immunoaffinity purifications of aminopeptidase P from guinea pig lungs, kidney and serum. *Biochem. Biophys. Res. Commun.* **205,** 1796-1802.

Sachs, A.B. (1993) Messenger RNA degradation in eukaryotes. *Cell* **74,** 413-421.

Saklatvala, J., Dean, J. and Finch, A. (1999) Protein kinase cascades in intracellular signalling by interleukin-1 and tumour necrosis factor. *Biochem. Soc. Symp.* **64,** 63-77.

Salvino, J.M., Seoane, P.R., Douty, B.D., Awad, M.M.A., Dolle, R.E., Houck, W.T., Faunce, D.M. and Sawutz, D.G. (1993) Design of potent non-peptide competitive antagonists of the human bradykinin B2 receptor. *J. Med. Chem.* **36,** 2583-2584.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci U.S.A.* **74,** 5463-5467.

Sassone-Corsi, P. (1995) Transcription factors responsive to cAMP. *Annu. Rev. Cell Dev. Biol.* **11,** 355-377.

Sato, F. and Nagasawa, S. (1988) Mechanism of kinin release from human low-molecular-mass-kininogen by the synergistic action of human plasma kallikrein and leucocyte elastase. *Biol. Chem. Hoppe-Seyler* **369,** 1009-1017.

Sawutz, D.G., Salvino, J.M., Dolle,R.E., Casiano, F., Ward, S.J., Houck, W.T., Faunce, D.M., Douty, B.D., Baizman, E., Awad, M.M.A., Marceau, F. and Seoane, P.R. (1994) The nonpeptide WIN 64338 is a bradykinin B2 receptor antagonist. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4693-4697.

Sawutz, D.G., Singh, S.S., Tiberio, L., Koszewski, E., Johnson, C.G. and Johnson, C.L. (1992) The effect of TNFα on bradykinin receptor binding, phosphatidylinositol turnover and cell growth in human A431 epidermoid carcinoma cells. *Immunopharmacology* **24,** 1-10.

Schanstra, J.P., Bataille, E., Marin, C.M., Barascud, Y., Hirtz, C., Pesquero, J.B., Pecher, C., Gauthier, F., Girolami, J.P. and Bascands, J.L. (1998) The B1-agonist [des-Arg^{10}]-kallidin activates transcription factor NF-kappaB and induces homologous upregulation of the bradykinin B1-receptor in cultured human lung fibroblasts. *J.Clin.Invest.* **101,** 2080-2091.

Schmidlin, F., Scherrer, D., Daeffler, L., Bertrand, C., Landry, Y. and Gies, J. (1998) Interleukin-1β induces bradykinin B_2 receptor gene expression through a prostanoid cyclic AMP-dependent pathway in human bronchial smooth muscle cells. *Mol. Pharmacol.* **53,** 1009-1015.

Schneck, K.A., Hess, J.F., Stonesifer, G.Y. and Ransom, R.W. (1994) Bradykinin B_1 receptors in rabbit aorta smooth muscle cells in culture. *Eur. J. Pharmacol.* **266,** 277-282.

Schremmer, D.E., Offner, A., Siebeck, M., Heinz, E.P., Gais, P. and Roshcer, A.A. (1996) Autoradiographic visualization of B1 bradykinin receptors in porcine vascular tissues in the presence or absence of inflammation. *Immunopharmacology* **33,** 95-100.

Schremmer, D.E., Offner, A., Siebeck, M. and Roshcer, A.A. (1998) B1 bradykinin receptors and carboxypeptidase M are both upregulated in the aorta of pigs after LPS infusion. *Biochem. Biophys. Res. Commun.* **243,** 246-252.

Schroeder, C., Beug, H. and Muller-Esterl, W. (1997) Cloning and functional characterization of the ornithokinin receptor. Recognition of the major kinin receptor antagonist, HOE140, as a full agonist. *J. Biol. Chem.* **272,** 12475-81.

Schul, W.R., van Dreil, R. and de Jong, L. (1998) A subset of poly(A) polymerase is concentrated at sites of RNA synthesis and is associated with domains enriched in splicing factors and poly(A) RNA. *Exp. Cell. Res.* **238,** 1-12.

Schulze-Osthoff, K., Ferrari, D., Riehemann, K. and Wesselborg, S. (1997) Regulation of NF-κB activation by MAP kinase cascades. *Immunobiol.* **198,** 35-49.

Seabrook, G.R., Bowery, B.J., Heavens, R., Brown, N., Ford, H., Sirinathsinghi, D.J.S., Borkowski, J.A., Hess, J.F., Strader, C.D. and Hill, R.G. (1997) Expression of B_1 and B_2 bradykinin receptors mRNA and their functional roles in sympathetic ganglia and sensory dorsal root ganglia neurones from wild-type and B_2 receptor knockout mice. *Neuropharmacology* **36,** 1009-10117.

Seabrook, G.R., Bowery, B.J. and Hill, R.G. (1995) Bradykinin receptors in mouse and rat isolated superior cervical ganglia. *Br. J. Pharmacol.* **115,** 368-372.

Shaw, G. and Kamen, R. (1986) A conserved AU sequence AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46,** 659-667.

Sheng, M., Thompson, M.A. and Greenberg, M.E. (1991) CREB: A Ca^{2+} -regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252,** 1427-1430.

Shibata, M., Ohkubo, T., Takahashi, H. and Inoki, R. (1989) Modified formalin test: characteristic biphasic pain response. *Pain* **38,** 347-352.

Shirasaki, H., Kojima, T., Asakura, K., Kataura, A., Shimamoto, K. and Timura, O. (1989) The pathophysiological role of kinin and chemical mediators on experimental allergic rhinitis. *Adv. Exp. Med. Biol.* **247,** 375-378.

Siebeck, M., Eich-Rathfelder, S., Fautz, M., Hohenbleicher, F., Fritz, H., Cheronis, J.C. and Whalley, E.T. (1997) Bradykinin B_1 receptor agonist studies in porcine endotoxin shock. *Br. J. Pharmacol.* **120,** 182P.

Siebeck, M., Spannagl, E., Schorr, M., Stumpf, B., Fritz, H., Whalley, E.T., and Cheronis, J.C. (1996) Effect of combined B_1 and B_2 kinin receptor blockade in porcine endotoxin shock. *Immunopharmacology* **33,** 81-84.

Siebeck, M., Whalley, E.T., Hoffmann, H., Weipert J. and Fritz, H. (1989) The hypotensive response to des-Arg^9-bradykinin increases during *E.coli* septicemia in the pig. *Adv. Exp. Med. Biol.* **247B,** 389-393.

Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahon, C.J., Grubin, C.E., Wignall, J.M., Jackson, J.L., Call, S.M., Friend, D., Alpert, A.R., Gillis, S., Urdal, D.L. and Dower, S.K. (1988) cDNA expression cloning of the IL1 receptor, a member of the immunoglobulin superfamily. *Science* **241,** 585-589.

Singh, R., Wang, B., Shirvaikar, A., Khan, S., Kamat, S., Schelling, J.R., Konieczkowski, M. and Sedor, J.R. (1999) The IL-1 receptor and rho directly associate to drive cell activation in inflammation. *J. Clin. Invest.* **103,** 1561-1570.

Sizemore, N., Leung, S. and Stark, G. (1999) Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-κB p65/RelA subunit. *Mol. Cell. Biol.* **19,** 4798-4805.

Smith, J.A.M., Webb, C., Holford, J. and Burgess, G.M. (1995) Signal transduction pathways for B_1 and B_2 bradykinin receptors in bovine pulmonary artery endothelial cells. *Mol. Pharmacol.* **47,** 525-534.

Song, H.Y., Regnier, C.H., Kirsching, C.J., Goeddel, D.V. and Rothe, M. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 9792-9796.

Soskic, V., Nyakatura, E., Roos, M., Muller-Esterl, W. and Godovac-Zimmermann, J. (1999) Correlations in palmitoylation and multiple phosphorylation of rat bradykinin B_2 receptor in chinese hamster ovary cells. *J. Biol. Chem.* **274,** 8539-8545.

Stancovski, I. and Baltimore, D. (1997) NF-κB activation: The IκB kinase revealed. *Cell* **91,** 299-302.

Staszewska-Woolley J and Woolley G. (1991) Effects of neuropeptides, ruthenium red and neuraminidase on chemoreflexes mediated by afferents in the dog epicardium. *J. Physio. (Lond)* **436,** 1-13.

Stein, B., Baldwin Jr, A.S., Ballard, D.W., Greene, W.C., Angel, P. and Herrlich, P. (1993) Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potentiated biological function. *EMBO J.* **12,** 3879-3891.

Steranka, L.R., De Hass, C.J., Vavrek, R.J., Stewart, J.M., Enna, S.J. and Synder, S.H. (1987) Antinociceptive effects of bradykinin antagonists. *Eur. J. Pharmacol.* **136,** 261-262.

Steranka, L.R., Manning, D.C., Dehaas, C.J., Ferkany, J.W., Borosky, S.A., Connor, J.R., Vavrek, R.J., Stewart, J.M. and Snyder, S.H. (1988) Bradykinin as a pain mediator: receptors are localised to sensory neurones, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 3245-3249.

Stewart, J.M., Gera, L., Hanson, W., ZU.S.A.ck, J.S., Burkard, M., McCullough, R. and Whalley, E.T. (1996) A new generation of bradykinin antagonists. *Immunopharmacology* **31,** 51-60.

Strader, C.D., Fong, T.M., Tota, M.R. and Underwood, D. (1994) Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem.* **63,** 101-132.

Stylianou, E., O'Neill, L.A.J., Rawlinson, L., Edbrooke, M.R., Woo, P. and Saklatvala J. (1992) Interleukin 1 induces NF-κB through its type I but not its type II receptor in lymphocytes. *J. Biol. Chem.* **267,** 15836-15841.

Su, B. and Karin, M. (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.* **8,** 402-411.

Sueyoshi, T., Enjyoji, K., Shimada, T., Kato, H., Iwanaga, S., Bando, Y., Kominami, E. and Katunuma, N. (1985) A new function of kininogens as thiol proteinase inhibitors: inhibition of palpain and cathepsins B, H, and L by bovine, rat and human plasma kininogens. *Febs Lett.* **182,** 193-195.

Sufka, K.J. and Roach, J.T. (1996) Stimulus properties and antinociceptive effects of selective bradykinin B_1 and B_2 receptors antagonists in rats. *Pain* **66,** 99-103.

Sugo, T., Ikari, N., Kato, H., Iwanaga, S. and Fuji, S. (1980) Functional sites of bovine high molecular weight kininogen as a cofactor in kaolin-mediated activation of factor XII (Hageman factor). *Biochemistry* **19,** 3215-3220.

Sun, P., Enslen, H., Myung, P.S. and Maurer, R.A. (1994) Differential activation of CREB by Ca^{2+}/ calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* **8,** 2527-2539.

Suzuki, T., Iwanaga, S., Kato, T., Nagasawa, S., Kato, H., Yang, M. and Horiuchi, K. (1967) Biochemical properties of kininogens and kinin-releasing enzymes. In: International symposium on vasoactive peptides: Bradykinin and related kinins, 27-38. Edited by Rocha E Silva, M., Rothschild, H. A., Sao Paulo, Brazil., Society for Brazillian farmacology experimental therapeutics.

Swantek, J.L., Christerson, L. and Cobb, M.H. (1999) Lipopolysaccharide-induced tumor necrosis factor-α promoter activity is inhibitor of nuclear factor-κB kinase-dependent. *J. Biol. Chem.* **274,** 11667-11671.

Sweet, M.J. and Hume, D.A. (1996) Endotoxin signal transduction in macrophages. *J. Leuk. Biol.* **60,** 8-26.

Takeuchi, K., Alexander, R.W., Nakamura, Y., Tsujino, T. and Murphy, T.J. (1993) Molecular structure and transcriptional function of the rat vascular AT_1a angiotensin receptor gene. *Circ. Res.* **73,** 612-621.

Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P. and Comb, M.J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.* **15,** 4629-4642.

Teather, S. and Cuthbert, A.W. (1997) Induction of bradykinin B_1 receptors in rat colonic epithelium. *Br. J. Pharmacol.* **121,** 1005-1011.

Tiffany, C.W. and Burch, R.M. (1989) Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macorphages. *FEBS Letts.* **247,** 189-192.

Tokumasu, T., Ueno, A. and Oh-Ishi, S. (1995) A hypotensive response induced by des-Arg^9-bradykinin in young Brown/Norway rats pretreated with endotoxin. *Eur. J. Pharmacol.* **274,** 225-228.

Tonussi.C.R. and Ferreria, S.H. (1997) Bradykinin-induced knee joint incapacitation involves bradykinin B_2 receptor mediated hyperalgesia and bradykinin B_1 receptor-mediated nociception. *Eur. J. Pharmacol.* **326,** 61-65.

Torchia, J., Glass, C. and Rosenfeld, M.G. (1998) Co-activators and co-repressors in the integration of transcriptional responses. *Curr. Opin. Cell Biol.* **10,** 373-383.

Trautwein, C., van der Geer, P., Karin, M., Hunter, T. and Chojkier, M. (1994) Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulates its DNA binding activity to DNA recognition elements. *J. Clin. Invest.* **93,** 2554-2561.

Treismann, R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Biol.* **8,** 205-215.

Tsukagoshi, H., Shimizu, Y., Horie, T., Fukabori, Y., Shimizu, Y., Iwamae, S., Hisada, T., Ishizuka, T., Iizuka, K., Dobashi, K. and Mori, M. (1999) Regulation by interleukin-1β of gene expression of bradykinin B_1 receptor in MH-S murine alveolar macrophage cell line. *Biochem. Biophys. Res. Commun.* **259,** 476-482.

Tsukiyama, T. and Wu, C. (1997) Chromatin remodeling and transcription. *Curr. Opin. Gen. Dev.* **7,** 182-191.

Ulevitch, R.J. and Tobias, P.S. (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* **13,** 437-457.

Vassalli, P. (1992) The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol.* **10,** 411-452.

Vavrek, R. J. and Stewart, J.M. (1985) Competitive antagonists of bradykinin. *Peptides* **6,** 161-164.

Velarde, V., Ullian, M.E., Morinelli, T.A., Mayfield, R.K. and Jaffa, A.A. (1999) Mechanisms of MAPK activation by bradykinin in vascular smooth muscle cells. *Am. J. Physiol.* **277,** C253-C261.

Venge, P., Dahl, R. and Hallgren, R. (1979) Enhancement of factor XII reactions by eosinophil cationic protein. *Throm. Res.* **14,** 641-649.

Vianna, R.M. and Calixto, J.B. (1998) Characterization of the receptor and the mechanisms underlying the inflammatory response induced by des-Arg^9-BK in mouse pleurisy. *Br.J.Pharmacol.* **123**, 281-291.

Vlahos, C.F., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269,** 5241-5248.

Von Banchet, G.S., Petersen, M. and Heppelmann, B. (1996) Bradykinin receptors in cultured rat dorsal root ganglion cells: influence of length of time in culture. *Neuroscience* **75,** 1211-12118.

Wachtfogel, Y. T., Dela-cadena, R.A., Kunapuli, S.P., Rick, L., Miller, M., Schultze, R. L., Altleri, D. C., Edgington, T.S. and Colman, R.W. (1994) High molecular weight kininogen binds to Mac-1 on neutrophils by its heavy chain and its light chain (domain 5). *J. Biol. Chem.* **269,** 19307-19312.

Wadgaonkar, R., Phelps, K.M., Haque, Z., Williams, A.J., Silvermann, E.S. and Collins, T. (1999) CREB-binding protein is a nuclear integrator of nuclear factor-κB and p53 signalling. *J. Biol. Chem.* **274,** 1879-1882.

Walker, K., Dray, A. and Perkins, M.N. (1996) Development of hyperthermia following intracerebroventricular administration of endotoxin in the rat: effect of kinin B_1 and B_2 receptor antagonists. *Br. J. Pharmacol.* 117, 684-688.

Wang, D., Chao, L. and Chao, J. (1996) Cellular localization of bradykinin B_1 receptor mRNA in the human kidney. *Immunopharmacology* **33,** 151-156.

Wang, D.Z., Ma, J.X., Chao, L. and Chao, J. (1994) Molecular cloning and sequence analysis of rat bradykinin B2 receptor gene. *Biochim. Biophys. Acta* **13,** 171-174.

Ward, P.E., Chow, A. and Drapeau, G. (1991) Metabolism of bradykinin agonists and antagonists by plasma aminopeptidase P. *Biochem. Pharmacol.* **42,** 721-727.

Warren, S.H., Vogel, F.R. and Chedid, L.A. (1986) Current status of immunological adjuvants. *Annu. Rev. Immunol.* **4**, 369-388.

Webb, M., McIntyre, P. and Phillips, E. (1994) B_1 and B_2 bradykinin receptors encoded by distinct mRNAs. *J. Neurochem.* **62,** 1247-1253.

Weinreich, D. (1986) Bradykinin inhibits a slow spike after-hyperpolarization in visceral sensory neurones. *Eur. J. Pharmacol.* **132,** 61-63.

Weinreich, D. and Woderlin, W.F. (1987) Inhibition of calcium-dependent spike after hyper-polarisation increase excitability of rabbit visceral sensory neurones. *J. Physiol.* **394,** 415-427.

Werle, E., Gotze, W. and Keppler, A. (1937) Uber die wirkung des kallikreins auf den isolierten darm und uber eine neue darmkontrahierende substanz. *Biochem. J.* **289**, 217-233.

Wesche, H., Korherr, C., Kracht, M., Falk, W., Resch, K. and Martin, M.U. (1997) The interleukin 1 receptor accessory protein (IL-1AcP) is essential for IL-1-induced activation of IRAK and SAP kinases. *J.Biol. Chem.* **272,** 7727-7731.

Wesselborg, S., Bauer, M.K.A., Vogt, M., Schmitz, M.L. and Schulze-Osthoff, K. (1997) Activation of transcription factor NF-κB and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. *J. Biol. Chem.* **272,** 12422-12429.

Whalley, E.T., Clegg, S., Stewart, J.M. and Vavrek, R.J. (1987) The effect of kinin agonists and antagonists on the pain response of the human blister base. *Naunyn Shcmeidebergs Arch. Pharmacol.* **336,** 652-655.

Whalley, E.T., Modaferri, D., Loy, S.D. and Cheronis, J.C. (1993) Effect of antagonists at BK-1, BK-2 and interleukin-1 receptors on the expression of BK-1 receptors in the rabbit [abstract]. *Br. J. Pharmacol.* **109,** 55P.

Whalley, E.T., Solomon, J. and Modafferi, D. (1992) CP-0127, a novel potent bradykinin antagonist increases survival in rat and rabbit models of endotoxin shock *Agents Actions* **38,** 413-20.

Whiteside, S.T., Epinat, J.C., Rice, N.R. and Israel, A. (1997) IκB epsilon, a novel member of the IκB family, controls RelA and cRel NF-κB activity. *EMBO J.* **16,** 1413-1426.

Wirth, K., Alpermann, H.G., Satoh, R. and Inazu, M. (1992) The bradykinin antagonist Hoe 140 inhibits carrageenan- and thermically induced paw oedema in rats. *Agents Actions Suppl.* **38,** 428-431.

Wong, S.K-F. and Ross, E.M. (1994) Chimeric muscarinic cholinergic:β-adrenergic receptors that are functionally promiscuous among G proteins. *J. Biol. Chem.* **269,** 18968-18976.

Wood, I.C., Roopra, A., Harrington, C.A. and Buckley, N.J. (1995) Structure of the m4 cholinergic muscarinic receptor gene and its promoter. *J. Biol. Chem.* **270,** 30933-30940.

Woronicz, J.D., Gao, X., Cao, Z., Rothe, M. and Goeddel, D.V. (1997) IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. *Science* **278,** 866-869.

Wright, S.D., Ramos, R.A., Tobias, P.S. Ulevitch, R.J. and Mathison, J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249,** 1431-1433.

Xing, J., Ginty, D.D. and Greenberg, M.E. (1996) Coupling of the Ras-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273,** 959-963.

Xing, J., Kornhauser, J.M., Xiz, Z., Thiele, E.A. and Greenberg, M.A. (1998) Nerve growth factor activates extracellular signal-related kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. *Mol. Cell. Biol.* **18,** 1946-1955.

Xiong, W., Chen,L. and Chao, J. (1990) Purification and characterisation of a kallikrein-like T-kininogenase. *J. Biol. Chem.* **265,** 2822-2827.

Yang, R., Mark, M.R., Gurney, A.L. and Godowski, P.J. (1999) Signaling events induced by lipopolysaccharide-activated toll-like receptor 2. *J. Immunol.* **163,** 639-643.

Yang, R., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L. and Godowski, P.J. (1998b) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* **395,** 284-288.

Yang, X. and Polgar, P. (1996) Genomic structure of the human bradykinin B_1 receptor gene and preliminary characterisation of its regulatory regions. *Biochem. Biophys. Res. Commun.* **222,** 718-725.

Yang, X., Taylor, L. and Polgar, P. (1998a) Mechanisms in the transcriptional regulation of bradykinin B_1 receptor expression. Identification of a minimum cell-type specific enhancer. *J. Biol. Chem.* **273,** 10763-10770.

Yoza, B.K., Hu, J.Y.Q. and McCall, C.E. (1996) Protein-tyrosine kinase activation is required for lipopolysaccharide induction of interleukin 1β and NF-κB activation, but not NF-κB nuclear translocation. *J. Biol. Chem.* **271,** 18306-18309.

Zhang, F.X., Kirschning, C.J., Mancinelli, R., Xu, X., Jin, Y., Faure, E., Mantovani, A., Rothe, M., Muzio, M. and Arditi, M. (1999) Bacterial lipopolysaccharide activates nuclear factor-κB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J. Biol. Chem.* **274,** 7611-7614.

Zhang, S., Han J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R. and Bokoch, G.M. (1995) Rho family GTPase regulate p38 MAP kinase through the downstream mediator PAK1. *J. Biol. Chem.* **270,** 23934-23936.

Zhang, X., Jeyakumar, M. and Bagchi, M.K. (1996) Ligand-dependent cross-talk between steroid and thyroid hormone receptors. *J. Biol. Chem.* **271,** 14825-14833.

Zhao, J., Hyman, L. and Moore, C. (1999) Formation of mRNA 3' ends in eukaryotes: Mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Micro. Mol. Biol. Rev.* **63,** 405-445.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. (1997) The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89,** 413-424.

Zhong, H., Voll, R.E. and Ghosh, S. (1998) Phosphorylation of NF-κB p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell.* **1,** 661-671.

Zhou, X., Polgar, P. and Taylor, L. (1998) Roles for interleukin-1beta, phorbol ester and a post-transcriptional regulator in the control of bradykinin B1 receptor gene expression. *Biochem. J.* **330,** 361-366.

Zolfaghari, R., Little, D., Baker, C.R., Canizaro, P.C. and Behal, F.J. (1989) Human lung membrane-bound neutral-endopeptidase-catalysed hydrolysis of bradykinin. *Enzyme* **42,** 160-173.

Zuzack, J.S., Burkard, M.R., Cuadrado, D.K., Greer, R.A., Selig, W.M. and Whalley, E.T. (1996) Evidence of a bradykinin B1 receptor in human ileum: pharmacological comparison to the rabbit aorta B1 receptor. *J. Pharmacol. Exp. Ther.* **277,** 1337-1343.

Zwick, E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maley, K.and Ullrich, A. (1997) Critical role of calcium-dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signalling. *J. Biol. Chem.* **272,** 24767-24770.

Publications and abstracts

Jones, C., Phillips, E., Davis, C., Arbuckle, J., Yaqoob, M., Burgess, G., Docherty, R., Webb, M., Bevan, S., McIntyre, P. (1999) Molecular characterisation of cloned bradykinin B1 receptors from rat and human. *Eur. J. Pharm.* **374,** 423-433.

Huang, T-J., Haddad, E-B., Fox, A.J., Salmon, M., Jones, C., Burgess, G., Chung, K.F. (1999) Contribution of bradykinin BKB_1 and BKB_1 receptors in the allergen-induced bronchial hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* (In press).

Jones, C. and McIntyre, P. Cloning and characterisation of the rat bradykinin B_1 receptor. Oral presentation of abstract at Kallikreins and Kinins Gordon Research Conference, Ventura, California. Janurary 1999.

Phagoo, S.B., Yaqoob, M., McIntyre, P., Jones, C., Burgess, G.M. (1997) Cytokines increase B1 bradykinin receptor mRNA and protein levels in human lung fibroblasts [Abstract]. *Biochem. Soc. Trans.* Feb; 25(1):43S.

Appendix I

FIGURE A.1

GGAATTCTCTCAACAAATCCTGGGCATCCCACAATGCATCAGCCTGGTGGCTGGTGTTGAAA
CTGTGACCATAATACAGGGAGAGATTAAACACACTTATTGCTGGGAGACCTCACACGCCCAC
TCAGGGCGCAGCAAATGAGAGCTAACACGTGTGTATAGAATACATGGAAAGTGGCAAGCGG
AATGAAGATCCAGGAAGCCAGGGAGGAGGAGGAGGGGGGAGGAGAGGGGGAGGGGGAGG
GGGAGGGGGAGGAAAAGGCTCCAAAAATGGCTGCATCTCCAGACATCTTGTAGAAACAGGA
AGACCTGAGGAGGTAAGCGTGAGGCTTCTTGGGTAAGCCGCTCCTCGGCAGAGGAAGCCGG
CAATCCCAAGGCTCTGTGGTGAAAGATGTGTCAGCTCTAAGACGGTTGAGGAGGTCTGGAGG
GCCTGTGAGAGCATCAGGACTGGCATGGGTGGCCTTGAAGACCGCCATCCTGCCTTAGTTCC
TGCTGGAGACAGAGGGTCAATGTTTCAAGGGTATAAAGGTCTTAGATTCCAAAAGATAAAGA
AGCAGAGTTGATTGTCCAGGTTCAGACCCCGATAGGGCCTTGGAGAAGTTTCCTCTTGAGGA
CTGGAACCCAGGGCTGAGATTCCCTTGTGAGGAGCACCTGGGAGACCCCATGTCCTTTTCCA
AGCAGCGCAGAACAGGGTCCAAGAGCACAGGTGGGTTCCTTATAAACTCCCCCTGGAGCATG
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CCCCCCTGCCTCAGGCTAGCTGCAGGTGGCTCAGGCCAGGGCCACACCTAGCATGAAGACGG
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CAGCCTCCACAAGGCTTCTGAGTGCGGTCCCACCCAAATCTGGCCGTCTGCCCTCGGCTGTTC
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CACATATATGAAATAAATAAATCTTTTAAAAAAAACAAAAAAAAGGAAAACCTTTATTGGTT
TGGAACCagACCACAGCTGGATTTGACCTCCTGTACTGTGTCAACGTCAGGTCACTGTGG**ATG**
GCGTCCGAGGTCTTGTTGGAGCTCCAGCCCTCTAACCGAAGCCTGCAGGCCCCTGCCAACAT
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CAGCTGCTTGAACCCACTGATTTATGTCTTCGCAGGCCGGCTCCTTAAGACCAGGGTTCTGGG
GACTTTATAAA**TGA**TGCAACCGAGAAGCCTCATGCCCATGTGGCCCAACAGGAAGGGACTCT
TCCAATTATCCTGCTGGAATTCAAACAGCAGGGAGCCAAGAAGCCTGGCTTCTTGACCAACC
ATCTCTGGTATCATAAAGACCATCTGGTCAGCTGACCCACAGCCCACAAAGATACCCAGGGT
AAAAGTTATTAGCAGGGTAGTCGGGTAGTCGGGACCTTGAAGGGCAATTACTAGGGCTCTGG
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GCAGACATCCTCTCTGAAACCTCCAGGAAAGACCAGACTTGGCCAACGTGCCGATGCCAGCC
CTGCCCCCCAGGTACTGTGAGAGAGTAAAGCTGCACTCTGATTTGCCAAGTTTGTGGTTATT
GATACCATTTGGAAACTTATGCGAGTACCTGACCCCCGGCAAGCGTCTGCTGTTTGACGCGT
CCACTCACCCAGACCTTCCTAGGCTAACGTGAGGTGAGGTGGCTGCAGACCCTGTCCGCAAG
GAACTTGTAGAGCTCTAAGTAACCCCGAGTAAGAACAGGGAGCTCCCAGGGAACGGGCCAG
CTCCTCCCTCAGCTCCATGTAGGGTAGACGCTGGGGCCCATGGAGACAGACAGGAAGAGCTA
AGTTCCCACCCAATCCCTGCAGTACGGGGAAGGACCAGGCTGCTGCCTGGAGTTCGCAGGAA
TATTACCAAATGCTGGAGCCTCCATCCAATCTCTCTGTGGCCTCAAGGATCC

Figure A.1 Nucleic acid sequence of the 6006 bp Eco RI to Bam HI fragment containing the rat BKB_1 receptor gene.

The position of the TIS from which the nucleotide numbering in the text begins and the start and stop codons for the protein coding sequence are shown in bold. and underlined. The location of the exon-intron boundaries are shown in lower case and underlined. The sequence corresponding to the full length cDNA has been submitted to the EMBL database (Accession number AJ132230).

Appendix 2

Normalised radioactive signal data from Northern studies

Figure 3.2	B	B+	K	K+	S	S+	U	U+	L	L+	H	H+
Data 1	0.006	0.2	0.001	0.008	0	0.002	0.07	1.1	0.008	0.2	0.004	0.034
Data 2	0.005	0.163	0.008	0.006	0	0.001	0.06	0.96	0.008	0.22	0.003	0.026
Figure 3.4	Cont	IL-1β	**Figure 4.2**	Cont	DbcA MP	Forsko lin	**Figure 4.3**	Cont	Chx	DbcA MP	DbcA MP + chx	
Data 1	1.16	18.88		0.04	1.04	0.33		0.09	0.4	2	5.02	
Data 2	0.04	0.68		0.02	0.51	0.12		0.26	1.3	5.23	13.78	
Figure 4.4	Cont	LPS	DBK	DKD	DBK + LPS	DKD + LPS						
Data 1	0.152	0.71	0.14	0.154	1.06	0.85						
Data 2	0.02	0.3	0.017	0.016	0.025	0.022						
Figure 4.5	0	0.5	1	2	4	6	8	16	25			
Data 1	0.2	0.71	1.14	3.12	1.68	1.95	1.8	1.19	1.05			
Data 2	0.005	0.0185	0.0325	0.08	0.04	0.045	0.037	0.031	0.026			
Figure 4.6	0	0.5	1	2	3	4	6	8	10			
Data 1	0.001	0.014	0.03	0.05	0.038	0.014	0.013	0.009	0.011			
Data 2	0.002	0.026	0.052	0.08	0.064	0.024	0.02	0.015	0.011			
Figure 4.7	0	0.5	1	2	4	6	8	10				
Data 1	0.028	0.12	0.32	0.24	0.11	0.06	0.072	0.076				
Data 2	0.04	0.18	0.46	0.34	0.17	0.08	0.107	0.11				
Figure 4.8	Cont	IL-1β	TNF-α	LPS								
Data 1	0.063	0.87	1.2	1.58								
Data 2	0.09	1.26	1.755	2.34								
Figure 4.9	Cont	IL-1β (2)	IL-1β (4,2)	IL-1β (4)	Cont	LPS (1)	LPS (2,1)	TNF-α (2)	TNF-α (4,2)			
Data 1	0.35	6.6	3.83	3.67	0.05	1.06	1.02	0.59	0.79			
Data 2	0.045	0.77	0.55	0.51	0.03	0.75	0.65	0.47	0.52			
Figure 4.10	Cont	IL-1Ra	IL-1β	IL-1β + IL-1Ra	LPS	LPS + IL-1Ra						
Data 1	0.14	0.15	0.83	0.27	1.36	1.19						
Data 2	0.03	0.03	0.23	0.07	0.35	0.31						
Figure 4.11	IL-1b	0	1	2	3	4	Cont	0	1	2	3	4
Data 1		100	48.4	26.7	8.5	6.2		100	56.3	14.1	8.9	
Data 2		100	51.3	27.3	9.2	5.8		100	55.9	25.2	6.3	
Figure 4.12	LPS	0	1	2	4	Cont	0	1	2	4		
Data 1		100	51.24	24.7	2.5		100	61.2	31.4	4.4		
Data 2		100	50.46	22.5	1.1		100	58.4	30.2	5		
Figure 4.13	4 kb	0	1	2	3	4	8 kb	0	1	2	3	4
Data 1		100	49.19	28.72	13.45	12.5		100	48.6	23.4	24.3	13.16
Data 2		100	52.3	31.86	14.2	9.9		100	49.5	27.41	24.3	12.1
Figure 4.14	Cont	IL-1β	LPS	Act D + IL-1β	Act D + LPS							
Data 1	2.86	76.46	84.66	2.33	3.33							
Data 2	0.08	1.71	2.32	0.12	0.09							
Figure 4.15	Cont	IL-1β	Chx	IL-1β + chx	Cont	Chx	LPS + chx	LPS				
Data 1	0.4	10.17	0.92	12.87	0.1	0.43	2.73	2.3				
Data 2	1.3	31.4	3.31	39.33	1.5	4.88	49.78	41.48				
Figure 5.1	Cont	LPS	Cont Trans	LPS trans								
Data 1	0.095	1.4	0.12	0.78								
Data 2	0.03	0.45	0.05	0.32								