THE EXPRESSION AND REGULATION OF KININ RECEPTORS IN RAT BLADDER SMOOTH MUSCLE CELLS

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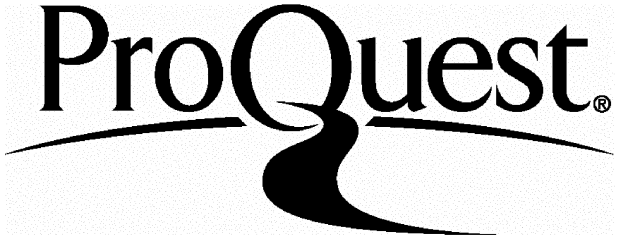

ABSTRACT

Kinins are important inflammatory mediators that have potent effects on a variety of cell types via B_1 and B_2 receptors. This thesis aimed to examine kinin receptor expression and regulation in rat bladder smooth muscle cells. The pharmacological profile of the responses of these cells to B_1 and B_2 agonists in a functional assay, measuring ^{45}Ca efflux, in conjunction with radioligand binding experiments, was consistent with the cells expressing both B_1 and B_2 receptors. In addition, the rat specific kinin, T-kinin, and its putative breakdown product, des-Arg^{11}-T-kinin, were potent and selective B_2 and B_1 agonists respectively, with selectivity for rat over human kinin receptors.

The pro-inflammatory cytokine IL-1ß upregulated the responsiveness of the rat bladder cells to B_1 agonists, but had no effect on responses to B_2 agonists. The effect of IL-1ß was inhibited by agents that inhibit protein synthesis such as cycloheximide and dexamethasone. This reflected increased expression of the B_1 receptor as IL-1ß increased B_1 receptor mRNA and specific binding of [^{3}H]-des-Arg^{10}-kallidin.

A novel mechanism by which B_1 responses can be modulated was elucidated. Treatment with dibutyryl cAMP also lead to an increase in B_1 receptor-evoked ^{45}Ca efflux with no effect on B_2 receptor-mediated responses. Although the increased responsiveness induced by dibutyryl cAMP was inhibited by cycloheximide and dexamethasone, there was no increase in B_1 receptor mRNA or in [^{3}H]-des-Arg^{10}-kallidin binding. This suggests that dibutyryl cAMP

stimulates the expression of a factor that acts downstream of the B_1 receptor to increase agonist-evoked ^{45}Ca efflux.

The results suggest that, during inflammation, the expression and function of B_1 receptors in the bladder may be increased by pro-inflammatory cytokines, whilst agents that elevate cAMP may increase B_1 receptor-mediated responses. In contrast B_2 mediated responses do not appear to be upregulated by inflammatory mediators in bladder smooth muscle cells.

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ABBREVIATIONS

ACE	angiotensin I converting enzyme
AP-1	activator protein 1
B9858	Lys-Lys-[Hyp^3,Igl^5,D-Igl^7,Oic^8]-des-Arg^9-bradykinin
BCG	*Mycobacterium bovis* bacillus calmette-guérin
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium
CPM	carboxypeptidase M
CPN	carboxypeptidase N
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
cAMP	cyclic AMP
CYP	cyclophosphamide
DAG	diacylglycerol
DbcAMP	N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate
DbcGMP	N^6,2′-O-dibutyrylguanosine 3′:5′-cyclic monophosphate

DMEM	Dulbecco's Modified Eagle Medium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FCS	foetal calf serum
GRE	glucocorticoid response element
HBSS	Hank's Balanced Salt Solution
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]
HMW kininogen	high molecular weight kininogen
HOE140	D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin
Hyp	trans-4-hydroxyproline
IC	interstitial cystitis
Igl	α-(2-indanyl)glycine
IKK	IκB kinase
IL-1ß	interleukin-1ß
IL-1R$_1$	type I interleukin-1 receptor
IL-1ra	interleukin-1 receptor antagonist

$Ins(1,4,5)P_3$	inositol-(1,4,5)-trisphosphate
IRAK	interleukin-1 associated kinase
JAK	Janus kinase
JNK	NH_2-terminal c-Jun kinase
kb	kilobase
LMW kininogen	low molecular weight kininogen
LPS	lipopolysaccharide
MAP kinase	mitogen activated protein kinase
MDP	muramyl dipeptide
MERGEPTA	D,L-2-mercaptomethyl-3-guanidinoethylthio-propanoic acid
NEP	neutral endopeptidase
NF-κB	nuclear factor κB
NIK	NF-κB inducing kinase
Oic	octahydroindolecarboxylic acid
PBS	phosphate buffered saline
PDTC	pyrrolidinedithiocarbamate
PGE_2	prostaglandin E_2

PGI_2	prostacyclin
PIC	phosphoinositidase C
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
PMCA	plasma membrane Ca^{2+}-ATPase
PtdIns(4,5)P_2	phosphatidylinositol-(4,5)-bisphosphate
RT-PCR	reverse transcriptase polymerase chain reaction
Sar	sarcosine
SDS	sodium dodecyl sulphate
SERCA	sarcoplasmic reticulum Ca^{2+}- ATPase
STAT	signal transducers and activators of transcription
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
Thi	ß-(2-thienyl)alanine
Tic	D-tetrahydroisoquinoline carboxylic acid

TNFα	tumour necrosis factor α
TRAF	TNF receptor associated factor
TRP	transient receptor potential
Tris	tris(hydroxymethyl)aminoethane
UV	ultra-violet

1 INTRODUCTION

1.1 INTRODUCTION TO KININS

Kinins, of which bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is the most widely studied, are peptide mediators released from inactive precursors called kininogens by the action of serine proteases known as kallikreins (Bhoola et al., 1992). They evoke pain and have a variety of other inflammatory actions including vasodilatation, increased vascular permeability and the release of pro-inflammatory substances such as prostanoids, cytokines and neuropeptides (Regoli and Barabé, 1980, Dray and Perkins, 1993). They also mediate some non-inflammatory effects including regulation of smooth muscle tone, water and electrolyte transport and sperm motility (Regoli and Barabé, 1980). The effects of kinins are mediated via interactions with two classes of cell surface receptors, the B_1 and B_2 bradykinin receptors. The B_2 receptor is thought to mediate the majority of the actions of kinins and is widely distributed on a variety of tissues including vascular smooth muscle, intestinal epithelium and smooth muscle, airway smooth muscle, ocular and neural tissues, see Hall (1992) for a review. The B_1 receptor is not widely expressed under normal conditions, but following tissue injury it is induced in a variety of tissues, especially smooth muscle, see Marceau (1995) for a review.

Although both classes of kinin receptor are involved in inflammatory processes, the B_2 receptor is thought to be involved primarily in the acute phase, whereas the B_1 receptor has a role in chronic inflammatory conditions, see Dray and Perkins (1993). This is supported by evidence from behavioural experiments that suggests B_1 receptors are expressed following the induction of chronic

inflammatory pain conditions (Perkins and Kelly, 1993, Davis and Perkins, 1994). These studies suggest that antagonists of the B_1 receptor may prove to be useful therapies for inflammatory diseases.

Kinins have been shown to produce powerful biological actions in the urinary tract, including smooth muscle contraction, plasma protein extravasation and release of neuropeptides such as CGRP (Saria et al., 1983, Maggi et al., 1989). The majority of these actions are thought to be mediated by B_2 receptors (Maggi et al., 1993). However, following the induction of inflammation, B_1-evoked responses, such as smooth muscle contraction, have been observed (Belichard et al., 1999). In inflammatory diseases of the bladder, such as interstitial cystitis there is evidence that levels of kinins and kallikreins in the bladder wall and urine are elevated (Zuraw et al., 1994, Rosamilla et al., 1999) and there is evidence that levels of the B_1 receptor are increased (Ruggieri et al., 1999).

In order to gain understanding of the role of kinins and their receptors in the bladder, the expression and regulation of kinin receptors in primary cultures of smooth muscle cells isolated from the rat bladder has been investigated. The pharmacological profile of kinin receptors on bladder smooth muscle cells was characterised and the effect of inflammatory mediators on kinin receptor levels and responses was investigated. In a separate, but related, study the rat B_1 receptor from the bladder was cloned in order to examine the regulatory elements in more detail (Jones et al., 1999). Together, these studies will help to elucidate the role of kinin receptors in bladder smooth muscle *in vivo* and provide evidence on how the expression of these receptors is controlled.

1.2 DISCOVERY OF KININS

Kinins were first investigated in 1909 when two French surgeons, Abelous and Bardier (Abelous and Bardier, 1909) observed a transient fall in the blood pressure in man following intravenous injections of fractions extracted from human urine. This factor was later given the name kallikrein (Kraut et al., 1930) and was found to release a smooth muscle-contracting substance from serum that Werle named "kallidin" (Werle and Berek, 1948). Around the same time Rocha e Silva et al. (1949) found that incubating blood with trypsin released an agent that contracted the guinea-pig ileum. As this response developed slowly compared to histamine he named the factor bradykinin. The sequence of bradykinin was elucidated in 1960 by Boissonnas and co-workers thus enabling further characterisation of its roles. The sequence of kallidin was determined as Lys-bradykinin by Werle et al. (1961).

1.3 FORMATION OF KININS

Bradykinin and kallidin are cleaved from precursor proteins known as kininogens by the action of enzymes known as kallikreins, for reviews see Proud and Kaplan (1988) and Bhoola et al. (1992). There are two separate pathways for the generation of kinins (Figure 1-1). In the plasma, prekallikrein, the inactive precursor of kallikrein, and high molecular weight kininogen (HMW kininogen) circulate in a complex. Prekallikrein is activated by another plasma protein known as the Hageman factor, which is itself activated by contact with negatively charged surfaces such as collagen, basement membrane and bacterial lipopolysaccharides (LPS). As a result of the increased vascular permeability that occurs during inflammation, Hageman factor, prekallikrein and kininogen leak out of the vessels. Contact with the negatively charged surface promotes the interaction of prekallikrein and Hageman factor, activating prekallikrein and leading to the production of bradykinin. The second pathway for kinin generation involves a distinct enzyme, tissue kallikrein, which is found in a variety of tissues such as the pancreas, salivary glands and kidney. Tissue kallikrein can act on HMW kininogen and low molecular weight kininogen (LMW kininogen) to generate kallidin. Tissue kallikrein is released from an inactive precursor (tissue prekallikrein) by the action of proteolytic enzymes. In the rat, due to a minor difference in the sequence of the cleavage site, tissue kallikrein generates bradykinin from kininogens. Tissue kallikrein is found in the bladder, and has been shown to be localised to the lamina propria, an area that expresses a high level of B_2 receptors, and to the smooth muscle (Manning and Snyder, 1989, Orfila et al., 1993).

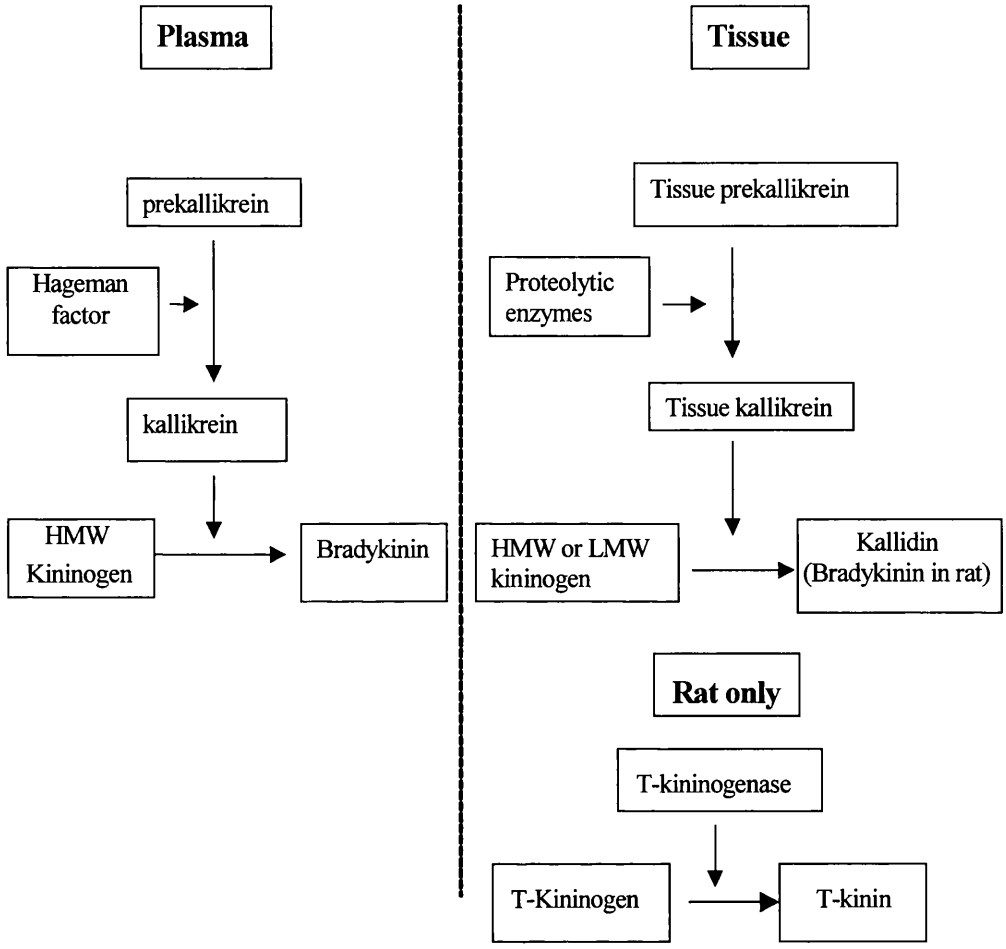

Figure 1-1 Formation of kinins

HMW and LMW kininogens are formed by the alternate splicing of a single gene; in the rat gene duplication has resulted in a third type of kininogen known as T-kininogen. It is broken down by the enzyme T-kininogenase to T-kinin (Ile-Ser-bradykinin) which has similar actions to bradykinin. T-kininogenase is expressed mainly in the submandibular gland and kidney of the rat (Ma et al., 1992). The expression of T-kininogen is increased following an inflammatory insult (Barlas et al., 1985a,b). In contrast, the expression of HMW and LMW kininogens is unaffected by inflammation (Barlas et al., 1985a).

1.4 DEGRADATION OF KININS

Bradykinin and kallidin are rapidly broken down by a number of peptidase enzymes, (for reviews see Bhoola et al., 1992 and Skidgel, 1992). The most important of these are carboxypeptidase N (CPN), carboxypeptidase M (CPM), angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) (Figure 1-2). CPN and CPM are sometimes referred to as kininase I and ACE and NEP as kininase II. CPN is synthesised by the liver and secreted into the circulation. It removes the C-terminal arginine from bradykinin to form des-Arg^9-bradykinin and is thought to account for 10% of the bradykinin destroying activity in human plasma. CPM is a membrane bound enzyme that has been found in human kidney, lung tissue and fibroblasts. It cleaves the C-terminal arginine from both bradykinin and kallidin to form des-Arg^9-bradykinin and des-Arg^{10}-kallidin. These actions of CPN and CPM are important because the des-Arg^9-metabolites of bradykinin and kallidin are selective agonists for the B_1 receptor. ACE and NEP both hydrolyse the Pro-Phe bond at the C-terminal end of bradykinin and kallidin and can metabolise the des-Arg^9-metabolites. The extra two amino-acids in T-kinin have been shown to confer resistance to ACE (Passaglio and Vieira, 1996), however, it is subject to degradation and can be converted to bradykinin by the action of an aminopeptidase (Vieira et al., 1994). It is also susceptible to carboxypeptidases (Passaglio and Vieira, 1996), but it is not known whether des-Arg^{11}-T-kinin occurs naturally or if it is always broken down to des-Arg^9-bradykinin via bradykinin. Kallidin can be converted to bradykinin by the action of aminopeptidase M, which is present in the plasma (Proud et al., 1987, Sheik and Kaplan, 1989).

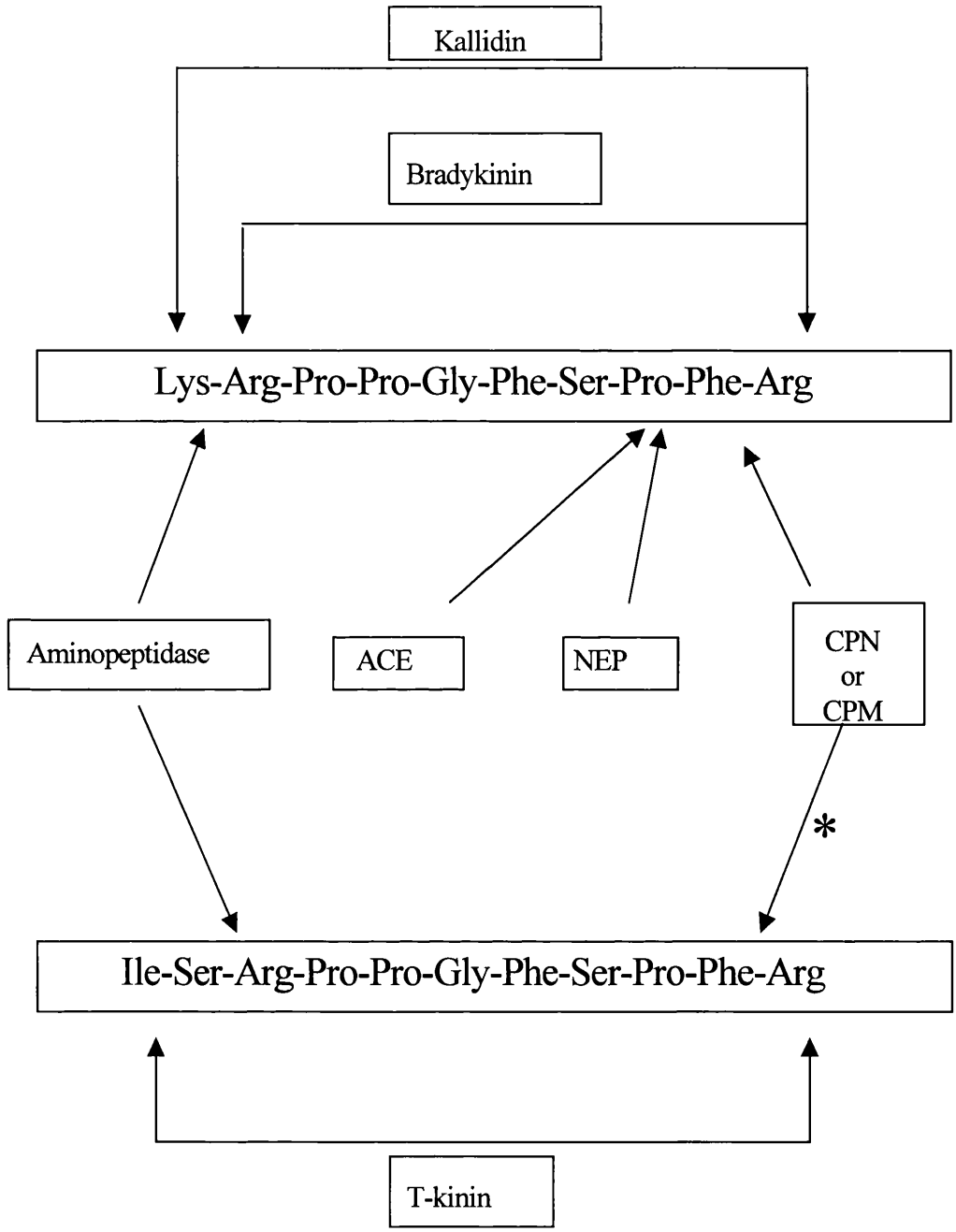

Figure 1-2 Degradation of kinins
* although this occurs *in vitro* it is not known if it occurs *in vivo*.

Other peptidases that will break down kinins include proline aminopeptidase, which specifically cleaves peptides with a penultimate proline at the N-terminus, prolyl endopeptidase which hydrolyses the same bond as ACE and NEP, endopeptidase 24.15 which hydrolyses the Phe-Ser bond of bradykinin and

meprin which can cleave the Gly-Phe bond of bradykinin. The extent to which any of these enzymes will inactivate kinin depends on their localisation and access to kinin substrate.

1.5 KININ RECEPTORS

The classification of kinin receptors into B_1 and B_2 was proposed by Regoli et al. (1977) based on pharmacological evidence from a variety of smooth muscle preparations. In contrast to tissues such as the rabbit jugular vein, rat uterus and dog carotid artery, which were more sensitive to bradykinin, it was found that the rabbit aorta was much more sensitive to des-Arg^9-bradykinin. The rabbit aorta receptor was designated B_1 and the receptor in the other tissues B_2. This classification system has become widely accepted and almost all actions of kinins can be attributed to one of these two receptors, although the existence of further subtypes has been proposed. For example, Farmer et al. (1989) claimed that bradykinin-induced contractions in the guinea-pig trachea were not inhibited by either B_1 or peptide B_2 antagonists (see Sections 1.5.1.2 and 1.5.2.2) and proposed that there might be a third type of kinin receptor. In addition, they claimed that the non-peptide B_2 antagonist WIN64338 inhibited bradykinin-induced contractions in the guinea-pig ileum but not in the trachea (Farmer and DeSiato, 1994). Subsequent studies have not provided any support for this hypothesis (Pruneau et al., 1995) and there is no evidence of any other receptor subtypes from cloning experiments.

1.5.1 THE B2 RECEPTOR

1.5.1.1 STRUCTURE

The rat B_2 receptor was the first kinin receptor to be cloned and sequenced (McEachern et al., 1991). Subsequently, the human (Hess et al., 1992), mouse (Ma et al., 1994) and guinea-pig (Farmer et al., 1998) B_2 receptor genes were cloned. The rat and mouse sequences are highly homologous, with approximately 90% homology at the amino-acid level. The human sequence has

about 80% homology with both the rat and mouse. The B_2 receptor shares limited homology with the B_1 receptor, for example the human B_1 and B_2 receptors are 36% identical, and the mouse B_1 and B_2 receptors 30%. There is also some homology (about 30%) between the B_2 receptor and the angiotensin II receptor. The B_2 receptor is a typical seven-transmembrane domain G-protein linked receptor with a predicted molecular weight of approximately 42,000Da. Analysis of the B_2 receptor protein purified from rat uterus revealed a protein with a molecular weight of 81,000Da (Yaqoob and Snell, 1994). The difference in predicted and actual molecular weights is likely to be due to glycosylation of the receptor; the sequence contains three potential N-linked glycosylation sites (Yaqoob et al., 1995). There are also consensus sites for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC). Analysis of genomic clones of the B_2 receptor shows that the gene has three exons separated by two introns. In the rat and mouse the coding domain is located solely on the third exon (Ma et al., 1994, Wang et al., 1994), whereas the human coding domain is split between the second and third exons (Kammerer et al., 1995). Various transcription factor consensus sequences, including the IL-6 response element and cyclic AMP response element (CRE), are found in the 5′ flanking region (Ma et al., 1994, Wang et al., 1994).

1.5.1.2 PHARMACOLOGY

B_2 receptors are defined as having the rank order of agonist potency [Tyr(Me)^8]-bradykinin > bradykinin > des-Arg^9-bradykinin. In addition, kallidin is more potent than des-Arg^{10}-kallidin. The first B_2 receptor antagonists were developed when it was found that replacing the proline at position 7 with a D-aromatic amino such as D-phenylalanine acid yielded an antagonist (Vavrek and Stewart,

1985). This modification also conferred resistance to ACE breakdown. Further modifications such as replacement of phenylalanine at positions 5 and 8 with isoteric ß-(2-thienyl)alanine (Thi) and replacement of one or both prolines at positions 2 and 3 with trans-4-hydroxyproline (Hyp) lead to enhanced potency. Extension of the N-terminus with Lys-Lys or D-Arg is thought to confer resistance to aminopeptidases (Bathon and Proud, 1991). These structures were, however, still subject to degradation by CPN and NEP. The next generation of B_2 antagonists, such as HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin), were more stable and showed a great increase in potency (Hock et al., 1991). Oic (octahydroindolecarboxylic acid) confers resistance to CPN whereas the bulky Tic (D-tetrahydroisoquinoline carboxylic acid) strongly restricts the conformational freedom of the molecule to a shape that fits the receptor (Kyle et al., 1993). Non-peptide B_2 antagonists have also been developed such as WIN64338 (Sawutz et al., 1994), FR173657 (Asano et al., 1997) and bradyzide (Burgess et al., 2000).

1.5.1.3 SIGNAL TRANSDUCTION

B_2 receptor activation has been most consistently linked with activation of phosphoinositidase C (PIC), in most cases PICß, leading to the breakdown of phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P_2) to inositol-(1,4,5)-trisphosphate (Ins(1,4,5)P_3), leading to the release of calcium from intracellular stores. This has been demonstrated for the B_2 receptor in a variety of cell types including NG108-15 neuroblastoma cells (Yano et al., 1984), cultured sensory neurones (Burgess et al., 1989), smooth muscle cells (Tropea et al., 1993) and guinea pig macrophages (Böckmann and Paegelow, 1995). It is thought that bradykinin-induced activation of PIC occurs via the G_q class of G protein

(Gutowski et al., 1991), although there is some evidence that bradykinin activates a cytoplasmic tyrosine kinase which can then activate another form of PIC (PICγ) (Venema et al., 1998). The breakdown of PtdIns(4,5)P_2 will also lead to the production of diacylglycerol (DAG), an activator of PKC. Bradykinin has most frequently been linked to the translocation of PKC-ε (Cesare et al., 1999, Lal et al., 1998), although bradykinin-induced translocation of other subtypes, including PKC-α, δ and ξ has been reported (Tippmer et al., 1994, Graness et al., 1997).

In several cell types such as fibroblasts, the renal cell line MDCK and keratinocytes (Kast et al., 1993, Kennedy et al., 1997, McAllister et al., 1993), activation of the B_2 receptor leads to arachidonic acid production. This can result from the action of diacylglycerol lipase on DAG or phospholipase A_2 (PLA_2) on phospholipids. Although G-proteins can activate PLA_2 directly, bradykinin is thought to activate the calcium-stimulated cytosolic PLA_2, as bradykinin-induced arachidonic acid production has been shown to be dependent on calcium (Kast et al., 1993, Kennedy et al., 1997, McAllister et al., 1993). Arachidonic acid serves as a substrate for cyclooxygenase and lipoxygenases, activation of which leads to the production of prostanoids and leukotrienes.

Bradykinin has also been reported to activate enzymes in the mitogen activated protein kinase (MAP kinase) pathway (El-Dahr et al.,1998). On activation MAP kinases translocate to the nucleus and phosphorylate a number of transcription factors, leading to changes in gene transcription.

1.5.1.4 EXPRESSION AND REGULATION

The B_2 receptor is constitutively expressed on a wide variety of tissues. This has been shown by Northern and Southern blot analysis (Ma et al., 1994, McEachern et al., 1991) and also by binding and functional studies (Hall, 1992). Tissues that are known to express high levels of B_2 receptor mRNA include uterus, ileum, pancreas and lung. There are, however, a variety of factors that have been shown to regulate B_2 receptor expression. In particular, agents that increase cyclic AMP (cAMP) have been shown to upregulate B_2 receptor levels and increase functional responses to bradykinin. In cultured canine smooth muscle cells, treatment with cholera toxin, forskolin and dibutyryl cyclic AMP (dbcAMP) increased the number of bradykinin binding sites and enhanced bradykinin-induced increases in intracellular calcium ($[Ca^{2+}]_i$) and $Ins(1,4,5)P_3$ accumulation (Yang et al., 1994). In rat mesangial cells, forskolin, prostaglandin E_2 (PGE_2) and 8-bromo cyclic AMP treatment resulted in increases in B_2 receptor mRNA, bradykinin binding sites and an increase in bradykinin-stimulated PGE_2 release, but not an increase in bradykinin-induced $Ins(1,4,5)P_3$ production or rise in $[Ca^{2+}]_i$ (Castaño et al., 1998).

Cytokines have also been shown to enhance B_2 mediated responses. In human synovial cells interleukin-1α increased the number of B_2 receptors (Bathon et al., 1992) and interleukin-1ß (IL-1ß) amplified bradykinin-induced PGE_2 production (Angel et al., 1994). In bronchial smooth muscle cells IL-1ß increased B_2 receptor density and B_2 receptor mRNA; the mechanism for this appeared to be mediated via PGE_2-induced cAMP production (Schmidlin et al., 1998). Tumour necrosis factor-α (TNFα) has been shown to potentiate

bradykinin-induced increases in $[Ca^{2+}]_i$ in tracheal smooth muscle cells (Amrani and Bronner, 1993).

An important characteristic of the B_2 receptor is that it exhibits rapid desensitisation, i.e. a second stimulation with bradykinin gives a much reduced response (Burgess et al., 1989, Roberts and Gullick, 1990). This has not been observed with the B_1 receptor and there is no evidence for cross-desensitisation between B_1 and B_2 receptors (Smith et al., 1995). In Chinese hamster ovary cells transfected with cDNA for the B_2 receptor, stimulation with bradykinin lead to rapid internalisation of the receptor suggesting that receptor sequestration may be involved in desensitisation. In contrast, the B_1 receptor expressed in the same cells was not internalised on stimulation with des-Arg^9-bradykinin. Chimeric receptors consisting of the B_2 receptor with the C-terminal region of the B_1 receptor did not internalise, but B_1 receptors containing the C-terminal region of the B_2 were internalised suggesting that the C-terminal tail is critical for ligand-induced receptor internalisation (Faussner et al., 1998). A recent report (Pizard et al., 1999) suggested that phosphorylation is important in the internalisation and identified three serines and two threonines as potential phosphorylation sites in the C-terminal tail region. A role for cyclic GMP in B_2 receptor desensitisation has been proposed. In dorsal root ganglion cells bradykinin induced a rise in cyclic GMP and exogenously applied cyclic GMP decreased the bradykinin-induced increase in $Ins(1,4,5)P_3$, but the number of $[^3H]$-bradykinin binding sites and the affinity of $[^3H]$-bradykinin for the receptor were not affected (Harvey and Burgess 1996), implying an effect on the coupling between the G-protein and receptor. Similar findings have also been reported for vascular endothelial cells (Miyamoto et al., 1997).

1.5.2 THE B1 RECEPTOR

1.5.2.1 STRUCTURE

The B_1 receptor has been cloned from a variety of species including human, mouse rabbit and rat (Menke et al., 1994, Pesquero et al., 1996, MacNeil et al., 1995, Ni et al., 1998a). The sequence shows that the receptor is a typical G-protein linked receptor with seven transmembrane domains. There is some homology between the B_1 and B_2 receptors (see Section 1.5.1.1) and the B_1 receptor also shares approximately 30% homology with the angiotensin II receptor. Two conserved Cys residues that are proposed to form a disulphide bond between the second and third extracellular domains of nearly all G-protein-coupled receptors are present in the sequence of all species that have been cloned so far. There are two consensus sites for N-linked glycosylation in the NH_2-terminal domain and one in the third extracellular domain. Potential phosphorylation sites for PKC and PKA are present in intracellular domains 2 and 3 and the carboxy terminal domain (Menke et al., 1994). The rabbit protein sequence has 78% homology with the human sequence and has a very similar structure. The mouse and rat sequences have 67% and 71% homology to the human sequence respectively, but there are two key differences. In the mouse and rat sequences there is an 8-amino acid insertion in the first intracellular loop and the C-terminus is reduced from 36 amino acids in the human to 11 in the mouse and rat (Pesquero et al., 1996, Ni et al., 1998a). The truncation of the C-terminal tail means that the rat and mouse receptors lack both a phosphorylation site and a cysteine that is a putative palmitoylation site in the human and rabbit sequences. Palmitoylation is thought to affect localisation of the receptor in the membrane and be implicated in protein:protein interactions. These variations in sequence may cause differences between species in the coupling to G-proteins.

The organisation of the human B_1 gene has also been studied (Bachvarov et al., 1996, Yang and Polgar 1996). The gene is present as a single copy and spans more than 10 kilobases (kb) containing three exons interrupted by two introns. While the 5' untranslated region is distributed on all three exons, the coding domain is located entirely on the third exon. Sequences for important transcription factors such as activator protein 1 (AP-1), simian virus-40 protein 1, nuclear factor κB (NF-κB), CCAAT/enhancer protein and cyclic AMP response element binding protein (CREB) are found in the 5'-flanking region and also in intron II. Two promoter regions have also been found, one located in the 5'-flanking region, the other in the intron II region. The structure of the rat gene is similar to the human except that there are only two exons and the entire coding domain is within the second exon. The 5' flanking region of the rat B_1 gene also contains a sequences for a variety of transcription factors including CREB, AP-1 and NF-κB (Ni et al., 1998a).

1.5.2.2 PHARMACOLOGY

The rank order of potency for agonists at the B_1 receptor is usually defined as des-Arg^9-bradykinin > Tyr(Me^8)-bradykinin > bradykinin. In addition, des-Arg^{10}-kallidin is more potent than kallidin. The relative potencies of des-Arg^9-bradykinin and des-Arg^{10}-kallidin depend on the species. At the rabbit (MacNeil et al., 1995) and human (Menke et al., 1994, Jones et al., 1999) receptors it was found that des-Arg^{10}-kallidin was 100-10000 times more potent than des-Arg^9-bradykinin, whereas at the mouse and rat receptors des-Arg^9-bradykinin and des-Arg^{10}-kallidin have similar potencies (Hess et al., 1996, Ni et al., 1998a, Jones et al., 1999). In addition, at the human and rabbit receptors kallidin, although less potent than des-Arg^{10}-kallidin, appeared to be more

potent than des-Arg^9-bradykinin. This may suggest that kallidin and des-Arg^{10}-kallidin are the important physiological kinins *in vivo* in these species, although it is possible that the apparent potency of kallidin in these studies was due to conversion to des-Arg^{10}-kallidin by CPN or CPM. In some studies, when the activity of CPN and CPM was inhibited with D,L-2-mercaptomethyl-3-guanidinoethylthio-propanoic acid (MERGEPTA), the apparent activities of kallidin and bradykinin at the B_1 receptor were significantly reduced (Regoli et al., 1990, Pesquero et al., 1996).

Other B_1 agonists have been developed by modification of the peptide sequence. Replacement of Phe^8 of des-Arg^9-bradykinin with D-Phe reduces degradation by carboxypeptidases and extension of the N-terminal with sarcosine (Sar) increases affinity and selectivity for the B_1 receptor, thus Sar-[D-Phe^8]-des-Arg^9-bradykinin is a stable, potent and selective B_1 agonist (Rhaleb et al., 1990).

The first kinin antagonist to be developed was actually a B_1 antagonist. It was found that replacement of Phe^8 of des-Arg^9-bradykinin with a hydrophobic aliphatic amino acid such as leucine yielded an antagonist (Regoli et al., 1977). Since then [Leu^8]-des-Arg^9-bradykinin has become the most widely used B_1 antagonist. The next B_1 antagonist to become widely available was the des-Arg^{10} form of the very potent B_2 antagonist, HOE140 (des-Arg^{10}-HOE140). The latest generation of B_1 antagonists incorporate some of the features that made development of stable B_2 antagonists possible (see section 1.5.1.2) and also introduce α-(2-indanyl)glycine (Igl) into the structure resulting in a great increase in potency (Stewart et al., 1996) in both B_1 and B_2 type structures. Of these Lys-Lys-[Hyp^3,Igl^5,D-Igl^7,Oic^8]-des-Arg^9-bradykinin (B9858) is a highly

potent and selective B_1 antagonist. Screening of combinatorial libraries has recently lead to the discovery of a non-peptide B_1 antagonist (Horlick et al., 1999).

1.5.2.3 SIGNAL TRANSDUCTION

In common with the B_2 receptor, the primary mechanism of B_1 receptor signal transduction is thought to be activation of PIC leading to the formation of $Ins(1,4,5)P_3$, with a subsequent increase in $[Ca^{2+}]_i$, and DAG. An increase in $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ following B_1 receptor activation has been demonstrated in a variety of cell types including vascular smooth muscle cells (Levesque et al., 1993, Schneck et al., 1994, Mathis et al., 1996), mesangial cells (Bascands et al., 1993), rabbit urinary bladder (Butt et al., 1995) and endothelial cells (Smith et al., 1995). The activation of PKC following B_1 receptor activation has not been widely investigated, however B_1-mediated translocation of PKC isoforms α, δ and ε to the membrane has been demonstrated in mesangial cells (Mouledous et al., 1997).

In many cell types including smooth muscle (Levesque et al., 1993, Galizzi et al., 1994), gastric mucosa (Brown et al., 1992) and endothelial cells (D'Orléans-Juste, et al., 1989) activation of the B_1 receptor has been associated with production of prostanoids including PGE_2 and prostacyclin (PGI_2). Arachidonic acid can be formed from phospholipids by PLA_2 or from DAG by diacylglycerol lipase. PLA_2 can be activated directly by G-protein coupled receptors (Axelrod et al., 1988) or stimulated by calcium, as described for the B_2 receptor. As B_1 agonists also increase $[Ca^{2+}]_i$, it is likely that this is the mechanism for B_1

receptor-induced production of prostanoids, although this has not been investigated.

Prostanoids can cause smooth muscle contraction, but it is unclear to what extent they play a role in the contraction of smooth muscle induced by B_1 receptor activation. In the rabbit aorta des-Arg^9-bradykinin-induced contractions were not inhibited by the cyclooxygenase inhibitor indomethacin and it was proposed that the rise in $[Ca^{2+}]_i$ induced by B_1 receptor activation was the primary mechanism of smooth muscle contraction (Levesque et al., 1993). However, in the rat bladder, B_1 receptor-induced contractions were inhibited by the cyclooxygenase inhibitor S-(–)-ketoprofen (Meini et al., 1998).

There is also a report that B_1 receptor activation can lead to activation of the MAP kinase pathway (Naraba et al., 1998). However this study was carried out in HEK 293 cells over-expressing the B_1 receptor and may not reflect the situation in cells which express the receptor naturally.

1.5.2.4 EXPRESSION AND REGULATION

The B_1 receptor is generally considered an "inducible" receptor which is not expressed, or expressed at very low levels, under physiological conditions and upregulated after tissue damage or exposure to inflammatory agents. Since the original discovery of B_1 receptors on the isolated rabbit aorta (Regoli et al, 1977), several other vascular tissues have also been shown to express the B_1 receptor following a lengthy incubation *in vitro*. For example, following incubation for 4.5h, the rat portal vein contracted in response to des-Arg^9-bradykinin (Campos and Calixto, 1994), and in the human umbilical vein des-Arg^9-bradykinin-induced contractions were observed following 4-6h of

incubation (Gobeil et al., 1996). In other vascular preparations B_1-induced relaxation has been observed. For example, in the pig coronary artery des-Arg^9-bradykinin-induced relaxations were observed following a 6h incubation (Pruneau et al., 1996). Bovine coronary artery (Drummond and Cocks, 1995) and rabbit mesenteric artery (Deblois and Marceau, 1987) have also been shown to relax in response to des-Arg^9-bradykinin following incubation of the tissue for approximately 6h. In all of these studies the relaxation was dependent on the presence of the endothelium and, in studies where it was tested, inhibited by the nitric oxide synthase inhibitor N^G-nitro-L-arginine (Drummond and Cocks, 1995, Pruneau et al., 1996). This suggests that the B_1 receptor is expressed on the endothelial cells, and that activation of the receptor leads to activation of nitric oxide synthase, which is stimulated by calcium. NO can then diffuse from the endothelial cells to smooth muscle cells where it can activate guanylate cyclase, leading to the production of cyclic GMP, which causes relaxation of smooth muscle. In tissues such as the rabbit aorta, where des-Arg^9-bradykinin evokes a contraction even in the presence of the endothelium, the smooth muscle contraction must outweigh the relaxant effect of the endothelium. This could be due to damage to the endothelium or low expression of B_1 receptors on the endothelium of that tissue.

Some non-vascular smooth muscle preparations have also been shown to express the B_1 receptor under similar conditions to vascular smooth muscle. For example, following an overnight incubation *in vitro*, the human ileum was shown to contract in response to des-Arg^9-bradykinin (Zuzack et al., 1996). The rabbit (Butt et al., 1995) and mouse (Busser et al., 1998) bladders have both been shown to contract in response to des-Arg^9-bradykinin following incubation

of the tissue for 3-6h. The mouse trachea has been reported to relax in response to des-Arg^9-bradykinin following a 6h incubation (Trevisani et al., 1999).

The expression of the B_1 receptor in isolated vascular smooth muscle preparations is enhanced by treatment with cytokines. Treatment of the rabbit aorta with IL-1ß, IL-2 and epidermal growth factor (EGF) for 3-6h lead to an increase in the size of the contractile response evoked by des-Arg^9-bradykinin (Bouthillier et al., 1987, Deblois et al., 1988, Deblois et al., 1989). In the human umbilical vein treatment with IL-1ß for 75min followedd by a 5h incubation caused a leftward shift of the des-Arg^9-bradykinin concentration response curve, without affecting the maximal response (Sardi et al., 1998). Cytokines are produced by activated macrophages and it has been shown that agents that stimulate macrophages, such as muramyl-dipeptide (MDP), LPS and phorbol myristate acetate (PMA) (Baqui et al., 1998, Sugawara et al., 1996, Keicho et al., 1991), also increased the size of the contractile response to des-Arg^9-bradykinin in the rabbit aorta (Bouthillier et al., 1987). The effects of agents such as IL-1ß and LPS in non-vascular smooth muscle have not been as widely studied, however, in the isolated mouse bladder, LPS treatment upregulated B_1 receptor expression but had no effect on B_2 receptors (Busser et al., 1998) and, in the rat bladder, IL-1ß increased the contraction evoked by des-Arg^9-bradykinin (Roslan et al., 1995).

These inflammatory mediators also upregulate B_1 receptor expression if given *in vivo*. For example, administration of des-Arg^9-bradykinin to rabbits treated with LPS, MDP, PMA or IL-1ß resulted in a hypotensive effect that did not occur in control animals (Bouthillier et al., 1987, Deblois et al., 1989, Deblois et al.,

1991). The location of the B_1 receptor that mediates this effect is not known but it is more likely to be on endothelial cells, where activation of the receptor leads to the release of NO, than on smooth muscle, where activation results in contraction. It is known, however, that the B_1 receptor is induced on smooth muscle under these conditions, as aortas removed from rabbits treated with LPS or IL-1ß contracted in response to des-Arg^9-bradykinin after an unusually short *in vitro* incubation period (Deblois et al., 1989, Deblois et al., 1991). The presence of the endothelium *in vivo* probably reduces contact of the smooth muscle with B_1 agonists.

The mechanism by which the response to B_1 agonists is upregulated is not fully understood, but it is thought to involve synthesis of new receptor protein rather than to be an effect on existing receptor or an effect downstream of the receptor. This has been demonstrated *in vitro* using a range of modulators of protein synthesis. Continuous exposure of smooth muscle preparations to either the protein synthesis inhibitor cycloheximide (Bouthillier et al., 1987), the RNA synthesis inhibitor actinomycin D (Drummond and Cocks, 1995), the protein translocation inhibitor brefeldin A or the protein glycosylation inhibitor tunicamycin (Audet et al., 1994) has been shown to inhibit the development of a response to des-Arg^9-bradykinin. Glucocorticosteroids, such as dexamethasone, have been shown to inhibit both the spontaneous and IL-1ß-stimulated development of a contractile response to des-Arg^9-bradykinin in the rabbit aorta (Deblois et al., 1988). As glucocorticosteroids interfere with gene transcription (see Discussion for more details of their mechanism of action), these results are another indication that the increase in the level of the B_1 receptor activity is due to new receptor synthesis.

Experiments using cultured cells isolated from tissues that express the B_1 receptor have provided further evidence that the increase in B_1-mediated responses is due to an increased level of B_1 receptor protein. The presence of B_1 receptors in primary cultures of smooth muscle cells of vascular origin has been demonstrated functionally and in binding studies (Tropea et al., 1993, Mathis et al., 1996, Galizzi et al., 1994, Schneck et al., 1994). Treatment of rabbit aorta smooth muscle cells with IL-1ß lead to an increase in des-Arg^9-bradykinin-induced Ins(1,4,5)P_3 formation (Levesque et al., 1993) and an increase in the number of binding sites for [^{3}H]-des-Arg^{10}-kallidin (Galizzi et al., 1994). The number of B_1 receptors on rabbit aorta smooth muscle cells increased with time in culture and in the presence of EGF (Schneck et al., 1994). In rat vascular smooth muscle cells, B_1 receptor mRNA levels were increased by treatment with LPS, IL-1ß, TNFα and foetal calf serum (FCS) (Ni et al., 1998b). Cell lines of human lung fibroblast origin such as WI-38 and IMR-90 have also been used to study the regulation of the B_1 receptor. These cells express the B_1 receptor constitutively, however the level of expression can be increased by treatment with cytokines (Menke et al., 1994, Phagoo et al., 1997). The possibility that kinin agonists may themselves increase B_1 receptor expression has been investigated in IMR-90 cells where it was shown that treatment with des-Arg^{10}-kallidin (Schanstra et al., 1998, Phagoo et al., 1999) or bradykinin (Phagoo et al., 1999) increased both B_1 receptor mRNA and protein.

The cloning of the B_1 receptor gene has allowed the study of the regulatory regions which may be involved in the induction of new receptor expression. Study of the 5' flanking region has demonstrated the presence of both positive and negative regulatory elements (Yang, X. et al., 1998). In addition, there are

consensus sequences for a variety of transcription factors including AP-1, NF-κB and CREB in both the human and rat B_1 genes (Ni et al., 1998a,b). Transfection of this region of the human B_1 gene linked to the luciferase gene into rat vascular smooth muscle cells lead to basal, IL-1ß and LPS stimulated luciferase activity. Mutations of the consensus sequences demonstrated that the NF-κB site is important for LPS and IL-1ß stimulated activity, whereas the CRE site is involved in both basal and IL-1ß or LPS stimulated activity. The AP-1 site appeared not to be involved (Ni et al., 1998b). These results indicate that the NF-κB and CRE sites may have an important role in controlling expression of the B_1 receptor gene. Further evidence for a role for NF-κB came from the observation that the NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC) inhibited IL-1ß-stimulated B_1 receptor mRNA and protein expression in IMR-90 cells (Schanstra et al., 1998). Not all studies, however, agree on the role of NF-κB in IL-1ß-stimulated B_1 receptor expression. In IMR-90 cells PDTC inhibited PMA-stimulated B_1 mRNA production but not IL-1ß-stimulated production, which was inhibited by tyrosine kinase inhibitors (Zhou et al., 1998).

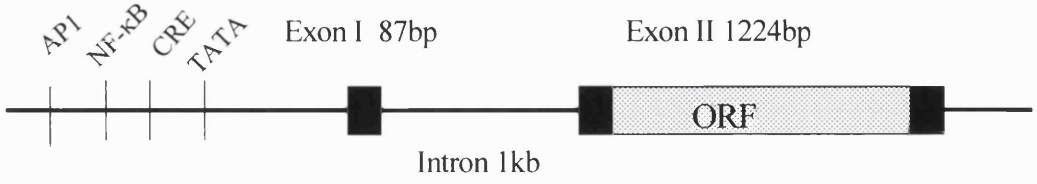

Figure 1-3 Structural organisation and regulatory elements of the rat B_1 receptor gene.
ORF – open reading frame

MAP kinases can be activated by cytokines such as IL-1ß and TNFα, and role for these enzymes in the upregulation of the B_1 receptor has been proposed (Larivée et al., 1998, Zhou et al., 1998, Haddad et al., 2000). MAP kinases can

be divided into three broad classes, the extracellular signal-regulated kinase (ERK), NH_2-terminal c-Jun kinase (JNK) and p38 MAP kinase. Specific inhibitors can be used to determine the extent to which each class of MAP kinase is involved in the regulation of the B_1 receptor. However, this aspect of B_1 receptor regulation was not investigated in the current study.

1.6 KININ RECEPTORS AND INFLAMMATION

Both B_1 and B_2 receptors are thought to have a role in inflammatory processes and also the pain associated with inflammation, see Dray and Perkins (1993) and Ahluwalia and Perretti (1999) for reviews. The B_2 receptor, but not the B_1 receptor, is involved in acute pain as B_2, but not B_1 agonists, elicit pain in the human blister base model (Whalley et al., 1987), and studies of acute nociceptor activation have shown no involvement of B_1 receptors (Dray et al., 1992, Mizumura et al., 1990). In addition B_2 antagonists are effective at reducing hyperalgesia in models of acute pain, such as acetic acid or kaolin-induced abdominal constrictions (de Campos et al., 1999, Asano et al., 1997). There is strong evidence for the expression of B_2 receptors on sensory neurones (Steranka et al., 1988, Burgess et al., 1989) and activation of these receptors leads to depolarisation (Burgess et al., 1989, Kano et al., 1994). However, it is likely that bradykinin-induced sensitisation is more important than the short-lasting activation in the overall production of pain. A likely mechanism of sensitisation involves bradykinin-induced prostanoid production, which has been demonstrated in a variety of cell types (Castaño et al., 1998, Angel et al., 1994, Modeer et al., 1990). Prostanoids sensitise sensory neurones to a variety of noxious stimuli, including thermal, mechanical and chemical stimuli, such as capsaicin and bradykinin itself (Rueff and Dray, 1993, Martin et al., 1987, Lopshire and Nicol, 1998, Mizumura et al., 1991, Smith et al., 2000).

In contrast to models of acute pain, in models of chronic inflammatory hyperalgesia such as Freund's adjuvant-induced hyperalgesia and ultra-violet (UV)-induced hyperalgesia, B_1 agonists increase hyperalgesia and

administration of B_1 antagonists results in a decrease (Perkins et al., 1993, Perkins and Kelly, 1993, Davis and Perkins 1994). In addition, although des-Arg^9-bradykinin has no hyperalgesic effect in naïve rats, it produced a hyperalgesic response in animals that had been treated with IL-1ß (Perkins and Kelly, 1994). The involvement of the B_1 receptor in these models does not exclude a role for the B_2 receptor, and it has been shown that HOE140 and the non-peptide B_2 antagonist bradyzide also reduce Freund's adjuvant-induced hyperalgesia (Davis and Perkins, 1994, Burgess et al., 2000). The site of the B_1 receptor induced in these models is unclear, but it does not appear to be expressed on sensory neurones (Davis et al., 1996, Jones et al., 1999). One possibility is that the B_1 receptors responsible for inflammatory hyperalgesia are expressed on other resident or infiltrating cells which then release mediators such as prostanoids which can sensitise sensory neurones. For example smooth muscle cells and fibroblasts produce prostanoids when exposed to B_1 agonists (Galizzi et al., 1994, Cahill et al., 1988). It is likely that the expression of the B_1 receptor on these cells would be increased by cytokines released from activated macrophages that would be present in the inflamed tissue. Macrophages themselves express the B_1 receptor, and can release cytokines in response to B_1 agonists (Tiffany and Burch 1989). Therefore in the presence of B_1 agonists, cytokine release from macrophages could be enhanced, leading to further upregulation of the B_1 receptor. A summary of the possible roles of kinin receptors and their interactions with other inflammatory mediators in inflammatory pain is shown in Figure 1-4.

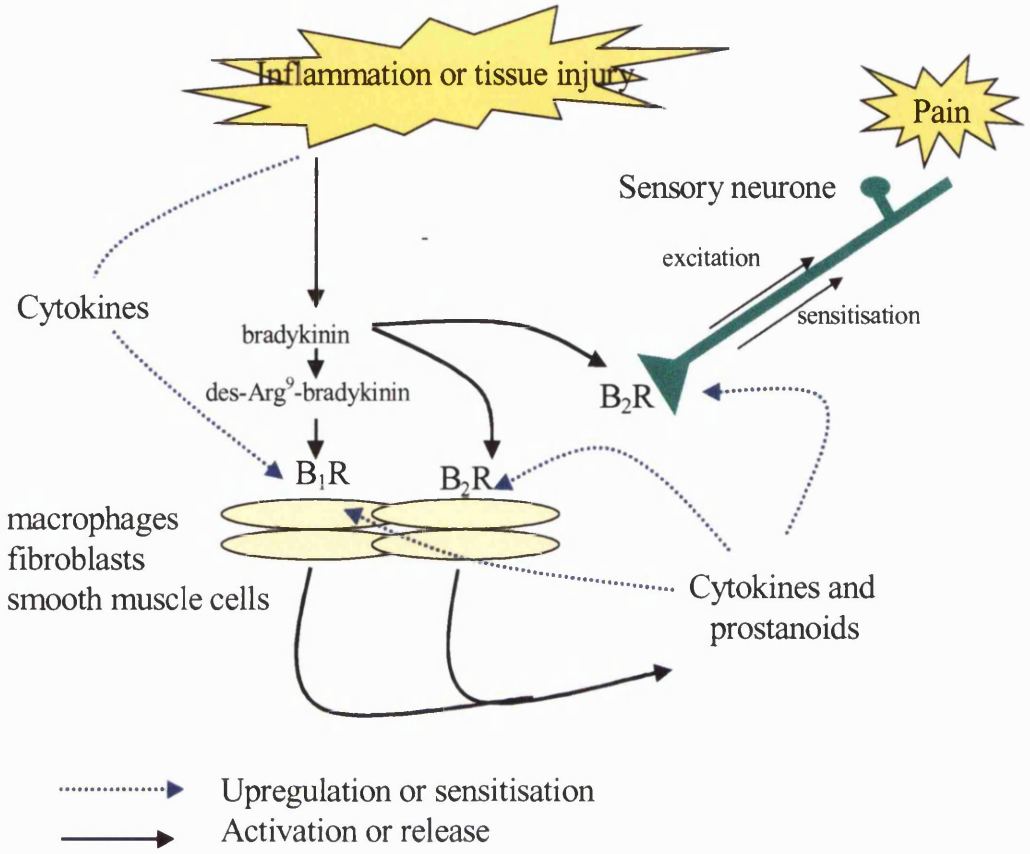

Figure 1-4 Role of kinin receptors in inflammatory pain

The B_2 receptor is desensitised by prolonged exposure to bradykinin, which means that in chronic inflammation the contribution of the B_2 receptor is likely to decrease. Consequently the B_1 receptor will have a more prominent role as the inflammation continues. In fact, in a model of rat paw oedema, desensitisation of the B_2 receptor correlated with an increase in B_1-mediated effects (Campos et al., 1995). This may have been because bradykinin itself induces upregulation of the B_1 receptor, a phenomenon reported in human lung fibroblasts and the rat kidney (Phagoo et al.,1999, Schanstra et al., 1999).

The recent development of mice lacking the B_2 (Rupniak et al., 1997) or B_1 receptor (Pesquero et al., 2000) has enabled the roles of kinin receptors in inflammatory pain to be investigated without the use of antagonists. In B_2

knockout mice carrageenan-induced thermal hyperalgesia was reduced compared to control animals, however, the induction of thermal hyperalgesia by Freund's adjuvant was intact, suggesting that the B_2 receptor plays a role in some, but not all, types of inflammatory pain (Rupniak et al., 1997). In B_1 knockout mice, LPS-induced hypotension and carrageenan-induced accumulation of polymorphonuclear leucocytes was reduced, indicating that the B_1 receptor plays a role in the initiation of inflammatory responses. In the tail-flick assay, which measures the acute nociceptive response initiated by noxious heat, there was no difference between wild-type and B_1 knockout mice. However, in the hot plate assay, B_1 knockout mice showed significant hypoalgesia in response to mildly painful stimuli, although there was no difference between wild-type and B_1 deficient mice with more painful stimuli. The hot plate assay measures a behaviour that is strongly modulated at spinal and supraspinal levels and consistent with this, in B_1 receptor-deficient mice there was a reduction in the activity-dependent facilitation (wind-up) of a nociceptive spinal reflex (Pesquero et al., 2000). These data are in contrast to reports suggesting that the B_1 receptor is not expressed in neuronal tissue (Davis et al., 1996, Jones et al., 1999).

1.6.1 KININS AND INFLAMMATION OF THE BLADDER

Kinins evoke a variety of responses in the urinary tract, including smooth muscle contraction, plasma protein extravasation, activation of sensory neurones and stimulation of neuropeptide release. Although kinins are present in the urine (Oza, 1988, Yamasu et al., 1989), under non-pathological conditions they are unlikely to have many effects because of the barrier formed by the urothelium and the high levels of proteases in the bladder mucosa. During inflammation,

however, levels of kinins in the bladder wall will increase and, if the urothelium is damaged, the kinins in the urine will have access to the tissues underneath.

There are a number of inflammatory conditions of the bladder. These include interstitial cystitis (IC), which is a chronic inflammatory disease characterised by severe urinary urgency, frequency, nocturia and pain. The prevalence of IC is difficult to estimate because of frequent misdiagnosis, with patients often waiting several years before receiving an accurate diagnosis (Slade et al., 1997). A recent study estimated the prevalence to be approximately 60 per 100,000 with the vast majority of cases being in women (Curhan et al., 1999). The incidence in men may, however, have been underestimated as IC is frequently misdiagnosed as non-bacterial prostatitis or prostadynia (Sant and Theoharides, 1999). The primary cause is not known, and although infection with a hard to culture organism has not been ruled out, other aetiologies such as autoimmunity or allergies have also been proposed. One of the key events in the disease process appears to be damage to the bladder epithelium or to the protective layer of glucosaminoglycan. The increased permeability of the bladder epithelium allows penetration of allergens, chemicals, potassium and bacteria into the bladder wall causing mast cell activation. Activated mast cells release a variety of vasoactive, nociceptive and proinflammatory agents causing immune cell infiltration, sensory nerve sensitisation and further activation of mast cells. The resultant vicious cycle of inflammation and nerve sensitisation leads to a long term disease that is very difficult to treat. Given the potential role of kinins in many of these processes it seems likely that they are involved in the inflammatory cascade of IC and it has been shown that there are increased levels of active kallikreins in IC patients compared to normal controls (Zuraw et al.,

1994, Rosamilla et al., 1999). Although the kinin receptor subtypes that may be involved in IC have not been extensively investigated, bladder biopsies from IC patients showed enhanced B_1 receptor expression (Ruggieri et al., 1999). B_2 receptor levels were not investigated in this study.

Current treatments for IC such as hydroxyzine, which is thought to inhibit mast cell activation through an anticholinergic mechanism (Minogiannis et al., 1998), immunosuppressants and sodium pentosan polysulphate, which stabilises mast cells and promotes urothelial stability, are only partially successful. There is therefore a clear need to develop better therapies and it is possible that kinin receptor antagonists could prove to be useful.

Bacterial cystitis is another disease of the bladder characterised by painful voiding symptoms. There are no reports on the effects of kinins in bacterial cystitis, however, given the known role of kinins in the pathophysiology of pain and inflammation, it seems likely that there would be some involvement.

Oral or intravenous administration of cyclophosphamide (CYP) is an effective form of treatment for a variety of malignant and non-malignant disease states. However, CYP therapy has several toxicity complications including bladder cystitis with symptoms ranging from painful voiding in the mildest cases to haemorrhagic cystitis in the most severe. Although the inflammation has been shown to be due to contact of the CYP metabolite acrolein with the urothelium, the exact mechanism of the inflammatory response is not clear. Kinins are thought to be involved and it is possible that kinin antagonists would reduce the severity of the CYP side effects.

Contractions of the bladder smooth muscle can be either local or reflex. Locally mediated contractions are small in amplitude (< 15mmHg) and result from direct activation of the smooth muscle. Reflex contractions are greater in amplitude (> 15mmHg) and are evoked when the volume in the bladder reaches a critical point, known as the V_{mic}. The reflex contractions are abolished by ablation of the pelvic ganglia. Both bradykinin (Lecci et al., 1995) and des-Arg^9-bradykinin (Lecci et al., 1999a,b) have been shown to evoke local and reflex contractions, but des-Arg^9-bradykinin-evoked contractions, both reflex and local, are greater in inflamed animals (Lecci et al., 1999b). Bradykinin is thought to activate the reflex response by stimulating capsaicin-sensitive afferent nerves with a contribution from prostanoids (Lecci et al., 1995). The mechanism by which des-Arg^9-bradykinin stimulates reflex contractions is not known. As attempts to demonstrate the presence of B_1 receptors on sensory neurones have been unsuccessful (Davis et al., 1996), it is more likely that B_1-stimulated prostanoid production is involved in B_1 receptor-evoked reflex contractions. Alternatively, as bladder mechanoreceptors are in series with smooth muscle fibres (Iggo, 1955) the local bladder contraction evoked by des-Arg^9-bradykinin could be a sufficient stimulus for evoking reflex responses

The role of kinins in inflammation of the bladder has been studied using a variety of animal models such as turpentine, CYP and ovalbumin-induced inflammation. Intravesical instillation of turpentine into the bladder leads to a sterile inflammatory response and a reduction in the V_{mic}, known as hyper-reflexia. This hyper-reflexia begins within 1h of instillation of turpentine and lasts at least 24h. Studies in this model with B_1 and B_2 receptor antagonists showed that, at an early time-point (2h), the B_2 antagonist HOE140 blocked the

reduction in V_{mic}, however at a later time-point (5h) both HOE140 and the B_1 antagonist [Leu^8]-des-Arg^9-bradykinin were effective (Jaggar et al., 1998). This suggests that the B_1 receptor had been induced by the later time-point. This is supported by evidence from a separate study in which des-Arg^9-bradykinin-mediated contractions in the isolated bladder were not seen in naïve bladders but were observed in bladders removed from turpentine treated rats (Roslan et al., 1995).

Animal studies have shown that intraperitoneal administration of CYP leads to inflammation of the bladder characterised by mucosal erosions and ulcers, inflammatory cell infiltration, haemorrhages, plasma extravasation, decreased bladder capacity and increased micturition frequency (Grinberg-Funes et al., 1990, Maggi et al., 1993, Ahluwalia et al., 1994). Studies with this model have shown that HOE140 inhibited the decrease in bladder volume, the increase in micturition frequency and plasma protein extravasation (Maggi et al., 1993, Ahluwalia et al., 1994). However, the increase in bladder tone associated with CYP-induced inflammation was not inhibited by either B_1 or B_2 antagonists (Lecci et al., 1999a). The effects of B_1 antagonists on the other effects of CYP-induced cystitis (i.e. bladder volume, increase in micturition frequency and plasma extravasation) have not been investigated, however, the B_1 receptor does appear to be induced following CYP treatment. For example, bladders removed from rats pre-treated with CYP were more responsive to des-Arg^9-bradykinin and had increased levels of [^{3}H]-des-Arg^{10}-kallidin binding and B_1 receptor mRNA compared to control animals, whereas B_2-mediated responses were unchanged (Lecci et al., 1999a,b, Belichard et al., 1999).

Ovalbumin-induced bladder inflammation has been used as an animal model of IC (Ahluwalia et al., 1998). In this model, local application of ovalbumin *in vivo* to ovalbumin-sensitised rats lead to inflammation of the bladder characterised by plasma protein extravasation and changes in smooth muscle reactivity. The B_2 antagonist HOE140, but not the B_1 antagonist B9858, inhibited the ovalbumin-induced plasma protein extravasation when given 2h following ovalbumin challenge. This may be because, as a peptide, B9858, is susceptible to degradation, however, it has several features which provide resistance to peptidases, including the N-terminal extension with Lys-Lys and the presence of the Oic residue. It may be, however, that B_1 antagonists would be effective at a later time-point, as was observed in the turpentine-induced hyper-reflexia model.

Inflammation of the bladder is also likely to lead to increased production of prostanoids. Under normal physiological conditions they are produced in response to distension of the bladder and have been shown to evoke both reflex and local contractions (Maggi, 1992). However, other stimuli such as mechanical irritation of the epithelium and application of inflammatory mediators also evoke prostanoid release (Downie and Karmazyn, 1984, Maggi et al., 1989). Thus under pathophysiological conditions there is likely to be increased prostanoid production leading to an increase in bladder excitability. This has been demonstrated in the ovalbumin-sensitised guinea-pig bladder where ovalbumin stimulated the release of PGE_2, PGD_2 and $PGF_{2\alpha}$ (Saban et al., 1994) and in bacterial cystitis the levels of PGE_2 in the urine were shown to correlate with the clinical symptoms (Farkas et al., 1980).

Both B_1 and B_2 agonists have been shown to evoke prostanoid release in the isolated bladder, and B_1 and B_2 evoked contractions are inhibited by cyclooxygenase inhibitors (Meini et al., 1998). As described in the previous section prostanoids can sensitise sensory neurones to a variety of stimuli and so they will probably have an important role in bladder pain. There also may be effects of prostanoids on the levels of kinin receptors similar to those described in Section 1.5.1.4 for mesangial and smooth muscle cells (Castaño et al., 1998, Schmidlin et al., 1998), although this has not been shown in bladder smooth muscle. Although the effect of prostanoids on B_1-receptor evoked responses has not been measured directly, the development of des-Arg^9-bradykinin-evoked contractions in the bladder following triton-induced cystitis and LPS treatment was inhibited by indomethacin (Marceau et al., 1980, Busser et al.,1998). It is therefore possible that in the inflamed bladder kinins, cytokines and prostanoids interact together, as described in Figure 1-4, leading to a self-perpetuating cycle of pain and inflammation.

1.7 OUTLINE OF THESIS

Although it is known that kinin receptors are expressed in the bladder, the location of the receptors, and whether the same cell type expresses both the B_1 and B_2 subtypes, is not known. As both B_1 and B_2 agonists mediate contraction of the bladder, it was thought likely that both subtypes would be expressed on smooth muscle cells. Smooth muscle cells derived from airway and vascular tissues have been widely used for the study of kinin receptors, however, smooth muscle cells derived from the bladder have not previously been used.

It is clear that kinins and their receptors play a key role in inflammatory processes in the bladder. However, it is not fully understood how inflammation modifies the expression of the receptors, and how the relative contribution of B_1 and B_2 receptors may change during inflammation. Current evidence suggests that immediately following an inflammatory insult in the bladder, kinins that are produced activate the constitutively expressed B_2 receptors, evoking hyper-reflexia and plasma protein extravasation (Maggi et al., 1993, Ahluwalia et al., 1994). The B_1 receptor appears to be induced some hours after the inflammation has started, and also appears to lead to hyper-reflexia (Belichard et al., 1999, Jaggar et al., 1998), however, the mechanisms of induction and the mediators involved have not been widely investigated.

The aim of this project was to investigate how kinins might be involved in bladder inflammation, and consequently in conditions such as IC. To do this the expression and regulation of kinin receptors on primary cultures of bladder smooth muscle cells was studied by:

- Developing a method for isolating and culturing smooth muscle cells from the rat bladder

- Characterising the pharmacology of kinin receptors on bladder smooth muscle cells using functional and binding assays.

- Investigating the regulation of B_1 and B_2 receptors by factors that are likely to be elevated during inflammation of the bladder, for example IL-1ß and cAMP.

2 METHODS

2.1 PREPARATION AND CULTURE OF BLADDER SMOOTH MUSCLE CELLS

Bladder smooth muscle cells were prepared by a modification of the method of Levesque et al. (1993). The urinary bladder was removed from female Sprague-Dawley rats weighing about 150-180g and washed in growth medium (Medium 199 containing 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin plus 10% FCS). The bladders were cut into small pieces, approximately 2 x 2mm, using a McIlwain tissue chopper. The chopped pieces were then transferred to a solution of growth medium containing 1mg/ml elastase (from porcine pancreas) and 3mg/ml collagenase (Type 4) and incubated for 90min on a rotary shaker at 37°C. The digested tissue was spun at 300g for 5min, the pellet was resuspended in growth medium and then triturated gently. The resulting cell suspension was passed through a 90μm muslin filter to remove undigested clumps of tissue and the cells transferred to 80cm^2 tissue culture flasks in growth medium and grown in 6% CO_2 at 37°C. The media was changed after one day to remove any debris. The cells multiplied in culture and were split by a ratio of 1:2 every 3-4 days using a solution containing 1mM ethylenediaminetetraacetic acid (EDTA) and 10mM N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulphonic acid] (HEPES) in calcium and magnesium free Hank's Banks Salt Solution (HBSS) to detach the cells. The cells were maintained in culture for up to 9 days.

2.2 IMMUNOFLUORESCENCE

Immunofluorescence was used to determine the proportion of the cell population that was smooth muscle. The cells were plated onto poly-D-ornithine coated 22mm glass coverslips at approximately 17,000 cells per coverslip in growth medium and grown overnight. The cells were then fixed in a solution of ice-cold methanol and glacial acetic acid (95/5% v/v) for 30min. After washing in phosphate buffered saline pH 7.2 (PBS) the cells were then incubated in blocking solution (5% sheep serum in PBS) for 15min before incubation with mouse monoclonal anti-α-smooth muscle actin at a dilution of 1:400, made up in blocking solution, for 2h at room temperature. Some coverslips were incubated in blocking solution containing no antibody or control ascites fluid. Chinese Hamster Ovary cells, which do not express smooth muscle actin were used as control cells. After extensive washing in PBS, fluorescein-linked anti-mouse Ig diluted 1:30 in blocking solution was applied for 2h in the dark. The coverslips were washed in PBS and placed on microscope slides with Citifluor mounting medium, sealed with nail varnish and examined under a fluorescence microscope. Images were captured using Image-Pro-Plus software.

2.3 MEASUREMENT OF ^{45}CALCIUM EFFLUX

2.3.1 TERASAKI BASED METHOD

Bladder smooth muscle cells were plated onto 60 well terasaki plates at approximately 1500 cells/well and grown overnight. The excess medium was removed and 10µl of 45µCi/ml [^{45}Ca]-calcium chloride made up in growth medium added to each well. For upregulation experiments, the agent being tested was added to the loading solution. The plates were incubated at 37°C for 4h and then washed for 17min in assay buffer, 10mM HEPES in HBSS pH 7.4, until the rate of efflux of ^{45}Ca from the cells was constant. Agonists were applied for 1min and antagonists were applied for the 4min prior to, and during, the 1min application of agonist. The amount of ^{45}Ca in each wash was measured by liquid scintillation counting on a Wallac liquid scintillation counter using Ready-Gel (a scintillant with a high volume capacity). The ^{45}Ca in the cells at the end of the experiment was determined by solubilising the cells with 0.2% sodium dodecyl sulphate (SDS). The rate of efflux for a given minute was defined as the amount of ^{45}Ca released divided by the mean of the total amount of ^{45}Ca in the cells at the beginning and end of that minute. The ^{45}Ca efflux-evoked in response to an agonist was calculated as the rate during the minute of agonist application minus the rate in the two preceding minutes (basal efflux). The basal efflux rate was 0.045 ± 0.002min^{-1} (n=24), and agonist-evoked efflux rate was 0.114 ± 0.002min^{-1} (n=24) for des-Arg^{9}-bradykinin (1µM) and 0.296 ± 0.045min^{-1} (n=6) for bradykinin (100nM) in 7-9 day old cells.

2.3.2 96 WELL PLATE BASED METHOD

Bladder smooth muscle cells were plated onto 96 well plates at 35,000 cells per well and grown overnight. The medium was removed from the cells and

replaced with 50µl of 50µCi/ml [^{45}Ca]-calcium chloride and incubated for 4h at 37°C. The cells were washed for 20min with assay buffer using a Denley cell washer and then incubated in 75µl of the agonist made up in assay buffer containing 0.1% bovine serum albumin (BSA). After 4min at 37°C, 50µl was transferred to a Packard picoplate and 100µl of Microscint-40 added to each well. Where antagonists were used, they were added for 4min before the addition of the agonist as well as during the application of agonist. The amount of ^{45}Ca in the cells was determined by solubilising the cells with 0.2% SDS. The picoplates were counted on a Packard Topcount. The amount of ^{45}Ca release was defined as (A x 1.5)/((A x 1.5) + B) where A is the number of counts in the 50µl removed from the well (out of a volume of 75µl) and B is the counts in the solubilised cells. Agonist-evoked ^{45}Ca efflux was defined as the amount of ^{45}Ca released in stimulated wells minus the ^{45}Ca released in control wells exposed to buffer only (basal efflux). Typically the basal efflux rate was 0.016 ± 0.001min^{-1} (n=6) and the agonist-evoked efflux rate was 0.030 ± 0.005min^{-1} (n=7) for des-Arg^{9}-bradykinin (1µM) and 0.091 ± 0.005min^{-1} (n=9) for bradykinin (100nM) in 7-9 day old cells. In cases where it was necessary to combine data from these two methods, agonist-stimulated ^{45}Ca efflux was expressed as fold increase over the basal efflux.

2.4 MEASUREMENT OF [^{3}H]-DES-ARG^{10}-KALLIDIN BINDING IN MEMBRANES PREPARED FROM COS-7 CELLS TRANSFECTED WITH THE RAT B_1 cDNA.

2.4.1 TRANSFECTION

Cos-7 cells were grown in 175cm^2 flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100μg/ml streptomycin and 10% FCS. Transfections were carried out using the Promega ProFection calcium phosphate transfection kit according to manufacturers instructions using 150μg/ml rat B_1 cDNA and 100μg/ml of human B_1 cDNA. On the day following transfection the precipitate was removed, fresh media added and the cells were then grown for a further two days.

2.4.2 PREPARATION OF MEMBRANES

The cells were detached from the flasks using 1mM EDTA made up in calcium and magnesium free HBSS containing 10mM HEPES pH 7.4. The cells were washed three times by centrifugation (300g for 5min) in the same solution. The final pellet was resuspended in homogenisation buffer (50mM tris(hydroxymethyl)aminoethane (Tris), pH 7.4, 1mM EDTA, 140μg/ml bacitracin, 50μg/ml chymostatin, 4μg/ml leupeptin and 4μg/ml trypsin inhibitor) and homogenised at 10,000rpm for 30s using a Polytron homogeniser. The suspension was centrifuged at 40,000g for 30min in a Sorvall SS34 rotor. The pellet was rehomogenised and centrifuged as above a further two times. The final pellet was resuspended in 50mM Tris, pH 7.4 plus 10% glycerol and stored at -70°C.

2.4.3 BINDING ASSAY

Membranes (approximately 50μg/well) were incubated in 96-well deep well plates with a range of concentrations of [^{3}H]-des-Arg^{10}-kallidin in a buffer containing 10mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), pH 7.4, 1mM EDTA, 0.1% BSA, 1μM MERGEPTA, 1μM enalapril and 10μM thiorphan in a final volume of 500μl. Non-specific binding was determined with 3μM des-Arg^{10}-kallidin. Following incubation for 1h at 4°C the membranes were filtered on a Packard Filtermate onto GF/B filters pre-soaked in 0.6% polyethyleneimine. The filters were washed 6 times with ice cold 50mM Tris, pH7.4. Following addition of 50μl Microscint-40 per well, the filters were counted on a Packard Topcount. For the saturation experiments total and non-specific binding was measured at a range of concentrations of [^{3}H]-des-Arg^{10}-kallidin. For displacement experiments a range of concentrations of the displacer were incubated with approximately 1.5nM [^{3}H]-des-Arg^{10}-kallidin for the rat B_1 membranes and 0.15nM [^{3}H]-des-Arg^{10}-kallidin for the human B_1 membranes, and the percentage specific binding for each concentration of displacer calculated.

2.5 MEASUREMENT OF [^{3}H]-DES-ARG^{10}-KALLIDIN BINDING IN BLADDER SMOOTH MUSCLE CELLS

2.5.1 BINDING ASSAY

Bladder smooth muscle cells were plated at approximately 50,000 cells/well on 24 well plates in growth medium and grown overnight. The cells were incubated with a variety of concentrations of [^{3}H]-des-Arg^{10}-kallidin as described in the Results (Section 3.3.2) in a total volume of 500µl for 1h at 4°C. The assay buffer contained 10mM TES pH 7.4, 300mM sucrose, 0.1% BSA, 10µM thiorphan, 1µM enalapril and 1µM MERGEPTA. Non-specific binding was determined with 3µM unlabelled des-Arg^{10}-kallidin. Following the incubation, the cells were washed 3 times with 0.5ml wash buffer (50mM Tris, pH 7.4, 300mM sucrose) and the cells solubilised with 0.2% SDS. Following removal of a sample for determination of protein concentration, the samples were counted on a Wallac liquid scintillation counter using Ready-Micro scintillant.

2.5.2 ANALYSIS OF THE STABILITY OF [^{3}H]-DES-ARG^{10}-KALLIDIN IN THE BINDING ASSAY

In order to determine whether [^{3}H]-des-Arg^{10}-kallidin was stable during the course of the binding experiments, samples of assay buffer containing 1.5nM [^{3}H]-des-Arg^{10}-kallidin were taken before and after incubation with the cells. The samples were spiked with 10µM unlabelled des-Arg^{10}-kallidin and 400µl loaded onto a C18 HPLC column. The components were separated using a water (containing 5mM trifluoroacetic acid) and acetonitrile gradient as shown in Table 2-1.

Time (min)	Flow rate (ml/min)	% water	% acetonitrile
0	1.5	90	10
1	1.5	90	10
5	1.5	50	50
7	1.5	0	100
10	1.5	0	100
11	1.5	90	10
15	1.5	90	10

Table 2-1 HPLC gradient for [^{3}H]-des-Arg^{10}-kallidin.

The amount of des-Arg^{10}-kallidin in the eluate was detected using an online UV spectrophotometer with a wavelength of 218nm. The [^{3}H]-des-Arg^{10}-kallidin was detected using an online radiochemical detector.

2.6 MEASUREMENT OF [^{125}I]-IL-1ß BINDING

Bladder smooth muscle cells were plated at 500,000 cells/well on 6-well dishes and grown overnight. The cells were incubated 0.2nM [^{125}I]-IL-1ß for 1h at 37°C in a final volume of 800µl. The assay buffer was HEPES (25mM) buffered DMEM containing 2mg/ml BSA and 0.01% sodium azide. Non-specific binding was determined with 50nM IL-1ß. Following the incubation the cells were washed three times with 3ml of wash buffer (50mM Tris, pH 7.4, 300mM sucrose) and then solubilised with 0.2% SDS. The amount of [^{125}I]-IL-1ß in the sample was determined by counting in a gamma counter. The human lung fibroblast line IMR-90, used as a positive control, was grown in DMEM supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin and 10% FCS. Measurement of [^{125}I]-IL-1ß binding was carried out as described above but using 250,000 cells/well.

2.7 ANALYSIS OF THE LEVEL OF B_1 RECEPTOR mRNA IN BLADDER SMOOTH MUSCLE CELLS USING RT-PCR

The relative amounts of B_1 receptor mRNA in control and treated cells were compared using a Reverse-Transcriptase Polymerase Chain Reaction method (RT-PCR). Total RNA was prepared from cultured bladder smooth muscle cells and a cDNA copy prepared using reverse transcriptase. This product was then subjected to PCR using B_1 specific primers and cyclophilin primers as a control. The PCR reaction amplified the desired products and the amount produced was directly related to the amount of starting material for a limited number of cycles after which the amplification rate reached a plateau. The factors that contribute to this plateau include substrate saturation of the enzyme, product strand reannealing and incomplete strand separation. Samples were taken at cycle numbers during the exponential phase and after saturation, and the amount of product was visualised on an agarose gel.

2.7.1 PREPARATION OF RNA

Bladder smooth muscle cells, in 175cm^2 flasks, were washed briefly with PBS and then 10ml of TRI Reagent (a monophasic solution of phenol and guanidine isothiocyanate) was added for 2-3min. The cells detached from the flasks and were then passed through a pipette several times to form an homogenous lysate. The flasks were washed with a further 5ml TRI Reagent and then 0.2ml of chloroform per ml of TRI Reagent used was added. The samples were shaken vigorously, allowed to stand for 2-3min at room temperature and then spun at 12,000g in a Sorvall SC5C centrifuge for 15min at 4°C. The centrifugation separated the mixture into three phases: a red organic phase (containing protein),

an interphase (containing DNA) and a colourless upper aqueous phase which contained RNA. The aqueous phase was transferred to siliconised glass Corex tube and 0.5ml isopropanol per ml of TRI reagent used was added. The samples were kept at –20°C overnight and then centrifuged at 12,000g for 15min at 4°C. The supernatant was removed and the RNA pellet washed by adding 1ml of 75% ethanol per ml of TRI reagent used. The samples were vortexed and spun at 12,000g for 10min at 4°C. The supernatant was removed and the pellet allowed to dry and then resuspended in a small volume of sterile water. The RNA concentration was determined by measuring the optical density at 260nm. An RNA solution with an optical density of 1.0 at 260nm has a concentration of 40µg/ml. A estimation of the purity of the RNA was made by calculating the ratio of the optical density at 260nm to that at 280nm. Any contamination of the sample by protein will increase the 280nm reading leading to a reduction in this ratio. The ratio for a pure preparation should be approximately 1.8, less than 1.5 was considered unacceptable.

2.7.2 PREPARATION OF cDNA

Approximately 5-10µg of RNA was incubated with 0.5µg of Oligo$(dT)_{12-18}$ in a total volume of 12µl for 10min at 70°C. The oligo(dT) acts as a primer by binding to the poly A^+ tails of mRNA. After cooling the reaction mixture to 4°C, 1st strand buffer (containing a final concentration of 50mM Tris, pH 8.3, 75mM KCl and 3mM $MgCl_2$), DTT (final concentration 5mM) and dNTPs (containing a final concentration of 0.5mM of dATP, dCTP, dGTP and dTTP) were added and the mixture incubated at 42°C for 2min. Superscript II Reverse Transcriptase (200 units) was added, giving a final volume of 20µl, and the reaction allowed to proceed at 42°C for 50min. The enzyme was inactivated by

incubation at 70°C for 15min and then cooled to 4°. The samples were then ready for PCR but could be frozen at –20°C until required.

2.7.3 PCR

1-2µl of the cDNA was mixed with PCR buffer (containing final concentrations of 10mM Tris, pH 8.3, 50mM KCl and 1.5mM $MgCl_2$), 200µM dNTPs, 200nM Primer 1, 200nM Primer 2, 2.5 units of AmpliTaq Gold DNA polymerase. The volume was made up to 25µl with sterile water. The B_1 primers were as follows:

Sense primer: CTTTGGCCTCTTGGGGAACCT

Antisense Primer: CAAGCCTCGTGGGGGAAA

These gave a product of 481bp.

As a control, the level of cyclophilin mRNA was also measured. The cyclophilin primers were as follows:

Sense primer: CACCGTGTTCTTCGACATCAC

Antisense primer: GCTAGACTTGAAGGGGAATG

These gave a product of 638bp.

Once all the constituents had been mixed together the samples were incubated at 95°C for 5min. This stage was necessary for activation of the AmpliTaq Gold. The samples were then subjected to the required number of PCR cycles consisting of 30s at 95°C, 30s at 55°C and 60s at 72°C on a MJ Research Peltier Thermal Cycler. Typically samples were removed at 24, 26, 28, 30 cycles

(during which the reaction was still in the exponential phase) and 35 cycles when the reaction had reached a plateau.

2.7.4 ANALYSIS OF SAMPLES

The amount of DNA formed during PCR was visualised on an agarose gel. A 10µl aliquot of the sample was mixed with 2µl of loading buffer containing bromophenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v) and sucrose (40% w/v), and loaded onto a 1% agarose gel containing 1µg/ml ethidium bromide; 0.5µg of a 100bp DNA ladder was also loaded. The gel was run at 100V for approximately 1h and then viewed under UV light. The density of the bands was analysed using Bio-Rad MultiAnalyst software.

2.8 AEQUORIN-BASED ASSAYS

Aequorin-based assays were used to measure the activity of the cloned B_2 receptor from rat and human. Aequorin is a protein isolated from jellyfish that emits light when it interacts with calcium. The cDNA for aequorin can be transfected into cells, along with a receptor of interest, and agonist-evoked rises in $[Ca^{2+}]_i$ can be detected by measuring the amount of light emitted. To measure the activity of the human B_2 receptor, CHO cells stably expressing the receptor and aequorin were used. The cells were grown in MEM Alpha Medium without ribonucleosides and deoxyribonucleosides, supplemented with 10% dialysed FCS, 2mM L-glutamine, 100IU/ml penicillin, 100μg/ml streptomycin and 700μg/ml G418, this medium maintains the selective pressure required for expression of the aequorin and the human B_2 receptor genes. For the assay the cells were plated onto Packard Viewplates at 50,000cells/well, grown overnight and then incubated with 20μM coelenterazine (a co-factor required for full activity of aequorin) plus 30μM glutathione, made up in the growth media, for 2-3h at 37°C. The loading solution was removed and replaced with assay buffer (10mM HEPES in HBSS pH 7.4) and the plate was placed in a Luminoskan luminometer. Following injection of the agonist the luminescence was measured for 20s.

The rat B_2 receptor was assayed using Cos-7 cells (grown as described in Section 2.4.1) transiently transfected with the rat B_2 receptor and aequorin cDNA. Following transfection with 50μg aequorin cDNA and 20μg of rat B_2 receptor cDNA using electroporation (300V, 500μF using a Bio-Rad Gene Pulser) the cells were plated at 12,500cells/well in Packard Viewplates and

grown for 2 days. The cells were loaded with coelenterazine and the luminescence measured as described above for the human B_2 receptor.

2.9 CALCIUM IMAGING

Bladder smooth muscle cells were plated onto poly-D-ornithine coated 22mm glass coverslips at approximately 17,000 cells per coverslip in growth medium and grown overnight. A video imaging system (Imagemaster, Photon Technology International Inc.) was used for measurement of $[Ca^{2+}]_i$. The cells were loaded with 2μM Fura-2/AM for 30min at 37°C, rinsed, transferred to a perfusion chamber at room temperature and perfused (flow rate of 1ml/min) with buffer (HBSS containing 10mM HEPES pH 7.4) in the presence and absence of drugs. Fura-2 was excited alternately at 340 and 380nm and the emitted light monitored at 510nm. Images were captured every 4s and ratio images calculated using Imagemaster software.

2.10 PROTEIN ASSAY

The protein concentration of assay samples was determined using a Bio-Rad Detergent Compatible protein assay kit which is based on the method of Lowry et al. (1951). The kit contains three reagents: Reagent A, an alkaline copper tartrate solution; Reagent B, a dilute Folin Reagent and Reagent S, a solution of SDS. Protein levels were determined against a γ-globulin standard curve in 0.2% SDS. Assay samples (50µl) and standards were placed into a 96 well plate, 25µl of reagent A/S (made by adding 200µl of S to 10ml of A) and 150µl of reagent B were added and the plate incubated for 15min at room temperature. The absorbance of the samples at 690nm was read on a Labsystems iEMS plate reader and the protein concentration of the samples calculated from the standard curve.

2.11 DATA ANALYSIS

Unless otherwise indicated the data represent the means ± the standard error of the mean (s.e.m.) from at least three independent experiments. For comparison of two samples statistical analysis was done using unpaired, or paired Student's t-test as indicated, in Microsoft Excel 97. Statistical analysis on 3 or more samples was carried out using ANOVA, followed by Dunnett's test for comparison of a range of treatments with control, or Bonferroni analysis for comparison of selected pairs of treatments, using GraphPad Instat, GraphPad Software San Diego, California, USA. Results were considered significant if $p < 0.05$. For upregulation experiments the response was expressed as a percentage of the response in control cells. However, the statistical analysis was carried out on the raw data.

Concentration-response curves (for agonists and antagonists) were fitted in Microcal Origin (Version 5.0) and the EC_{50} or IC_{50} values calculated using a the Hill equation:

$$y = \frac{A1 - A2}{1 + (x / x_0)^p} + A2$$

where $A1$ = minimum response
$A2$ = maximum response
x_0 = EC_{50} or IC_{50}
p = slope factor

The value of p was allowed to vary, and the p values obtained for the data shown in the concentration-response curves are given in the figure legends. Using this equation the IC_{50} or EC_{50} values from at least three individual experiments were obtained and used to calculate the mean and s.e.m. presented in the text and in

Table 3-1. For comparison the EC_{50} or IC_{50} value obtained from the graph of the meaned data is given in the figure legends.

Saturation curves from binding experiments were plotted in Origin and fitted using a hyperbolic fit:

$$y = \frac{P_1 x}{P_2 + x}$$

where $P1$ $= B_{max}$
$P2$ $= K_D$

In displacement experiments K_I values were calculated from the IC_{50} values using the Cheng-Prusoff equation:

$$K_I = \frac{IC_{50}}{1 + ([RL]/K_D)}$$

where *[RL]* is the radioligand concentration (Cheng and Prusoff, 1973).

2.12 MATERIALS

2.12.1 Animals

Female Sprague-Dawley adult rats (150-200g) were maintained on a 12h light-dark cycle and fed and watered *ad libitum.*

2.12.2 Radiochemicals

The following radiochemicals were obtained either from Amersham Pharmacia Biotech. Ltd, Little Chalfont, Bucks., UK or NEN Life Sciences, Hounslow, UK as indicated.

[^{45}Ca]-calcium chloride (specific activity – 0.185-1.85GBq/mg calcium)	Amersham
[^{3}H]-des-Arg^{10}-kallidin (specific activity – 2.22-4.44TBq/mmol)	NEN
[^{125}I]-IL-1ß (specific activity – 2.96-6.66MBq/mmol)	NEN

2.12.3 Biochemicals

General laboratory chemicals were purchased as analytical grade from either BDH Chemicals Ltd., Poole, Dorset, UK or Sigma-Aldrich, Poole, Dorset, UK.

The following specialised biochemicals were obtained from the sources indicated:

Anti-α-smooth muscle actin	Sigma-Aldrich
Bacitracin	Sigma-Aldrich
Calcium phosphate transfection kit	Promega, Southampton, UK
Chymostatin	Sigma-Aldrich
Citifluor	Citifluor Ltd, City University, London, UK
Collagenase (Type 4)	Worthington Biochemical Corporation, Lakewood, NJ, USA
Control ascites fluid	Sigma-Aldrich
Detergent Compatible Protein Assay	Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK.

N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate	Sigma-Aldrich
N^6,2′-O-dibutyrylguanosine 3′:5′-cyclic monophosphate	Sigma-Aldrich
DNA ladder (100bp)	Life Technologies Ltd, Paisley, UK
DNA loading buffer	Sigma-Aldrich
dNTPs	Life Technologies Ltd, Paisley, UK
Elastase	Roche Diagnostics, Lewes, E. Sussex, UK
Enalapril	Sigma-Aldrich
Ethidium bromide	Sigma-Aldrich
Fluorescein-linked anti-mouse Ig	Amersham Pharmacia Biotech. Ltd
Forskolin	Calbiochem, Nottingham, UK
Fura –2/AM	Molecular Probes, Eugene, OR, USA
G418	Sigma-Aldrich
Interleukin-1ß	R & D Systems, Abingdon, Oxon, UK
Leupeptin	Sigma-Aldrich
lipopolysaccharide	Sigma-Aldrich
MERGEPTA	Calbiochem, Nottingham, UK
Microscint-40	Packard Bioscience Ltd, Pangbourne, Berks, UK
Oligo(dT)	Life Technologies Ltd, Paisley, UK
Poly-D-ornithine	Sigma-Aldrich
Ready-Gel and Ready Micro	Beckman Coulter Ltd, High Wycombe, Bucks., UK
Superscript II reverse transcriptase and components for reaction (1^{st} strand buffer, DTT)	Life Technologies Ltd, Paisley, UK
Sheep serum	Sigma-Aldrich
AmpliTaq Gold DNA polymerase and PCR buffer	Applied Biosystems, Warrington, UK
Thiorphan	Sigma-Aldrich
TNFα (rat)	R & D Systems, Abingdon, Oxon, UK
TRI reagent	Sigma-Aldrich
Trypsin Inhibitor	Sigma-Aldrich

Coelenterazine was synthesised at Novartis Institute for Medical Sciences, London, UK. The Fujisawa B_2 antagonist (FR173657) was synthesised by Dr David Xu at Novartis, Summit, USA.

The primers for B_1 and cyclophilin were custom synthesised by Sigma-Genosys Ltd, Cambridge, UK

The HPLC system and the C18 Symmetry Shield column (4 x 100mm, 3.5µm) was from Waters Ltd, Watford, Herts, UK. The solvents for HPLC were from Romil Ltd, Cambridge, UK

2.12.4 Peptides

The following kinin peptides were obtained from the sources indicated;

Bradykinin Des-Arg^9-bradykinin [Leu^8]-des-Arg^9-bradykinin Kallidin	Bachem (UK) Ltd, Saffron Walden, Essex, UK
Des-Arg^{10}-kallidin Des-Arg^{10}-HOE140 HOE140	Peninsula Laboratories Europe Ltd, St. Helens, Merseyside, UK
Lys-Lys-[Hyp^3,Igl^5,D-Igl^7,Oic^8]-des-Arg^9-bradykinin (B9858)	Phoenix Pharmaceuticals Inc, Mountain View, CA, USA
T-kinin Sar-[D-Phe^8]-des-Arg^9-bradykinin	Novabiochem, Nottingham, UK
Des-Arg^{11}-T-kinin	Peptide and Protein Research, Washington Singer Labs, University of Exeter, Exeter, UK

2.12.5 Tissue culture products

Salt solutions, tissue culture media and supplements were obtained from Life Technologies Ltd, Paisley, UK. Tissue culture flasks, multiwell plates and 60 well terasaki plates were Nunc Plasticware Products obtained from Life

Technologies Ltd, Paisley, UK. Glass coverslips and microscope slides were obtained from BDH, Poole, Dorset, UK.

2.12.6 Consumables

PCR tubes were obtained from Applied Biosystems, Warrington, UK and GF/B filters, picoplates and viewplates were from Packard Bioscience Ltd, Pangbourne, Berks, UK

3 RESULTS

3.1 ISOLATION OF BLADDER SMOOTH MUSCLE CELLS

Immediately following isolation, cells from rat bladder were primarily long, thin spindle shaped cells. They multiplied in culture and during that time (up to 9 days) their morphology changed to a flatter, more spread-out shape, showing the "hill and valley" pattern typical of cultured smooth muscle cells (Chamley-Campbell et al., 1979). Immunohistochemical studies on cells that had been in culture for 7-9 days indicated that approximately 90% of the cells (190/218 in one culture, 123/143 in a second and 96/100 in a third) stained positively with an antibody to the smooth muscle form of actin, indicating that the majority of the cells were smooth muscle in origin (Figure 3-1). It was not possible to determine the identity of the remaining cells, however, it is likely that fibroblasts would be present.

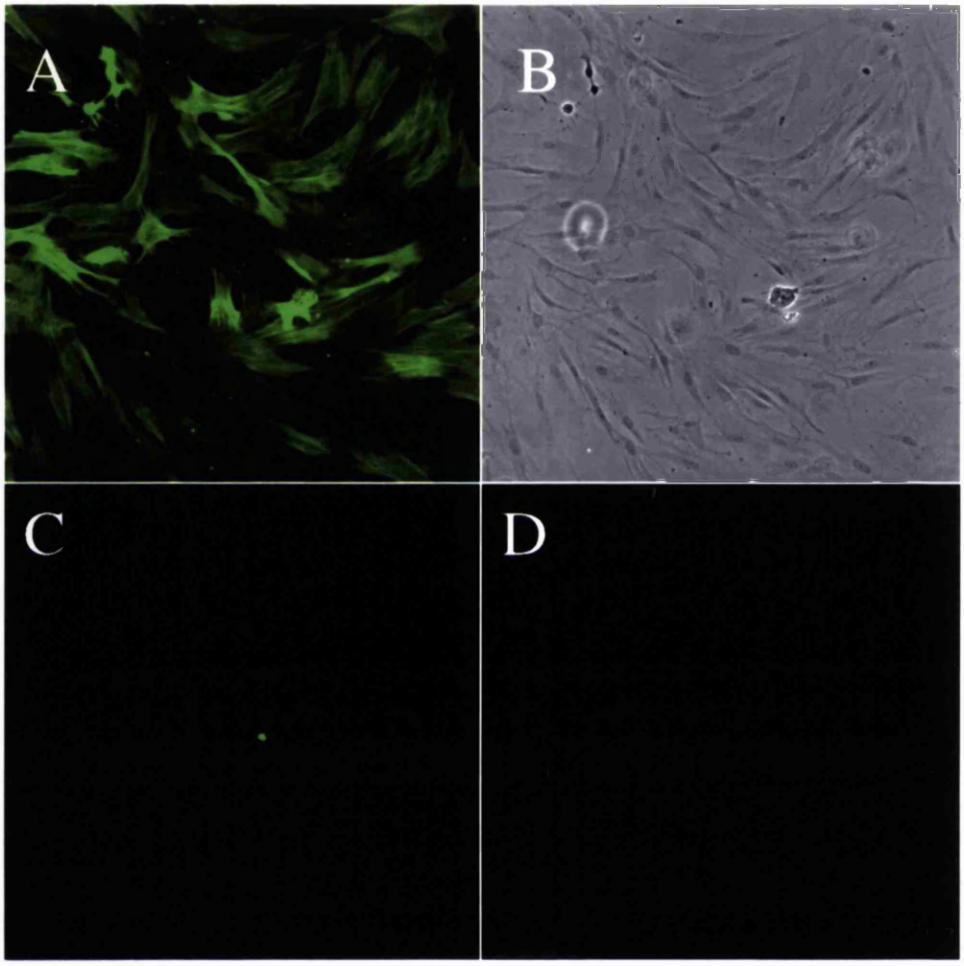

Figure 3-1 Staining of bladder smooth muscle cells with anti-α smooth muscle actin.
(A) bladder smooth muscle cells incubated with anti-α smooth muscle actin, (B) the same field of cells under phase contrast, (C) bladder smooth muscle cells incubated with control ascites fluid, (D) Chinese Hamster Ovary cells incubated with anti-α smooth muscle actin. Magnification x 400.

The anti-α smooth muscle actin did not stain control cells (Chinese Hamster Ovary cells), and no staining was observed using control ascites fluid.

3.2 PHARMACOLOGY OF KININ RECEPTORS ON BLADDER SMOOTH MUSCLE CELLS

The pharmacology of kinin receptors on bladder smooth muscle cells was studied using a combination of agonist-evoked functional responses and binding experiments with the B_1 specific ligand [^{3}H]-des-Arg^{10}-kallidin (see Section 3.3).

3.2.1 ^{45}CA EFFLUX

The second messenger pathways activated by B_1 and B_2 receptors in bladder smooth muscle cells were not known, however it was thought likely that both receptors would be coupled to PIC and receptor activation would thus lead to an increase in $[Ca^{2+}]_i$.

Figure 3-2 shows that both the B_2 agonist bradykinin and the B_1 agonist des-Arg^9-bradykinin evoked an increase in the rate of ^{45}Ca efflux from bladder smooth muscle cells pre-equilibrated with this radioisotope. This indicates that both agonists increased $[Ca^{2+}]_i$ and confirmed that both B_1 and B_2 receptors were coupled to PIC.

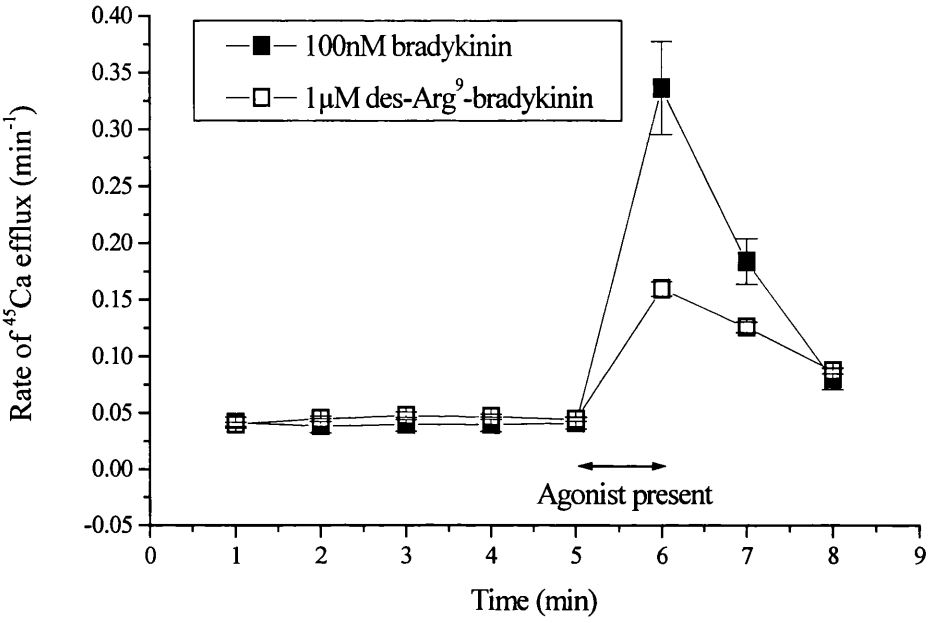

Figure 3-2 Stimulation of ^{45}Ca efflux by bradykinin and des-Arg^9-bradykinin in bladder smooth muscle cells.
The data show the rates of ^{45}Ca efflux prior to and during the application of 100nM bradykinin or 1μM des-Arg^9-bradykinin and are the means and s.e.m.s of 6 (bradykinin) and 24 (des-Arg^9-bradykinin) experiments. The arrow ↔ indicates the presence of the agonist for 1min.

Comparison of the responses to both of these agonists in 1 day old cells and in 7-9 day old cells showed that the responses were significantly greater ($p < 0.001$, unpaired Student's t-test) in cells that had been in culture for 7-9 days (Figure 3-3).

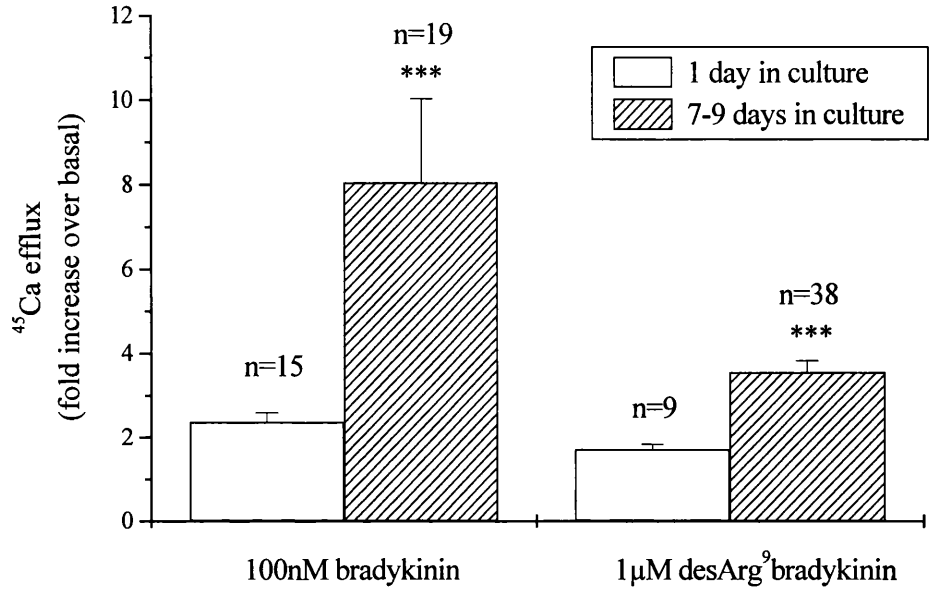

Figure 3-3 Stimulation of ^{45}Ca efflux in bladder smooth muscle cells by bradykinin and des-Arg^{9}-bradykinin.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by bradykinin and des-Arg^{9}-bradykinin in 1 day old and 7-9 day old cells, and are the means and s.e.m.s of the number of experiments shown. *** $p < 0.001$ compared to 1 day old cells, unpaired Student's t-test.

Because more cells were available and the responses to the agonists were increased in magnitude it was decided to use cells that had been in culture for 7-9 days for the pharmacological characterisation of the responses to kinin agonists.

The B_2 receptor agonists bradykinin and kallidin evoked ^{45}Ca efflux in bladder smooth muscle cells with EC_{50} values of 2.7 ± 1.6nM (n=6) and 3.2 ± 1.1nM (n=4) respectively (Figure 3-4). The maximum response evoked by bradykinin was an 8.0 ± 1.6 (n=6) fold increase in ^{45}Ca efflux over the basal level. For kallidin an 8.4 ± 0.9 (n=4) fold increase was observed which was not significantly ($p > 0.1$, unpaired Student's t-test) different from the response evoked by bradykinin.

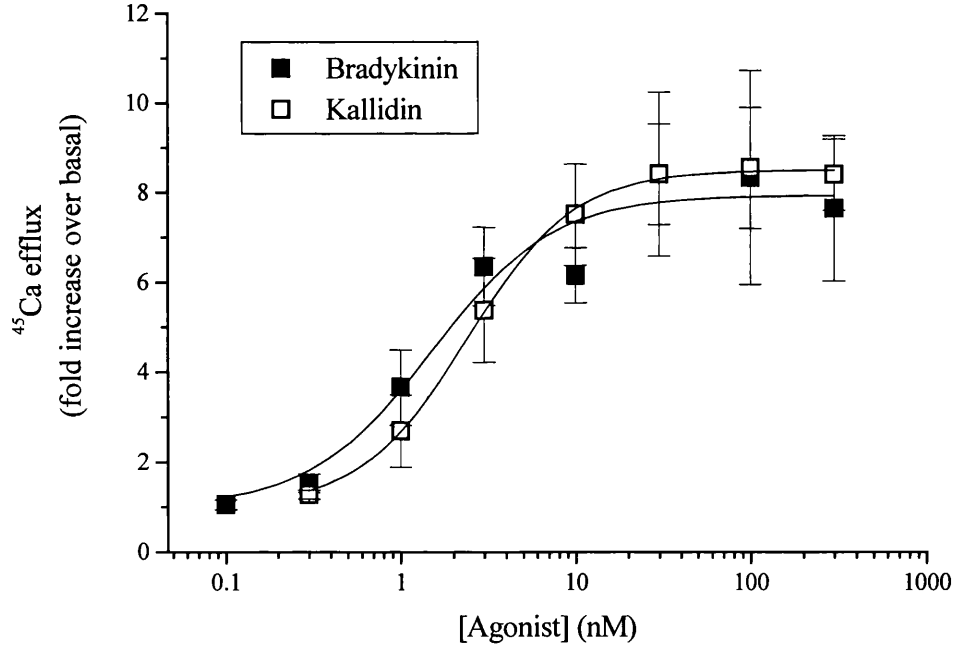

Figure 3-4 Stimulation of ^{45}Ca efflux in bladder smooth muscle cells by bradykinin and kallidin.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by bradykinin and kallidin and are the means and s.e.m.s of 4-6 independent experiments. Slope factors (p): 1.2 (bradykinin), 1.4 (kallidin). EC_{50} values obtained from mean graph: 1.5nM (bradykinin) and 2.4nM (kallidin).

The B_1 agonist des-Arg^9-bradykinin also evoked ^{45}Ca efflux in bladder smooth muscle cells. Initial experiments showed that even at very high concentrations (100μM) the evoked efflux had not reached a maximum level and it was not possible to determine an EC_{50} value (Figure 3-5). When the experiments to measure the concentration-response relationship were repeated in the presence of 30nM HOE140, a selective B_2 antagonist, the response to des-Arg^9-bradykinin reached a maximum at approximately 3μM. This indicates that, in the absence of a B_2 antagonist, concentrations of des-Arg^9-bradykinin higher than 3μM activate B_2 receptors (Figure 3-5).

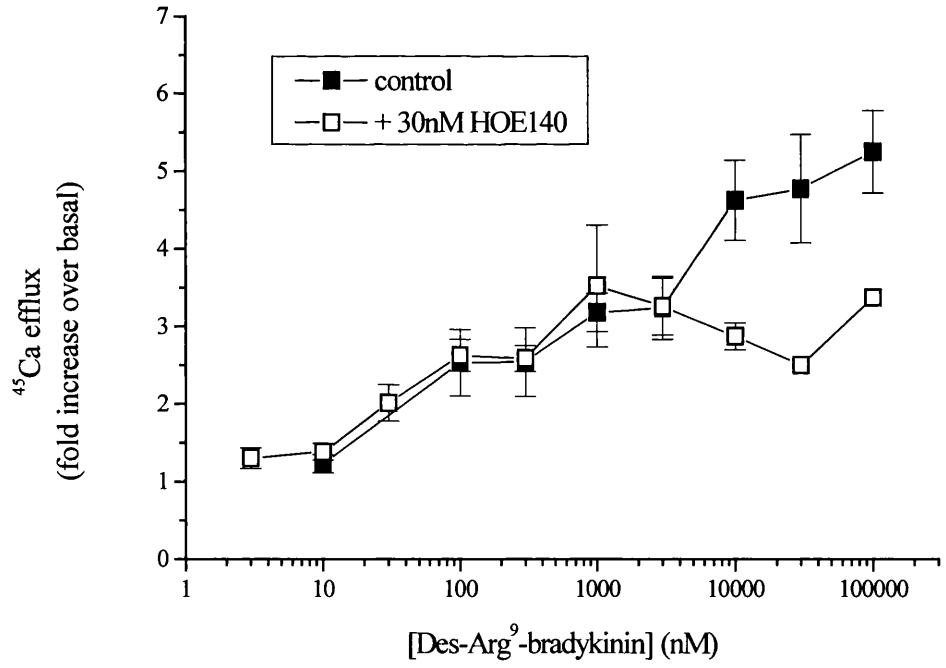

Figure 3-5 Stimulation of ^{45}Ca efflux in bladder smooth muscle cells by des-Arg^9-bradykinin in the presence and absence of HOE140.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by des-Arg^9-bradykinin in the presence and absence of 30nM HOE140 and are the means and s.e.m.s of 7 independent experiments.

In the presence of 30nM HOE140 the EC_{50} values for des-Arg^9-bradykinin and des-Arg^{10}-kallidin were 37 ± 9nM (n=10) and 39 ± 14nM (n=5) respectively (Figure 3-6). The maximum responses were 3.0 ± 0.2 (n=10) fold increase over basal for des-Arg^9-bradykinin and 2.8 ± 0.5 (n=5) for des-Arg^{10}-kallidin. These were not significantly ($p > 0.1$, unpaired Student's t-test) different from each other but were significantly less ($p < 0.01$, unpaired Student's t-test) than the responses obtained for both the B_2 agonists, bradykinin and kallidin.

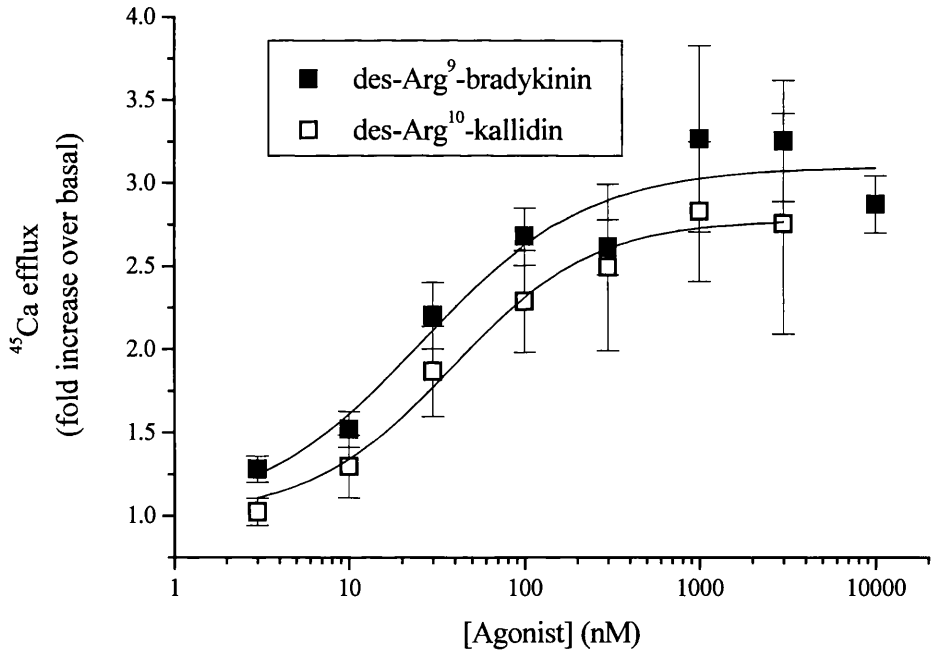

Figure 3-6 Stimulation of ^{45}Ca efflux in bladder smooth muscle cells by des-Arg^9-bradykinin and des-Arg^{10}-kallidin in the presence of HOE140.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by des-Arg^9-bradykinin and des-Arg^{10}-kallidin in the presence of 30nM HOE140 and are the means and s.e.m.s of 5-10 independent experiments. Slope factors (p): 0.92 (des-Arg^9-bradykinin), 1.1 (des-Arg^{10}-kallidin). EC_{50} values obtained from mean graph: 26nM (des-Arg^9-bradykinin), 38nm (des-Arg^{10}-kallidin).

In order to investigate the activity of des-Arg^9-bradykinin and des-Arg^{10}-kallidin at B_2 receptors further, the ^{45}Ca efflux evoked by these agonists was measured in the presence of 100nM [Leu^8]-des-Arg^9-bradykinin (a potent B_1 receptor antagonist) (Figure 3-7). Under these conditions, concentrations of des-Arg^9-bradykinin up to 300nM failed to evoke a response, however, concentrations greater than 1μM stimulated ^{45}Ca efflux. Although the concentration response curve had not quite reached a plateau, the EC_{50} value was estimated to be approximately 4μM. The other B_1 agonist des-Arg^{10}-kallidin caused only a very small response at 30μM, the highest concentration tested, in the presence of a B_1 antagonist.

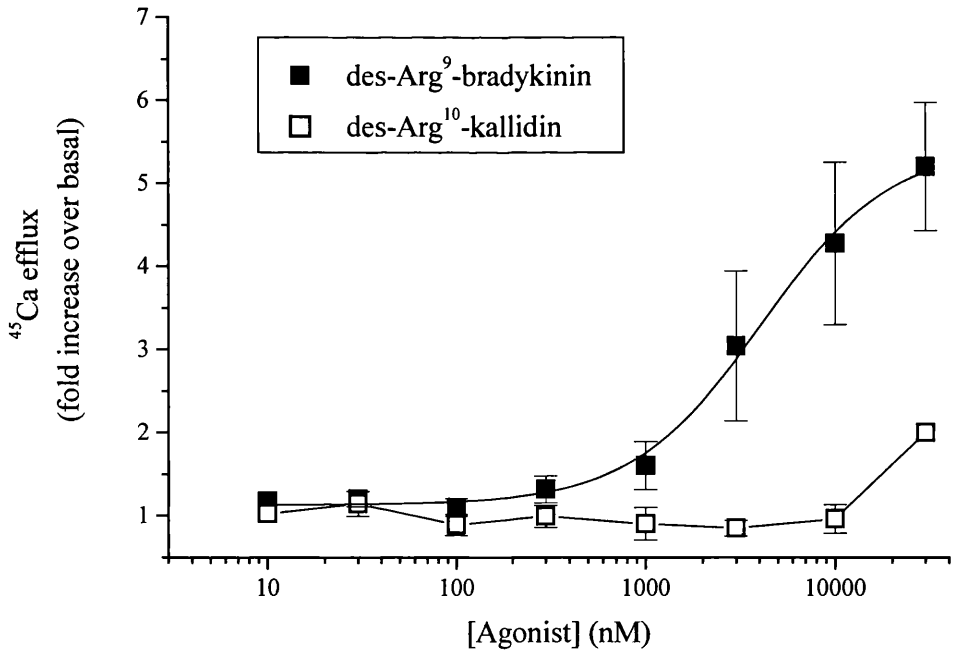

Figure 3-7 Activation of B_2 receptors in bladder smooth muscle cells by high concentrations of des-Arg^9-bradykinin and des-Arg^{10}-kallidin.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by des-Arg^9-bradykinin and des-Arg^{10}-kallidin in the presence of 100nM [Leu^8]-des-Arg^9-bradykinin and are the means and s.e.m.s of 4 independent experiments. Slope factor (p): 1.1. EC_{50} value obtained from mean graph: 4.1μM.

The ^{45}Ca efflux evoked by high concentrations of des-Arg^9-bradykinin, in the presence of 100nM [Leu^8]-des-Arg^9-bradykinin, could be blocked with the B_2 antagonist HOE140 confirming that it was mediated by activation of B_2 receptors (Figure 3-8).

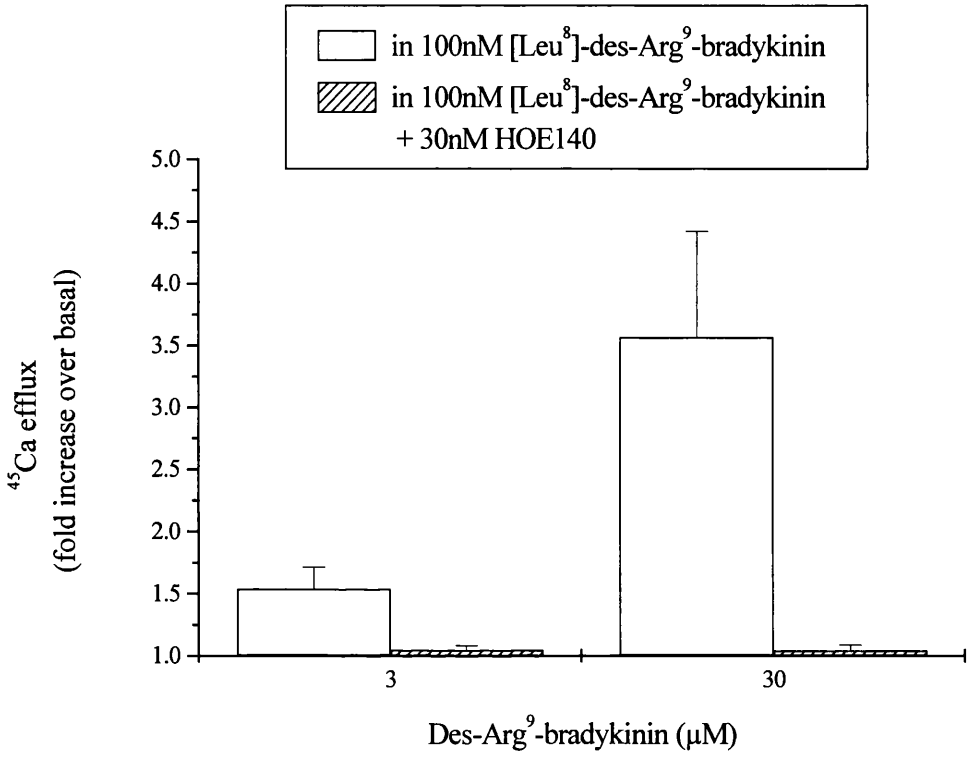

Figure 3-8 Activation of B_2 receptors in bladder smooth muscle cells by high concentrations of des-Arg^9-bradykinin.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by 3μM or 30μM des-Arg^9-bradykinin in the presence of 100nM [Leu^8]-des-Arg^9-bradykinin or 100nM [Leu^8]-des-Arg^9-bradykinin plus 30nM HOE140 and are the means and s.e.m.s of 5 independent experiments.

As des-Arg^9-bradykinin could activate B_2 receptors at high concentrations the possibility that high concentrations of bradykinin may activate B_1 receptors was investigated by examining the ability of bradykinin to evoke ^{45}Ca efflux in the presence of HOE140. The concentration-response curve for bradykinin in the presence of 30nM and 1μM HOE140 was shifted to the right and the maximum response was decreased (Figure 3-9). Although HOE140 has been reported to be a competitive antagonist in some tissues, this result was not entirely unexpected as HOE140 has been shown to behave non-competitively at the B_2 receptor in other tissues (Félétou et al., 1995).

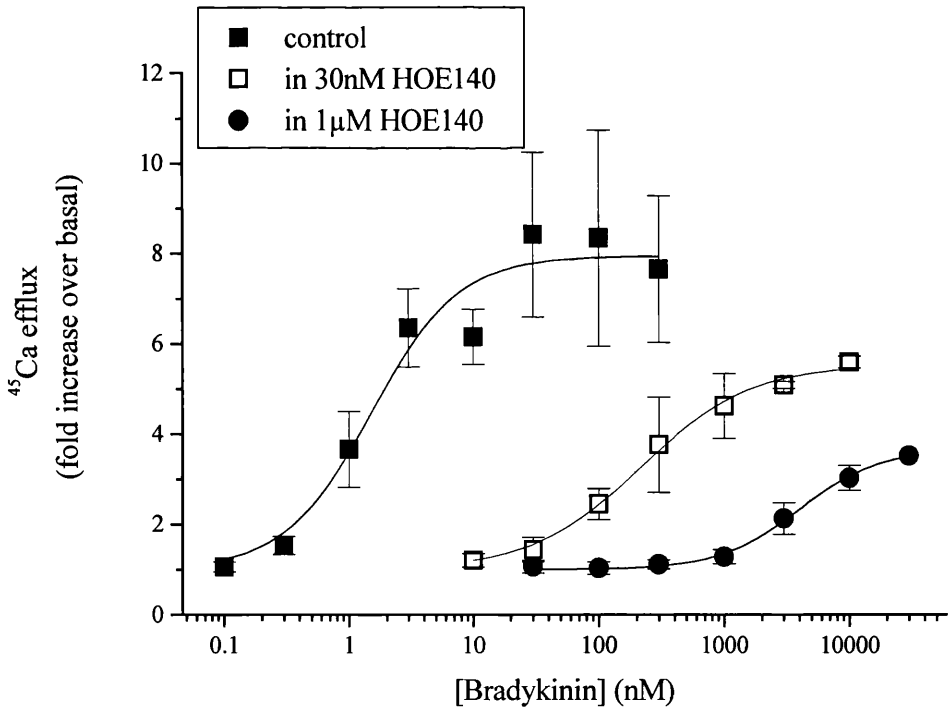

Figure 3-9 Bradykinin-induced ^{45}Ca efflux in the presence of HOE140.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by bradykinin in the presence of 30nM or 1μM HOE140 and are the means and s.e.m.s of 3-6 independent experiments. Slope factors (p): 1.2 (control), 1.0 (in 30nM HOE140), 1.2 (in 1μM HOE140). EC_{50} values obtained from mean graph: 1.5nM (control), 216nM (in 30nM HOE140), 3.9μM (in 1μM HOE140).

However, in the presence of 1μM HOE140, the response to 3μM and 30μM bradykinin was inhibited by the B_1 antagonist [Leu^8]-des-Arg^9-bradykinin (100nM) indicating that high concentrations bradykinin were capable of activating B_1 receptors (Figure 3-10). In contrast, the response to lower concentrations of bradykinin (e.g. 3nM) were completely blocked by HOE140 (30nM) and unaffected by [Leu^8]-des-Arg^9-bradykinin (up to 30μM), see Figure 3-11.

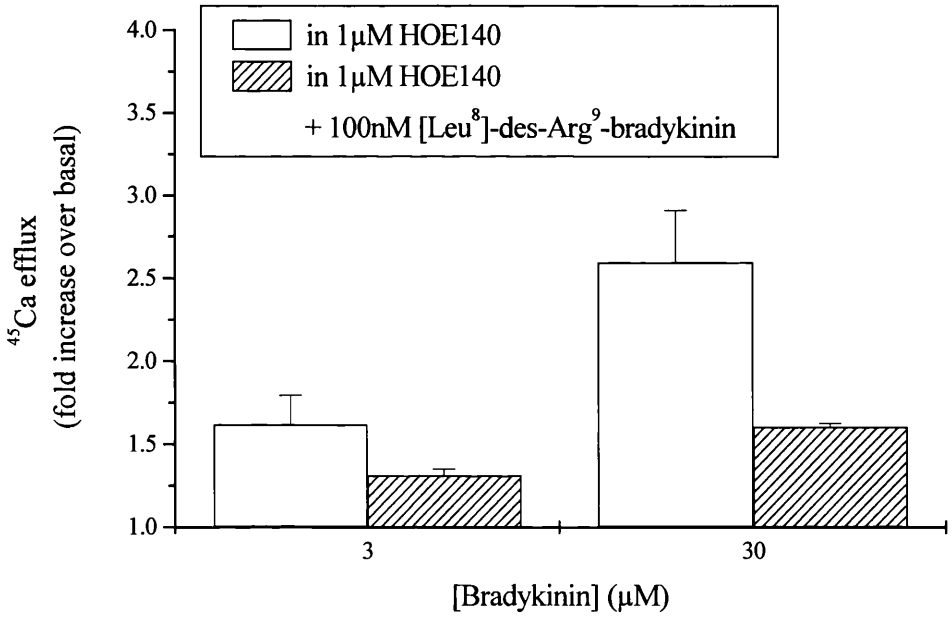

Figure 3-10 Activation of B_1 receptors by high concentrations of bradykinin.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase over basal, evoked by bradykinin in the presence of 1μM HOE140 or 1μM HOE140 plus 100nM [Leu^8]-des-Arg^9-bradykinin and are the means and s.e.m.s of 3-6 independent experiments.

The pharmacology of kinin receptors on bladder smooth muscle cells was investigated further using a range of kinin antagonists. The effect of these antagonists on the response evoked by concentrations of bradykinin and des-Arg^9-bradykinin close to their EC_{50} values (3nM and 40nM respectively) was tested (Figure 3-11 and Figure 3-12). The antagonists tested were the selective peptide B_2 antagonist HOE140 (described in Section 1.5.1.2) and its B_1

derivative des-Arg^{10}-HOE140, the non-peptide B_2 antagonist FR173657 and the peptide B_1 antagonists [Leu^8]-des-Arg^9-bradykinin and B9858 (see Section 1.5.2.2). The rank order of potency against 3nM bradykinin was HOE140 > FR173657> des-Arg^{10}-HOE140 = B9858 >> [Leu^8]-des-Arg^9-bradykinin, which is consistent with the known pharmacology of the B_2 receptor. Against 40nM des-Arg^9-bradykinin, the rank order of potency was [Leu^8]-des-Arg^9-bradykinin = B9858 > des-Arg^{10}-HOE140 > HOE140 > FR173657, which is consistent with B_1 receptor pharmacology.

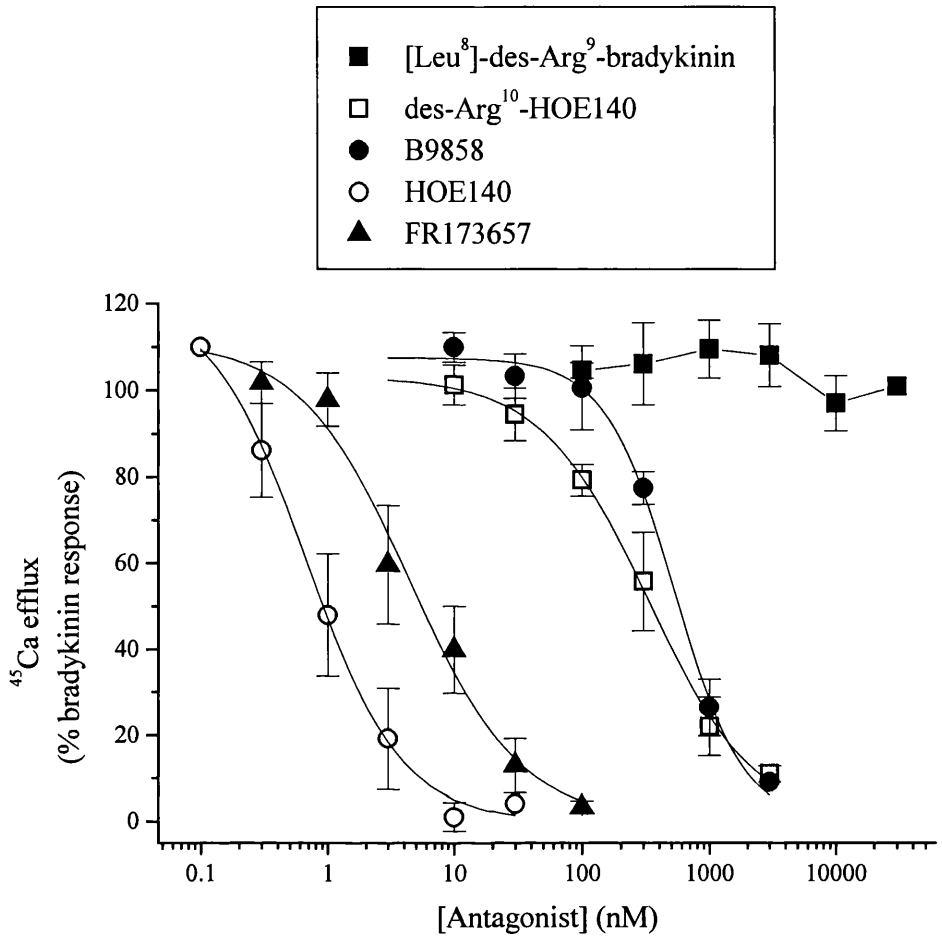

Figure 3-11 Inhibition of bradykinin-evoked ^{45}Ca efflux by a range of kinin antagonists.
The data represent the increase in the rate of ^{45}Ca efflux evoked by 3nM bradykinin in the presence of the antagonists. The results are calculated as a percentage of the control response to 3nM bradykinin and are the means and s.e.m.s of 3-4 independent experiments. Slope factors (p): 1.2 (HOE140), 0.98 (FR173657), 1.1 (des-Arg^{10}-HOE140), 1.7 (B9858). IC_{50} values obtained from mean graph: 0.67nM (HOE140), 4.4nM (FR173657), 346nM (des-Arg^{10}-HOE140), 547nM (B9858).

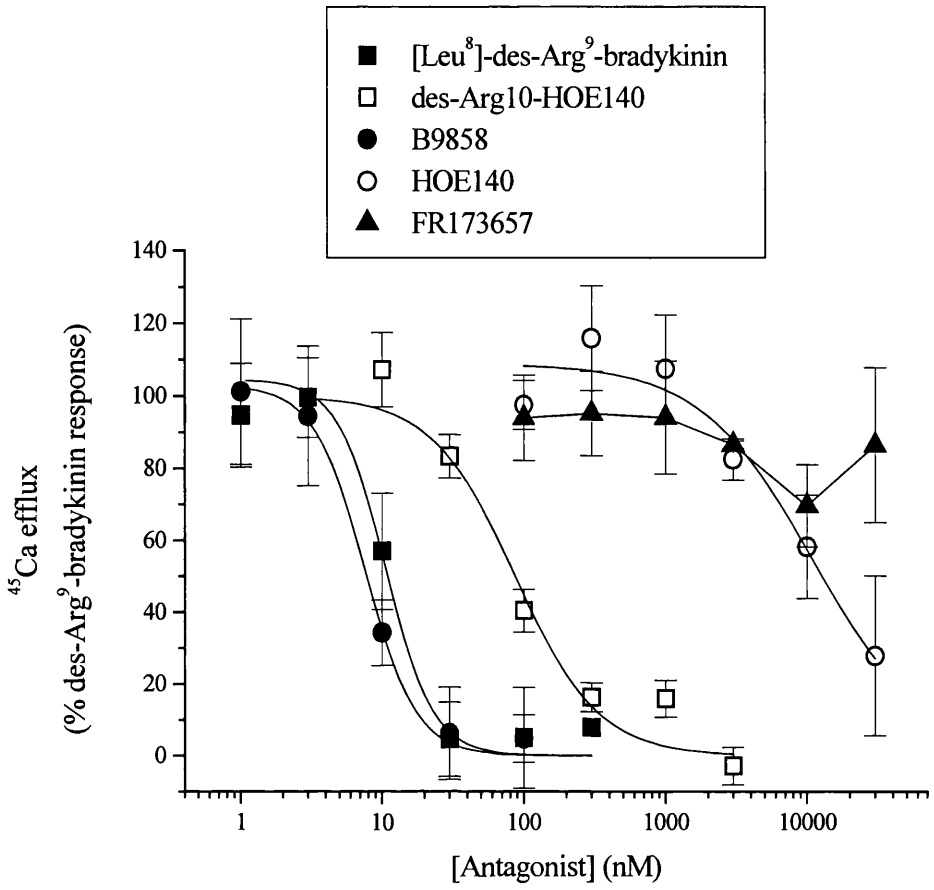

Figure 3-12 Inhibition of des-Arg^9-bradykinin-evoked ^{45}Ca efflux by a range of kinin antagonists.
The data represent the increase in the rate of ^{45}Ca efflux evoked by 40nM des-Arg^9-bradykinin in the presence of antagonists. The results are calculated as a percentage of the control response to 40nM des-Arg^9-bradykinin and represent the means and s.e.m.s of 3-4 independent experiments. Slope factors (p): 3.3 ([Leu^8]-des-Arg^9-bradykinin), 1.6 (des-Arg^{10}-HOE140), 2.4 (B9858), 1.1 (HOE140). IC_{50} values obtained from mean graph: 11nM ([Leu^8]-des-Arg^9-bradykinin), 84nM (des-Arg^{10}-HOE140), 7.6nM (B9858), 11μM (HOE140).

T-kinin (Ile-Ser-bradykinin) is formed uniquely in the rat and thought to act at the B_2 receptor. One of its possible breakdown products is des-Arg^{11}-T-kinin, which would be expected to act at B_1 receptors. It is not known if it occurs naturally and its pharmacology has not been studied. We investigated the pharmacology of both these peptides in bladder smooth muscle cells. T-kinin caused an increase in ^{45}Ca efflux with an EC_{50} value of 1.1 ± 0.3nM (n=4) making it the most potent of all the agonists investigated (Figure 3-13). The maximum response evoked by T-kinin was equivalent to a 5.7 ± 0.9 fold

increase over basal which was not significantly different ($p > 0.1$, unpaired Student's t-test) from the maximum response to bradykinin. Des-Arg^{11}-T-kinin also evoked ^{45}Ca efflux in bladder smooth muscle cells with an EC_{50} value of 40 ± 8nM (n=4) and a maximum response of 5.4 ± 1.0 fold increase over the basal level (in the presence of 30nM HOE140). This was significantly greater ($p < 0.05$, unpaired Student's t-test) than the response to the B_1 agonists des-Arg^9-bradykinin or des-Arg^{10}-kallidin but not significantly different ($p > 0.1$, unpaired Student's-test) from the response to bradykinin or T-kinin.

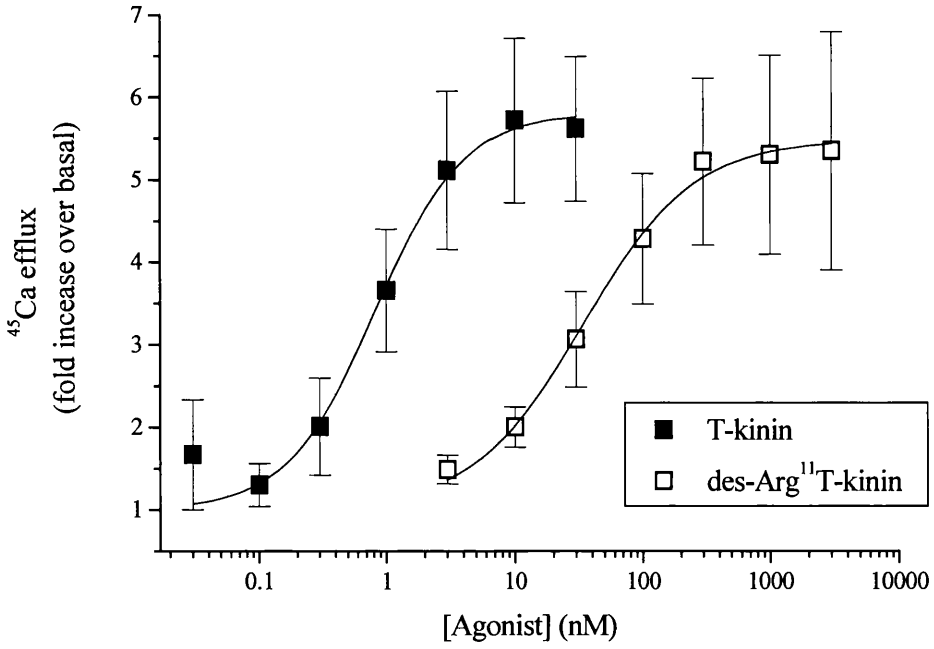

Figure 3-13 Stimulation of ^{45}Ca efflux in bladder smooth muscle cells by T-kinin and des-Arg^{11}-T-kinin.
The data represent the increase in the rate of ^{45}Ca efflux, expressed as fold increase above basal, evoked by the concentrations shown of T-kinin and des-Arg^{11}-T-kinin (in the presence of 30nM HOE140) and are the means and s.e.m.s of 4 independent experiments. Slope factors (p): 1.3 (T-kinin), 1.0 (des-Arg^{11}-T-kinin). EC_{50} values obtained from mean graph: 0.82nM (T-kinin), 34nM (des-Arg^{11}-T-kinin).

The pharmacology of the responses evoked by T-kinin and des-Arg^{11}-T-kinin was investigated using B_1 and B_2 antagonists. The ^{45}Ca efflux induced by 1nM T-kinin was inhibited by HOE140 with an IC_{50} value of 1.7 ± 0.5nM (n=3) but was unaffected by high concentrations of [Leu^8]-des-Arg^9-bradykinin (up to 3μM) (Figure 3-14).

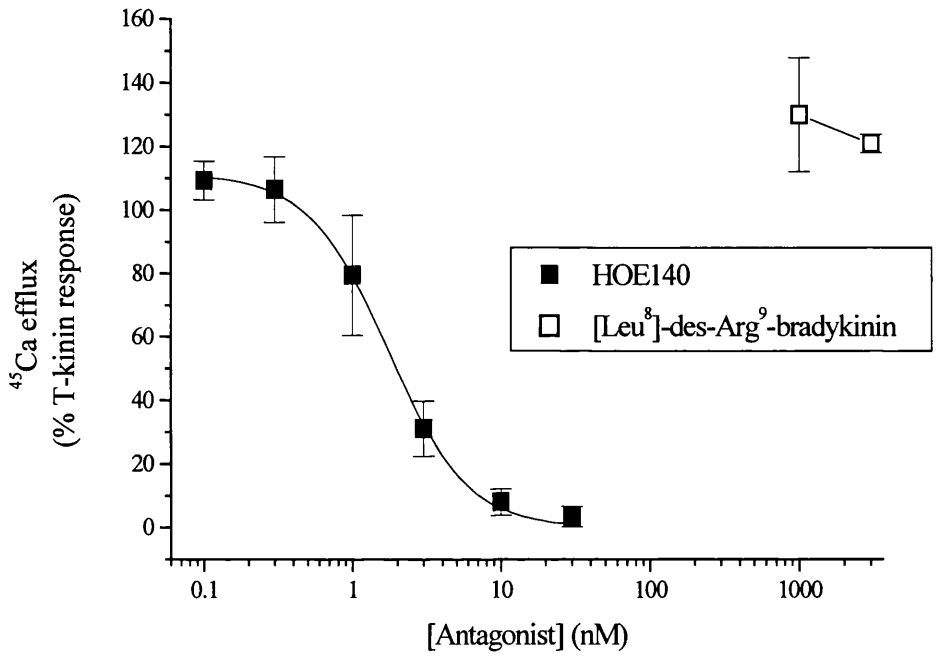

Figure 3-14 Inhibition of T-kinin-evoked ^{45}Ca efflux by HOE140 and [Leu^8]-des-Arg^9-bradykinin.
The data represent the increase in the rate of ^{45}Ca efflux evoked by 1nM T-kinin in the presence of the antagonists as a percentage of the control response to 1nM T-kinin and are the means and s.e.m.s of 3 independent experiments. Slope factor (p): 1.6. IC_{50} value obtained from mean graph: 1.6nM.

The response to 40nM des-Arg^{11}-T-kinin was inhibited by [Leu^{8}]-des-Arg^{9}-bradykinin with an IC_{50} value of 38 ± 13nM (n=4) but not by high concentrations of HOE140 (up to 3μM) (Figure 3-15). These data suggest that T-kinin activates B_2 receptors whereas des-Arg^{11}-T-kinin activates B_1 receptors.

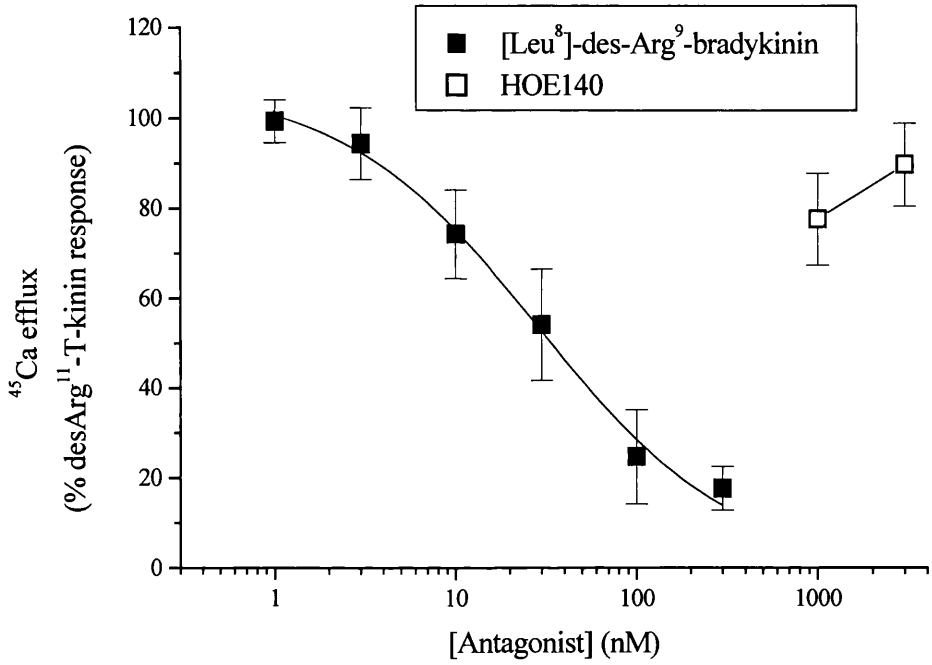

Figure 3-15 Inhibition of des-Arg^{11}-T-kinin-evoked ^{45}Ca efflux by HOE140 and [Leu^{8}]-des-Arg^{9}-bradykinin.
The data represent the increase in the rate of ^{45}Ca efflux evoked by 40nM des-Arg^{11}-T-kinin in the presence of the antagonists as a percentage of the control response to 40nM des-Arg^{11}-T-kinin and are the means and s.e.m.s of 5 independent experiments. Slope factor (p): 0.8. IC_{50} value obtained from mean graph: 29nM.

A summary of all the calcium efflux data is shown in Table 3-1. These data are consistent with bladder smooth muscle cells expressing a mixture of B_1 and B_2 kinin receptors, both of which activate PIC.

	Activity at bladder B_2 receptor	**Activity at bladder B_1 receptor**
Agonists	**EC_{50} (nM)**	**EC_{50} (nM)**
bradykinin	2.9 ± 1.9	
kallidin	3.2 ± 1.1	
T-kinin	1.1 ± 0.5	
des-Arg^9-bradykinin	~ 4000	37 ± 9
des-Arg^{10}-kallidin	>10000	39 ± 14
Sar-[D-Phe^8]-des-Arg^9-bradykinin	> 10000	42 ± 7
des-Arg^{11}-T-kinin	>10000	45 ± 8
Antagonists	**IC_{50} (nM)**	**IC_{50} (nM)**
FR173657.	7.0 ± 3.0	> 30000
HOE140	1.2 ± 0.6	16316 ± 7421
[Leu^8]-des-Arg^9-bradykinin	>30000	10 ± 2
B9858	536 ± 81	8.1 ± 2.3
des-Arg^{10}-HOE140	432 ± 180	41 ± 7

Table 3-1 Summary of kinin pharmacology in bladder smooth muscle cells.
For agonists the data represents the concentration required to evoke a response of 50% of the maximum (EC_{50} value) and for antagonists the concentration required to inhibit the response to 3nM bradykinin (B_2) or 40nM des-Arg^9-bradykinin (B_1) by 50% (IC_{50} value).

The results of the ^{45}Ca efflux assay suggest that bladder smooth muscle cells express both B_1 and B_2 receptors, but do not indicate whether B_1 and B_2 receptors are located on the same cell. This question could be addressed using an imaging system where the changes in $[Ca^{2+}]_i$ in response to application of B_1 and B_2 agonists can be monitored in individual cells. Although it was not possible to carry out a comprehensive study with an imaging system, a few experiments were carried out in order to determine the feasibility of such an approach. The experiment in Figure 3-16 shows that increases in $[Ca^{2+}]_i$ could be detected in bladder smooth muscle cells following exposure to both des-Arg^9-

bradykinin (300nM) and bradykinin (100nM). Furthermore, it is clear that both des-Arg^9-bradykinin and bradykinin, at concentrations selective for B_1 and B_2 receptors respectively, were able to evoke responses in the same cell. Out of 11 cells challenged with both agonists all responded to bradykinin and 8 responded to des-Arg^9-bradykinin. This indicates that B_1 and B_2 receptors are expressed on the same cell.

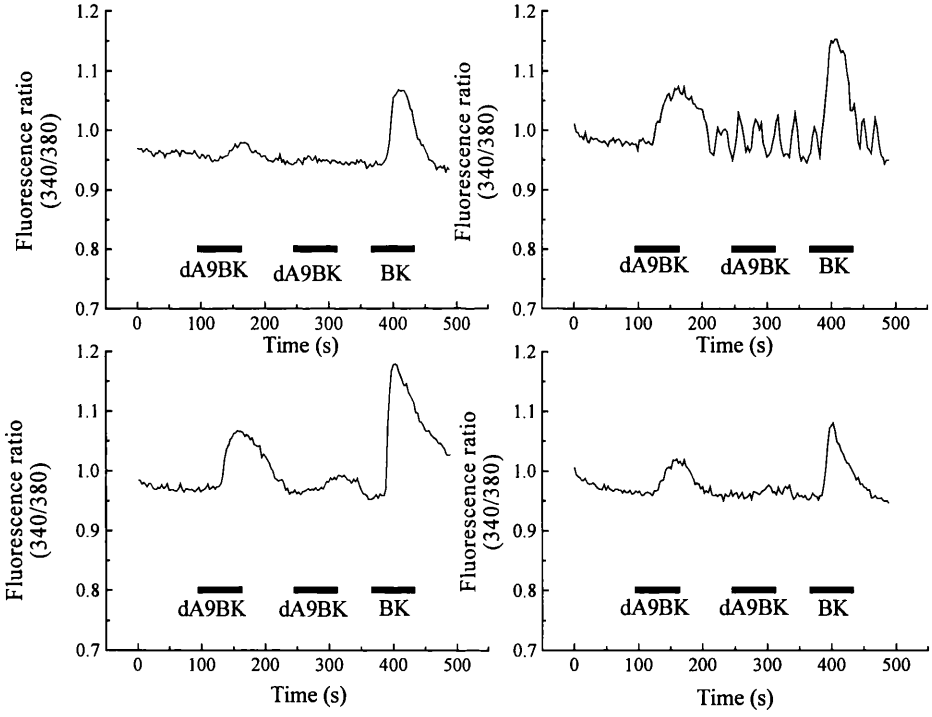

Figure 3-16 Increases in $[Ca^{2+}]_i$ evoked by des-Arg^9-bradykinin and bradykinin in bladder smooth muscle cells.
The data represent the ratio of the fluorescence emitted at 510nm, following excitation at 340 and 380nm, in individual bladder smooth muscle cells loaded with Fura-2/AM. The cells were stimulated with 300nM des-Arg^9-bradykinin (two applications of 1min) followed by 100nM bradykinin (one application of 1min). The bar (▬) indicates the presence of either des-Arg^9-bradykinin (dABK) or bradykinin (BK). The data represent the results from 4 individual cells measured in parallel.

One of the cells illustrated in Figure 3-16 appears to show oscillatory changes in $[Ca^{2+}]_i$ following application of des-Arg^9-bradykinin. Further experiments showed that these responses were only seen in cells challenged with des-Arg^9-bradykinin and not in cells that were exposed to bradykinin alone (Figure 3-17).

Overall, out of 15 cells exposed to bradykinin all 15 responded with transient increases in $[Ca^{2+}]_i$, and none of the responses were oscillatory in nature. Out of 17 cells exposed to des-Arg^9-bradykinin 14 responded, and, of these 3 were oscillatory responses.

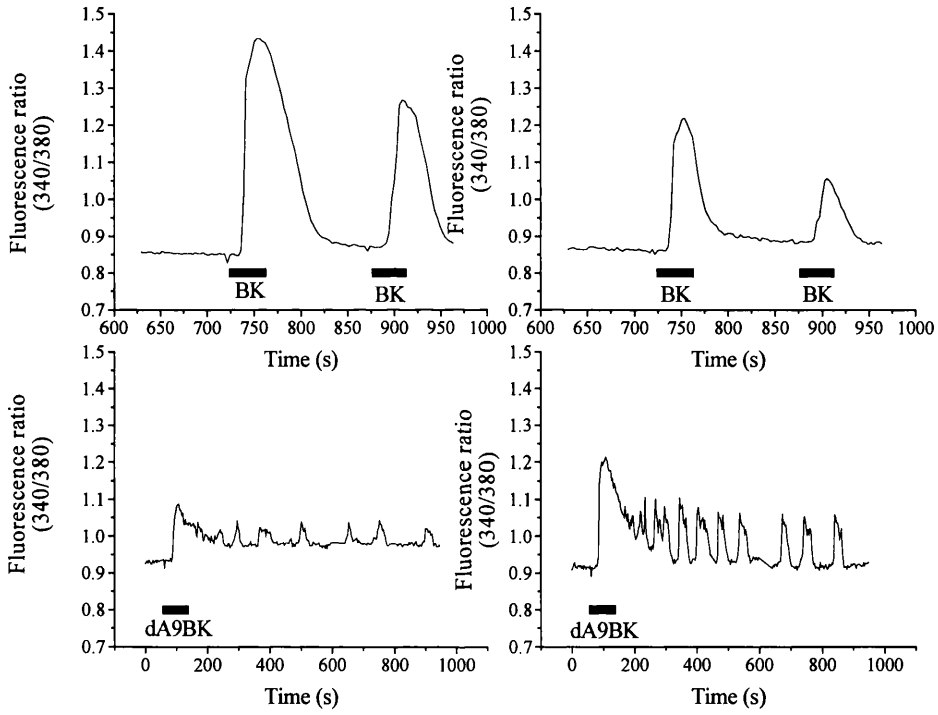

Figure 3-17 Oscillatory changes in $[Ca^{2+}]i$ evoked by des-Arg^9-bradykinin but not bradykinin in bladder smooth muscle cells.
The data represent the ratio of the fluorescence emitted at 510nm, following excitation at 340 and 380nm, in bladder smooth muscle cells loaded with Fura-2/AM. The cells were stimulated with 100nM bradykinin or 300nM des-Arg^9-bradykinin. The bar (▬) indicates the presence of either des-Arg^9-bradykinin (dABK) or bradykinin (BK). The data represent the results from 4 individual cells from 2 separate experiments.

3.3 BINDING STUDIES

The B_1 specific ligand [^{3}H]-des-Arg^{10}-kallidin has been widely used to measure levels of B_1 receptor in human and rabbit cells, however, there are no reports of specific binding using this ligand in rat cells and initial attempts in bladder smooth muscle cells were unsuccessful. Following the cloning of the rat B_1 receptor (Jones et al., 1999), a binding assay was successfully developed using membranes from Cos-7 cells transfected with the rat B_1 receptor. It was then possible to adapt this protocol to make it suitable for use with bladder smooth muscle cells.

In the binding assay the radioligand was incubated for a prolonged period of time (60min) with the cells. In order to confirm that no degradation of the ligand was occurring during the course of the binding assay in bladder smooth muscle cells, samples were taken before and after incubation of the radioligand with the cells and analysed using HPLC, with both UV and radiochemical detection. Figure 3-18A shows the UV and radiochemical detector traces for a sample prior to incubation with the cells. The UV detector trace in Figure 3-18A shows a sharp peak with a retention time of 6.3min corresponding to the unlabelled des-Arg^{10}-kallidin spiked into the samples, the next much larger peak in the UV trace was the BSA that was present in the binding buffer. In the radiochemical detector trace there was a single large peak with a retention time of 7.0min corresponding to [^{3}H]-des-Arg^{10}-kallidin, which ran later than the unlabelled des-Arg^{10}-kallidin because of the time delay between the UV detector and the radiochemical detector. Figure 3-18B shows the UV and radiochemical traces for the same sample following incubation with the cells for 1h at 4°C. No

additional peaks were observed suggesting that [^{3}H]-des-Arg^{10}-kallidin was not degraded during the binding assay.

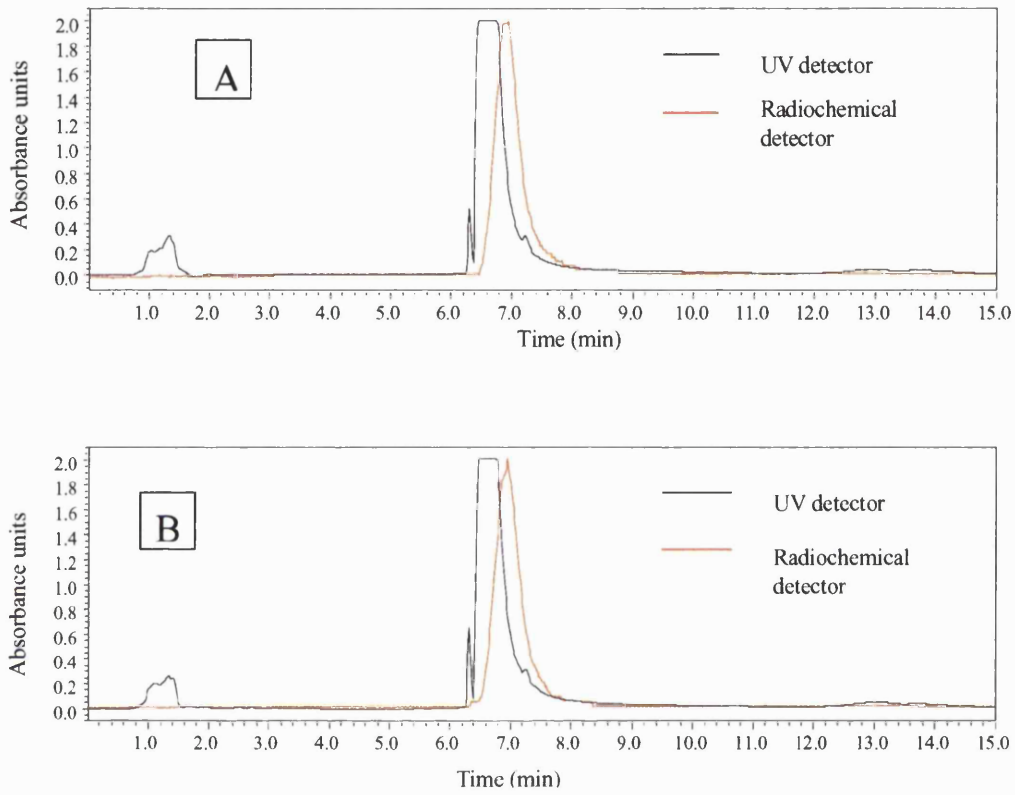

Figure 3-18 Analysis of the stability of [^{3}H]-des-Arg^{10}-kallidin in the binding assay with bladder smooth muscle cells.
The data represent the UV and radiochemical detector traces for 1.5nM [^{3}H]-des-Arg^{10}-kallidin, made up in binding buffer and spiked with 10μM unlabelled des-Arg^{10}-kallidin, analysed on a C18 column with a water and acetonitrile gradient (as described in Methods Section 2.5.2) from control samples (A) and following incubation with bladder smooth muscle cells for 1h at 4°C (B).

3.3.1 PHARMACOLOGY OF CLONED RAT B1 RECEPTOR

Membranes prepared from Cos-7 cells transfected with the rat B_1 receptor cDNA showed saturable binding of the B_1 specific ligand [^{3}H]-des-Arg^{10}-kallidin with a B_{max} of 276 ± 12fmol/mg and a K_D of 1.0 ± 0.2nM (n=7) (Figure 3-19).

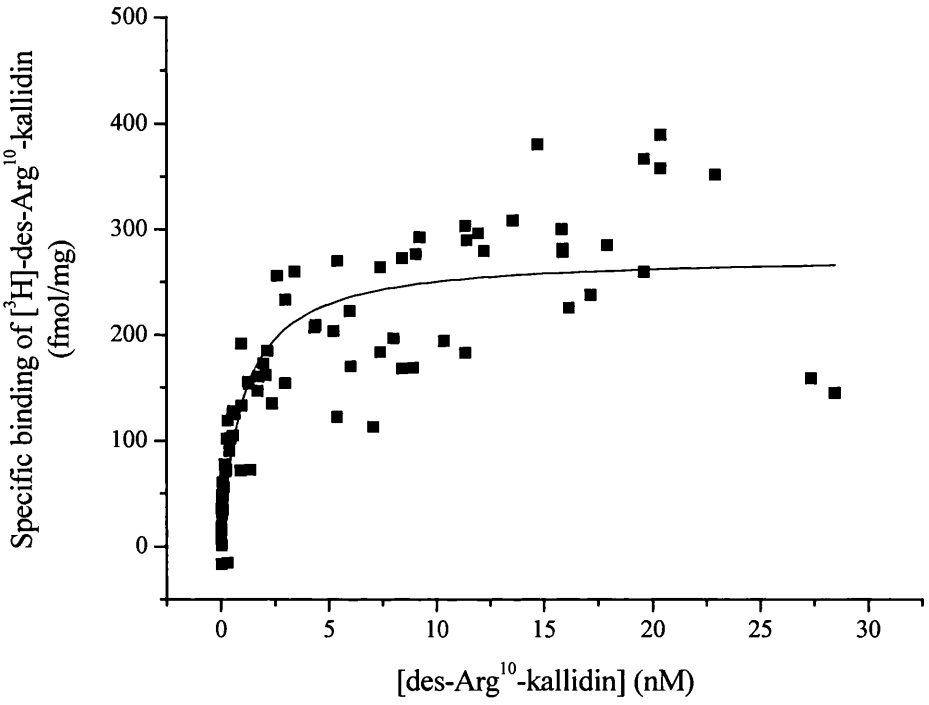

Figure 3-19 Binding of [^{3}H]-des-Arg^{10}-kallidin to membranes prepared from Cos-7 cells transfected with the rat B_1 cDNA.
The data represent specific binding at the concentrations shown of [^{3}H]-des-Arg^{10}-kallidin and are the combined results from 7 independent experiments.

The ability of a range of kinin compounds to displace the specific binding of 1.5nM [^{3}H]-des-Arg^{10}-kallidin (a concentration close to the K_D value) was investigated (Table 3-2).

Compound	K_I (nM) Cos-7 Rat B_1 membranes
des-Arg^{10}-kallidin	1.6 ± 0.2
B9858	3.0 ± 1.0
des-Arg^9-bradykinin	15 ± 5
[Leu^8]-des-Arg^9-bradykinin	31 ± 9
des-Arg^{10}-HOE140	35 ± 7
Des-Arg^{11}-T-kinin	46 ± 14
Sar-[D-Phe^8]-des-Arg^9-bradykinin	59 ± 19
kallidin	285 ± 38
HOE140	573 ± 169
bradykinin	6199 ± 920
T-kinin	8282 ± 1839
FR173657	> 30000

Table 3-2 K_I values in membranes prepared from Cos-7 cells transfected with rat B_1 cDNA.

The data represent the K_I values at the rat B_1 receptor, calculated from the IC_{50} values (the concentration giving 50% displacement of [^{3}H]-des-Arg^{10}-kallidin binding) using the Cheng-Prusoff equation ($K_I = IC_{50}/(1+ ([RL]/K_D))$ where [RL] is the concentration of radioligand, and are the means and s.e.m.s of 3-5 independent experiments.

The activities of the novel kinins, T-kinin and des-Arg^{11}-T-kinin, at the rat B_1 receptor were compared to their activity at the human B_1 receptor. The ability of T-kinin and des-Arg^{11}-T-kinin to displace 0.15nM [^{3}H]-des-Arg^{10}-kallidin binding (a concentration close to the K_D value of 0.064nM) in membranes prepared from Cos-7 cells transfected with the human B_1 receptor cDNA was measured. The K_I values obtained are shown in Table 3-3.

	K_I (nM) Cos-7 Rat B_1 membranes	K_I (nM) Cos-7 Human B_1 membranes
des-Arg^{11}-T-kinin	46 ± 14	1492 ± 355
T-kinin	8282 ± 1839	5898 ± 233

Table 3-3 Comparison of K_I values for T-kinin and des-Arg^{11}-T-kinin at human and rat B_1 receptors.

The data represent the K_I values, which are calculated from the IC_{50} values (the concentration giving 50% displacement of [^{3}H]-des-Arg^{10}-kallidin binding) using the Cheng-Prusoff equation ($K_I = IC_{50}/(1+ ([RL]/K_D))$ where [RL] is the concentration of radioligand, and are the means and s.e.m.s of 3-4 independent experiments.

This data confirms that des-Arg^{11}-T-kinin was selective for the rat B_1 receptor compared to the human. The B_2 ligand, T-kinin, had only weak affinity at both the human and rat B_1 receptors. In order to investigate whether T-kinin was more selective for the rat B_2 receptor over the human B_2 receptor, a functional assay using the calcium-sensitive probe aequorin was used to measure B_2 receptor-mediated increases in $[Ca^{2+}]_i$. T-kinin evoked an increase in aequorin-induced luminescence in Cos-7 cells transiently transfected with the rat B_2 receptor and aequorin cDNA, with an EC_{50} value of 42 ± 10nM (n=4). In contrast, in Chinese Hamster Ovary cells stably transfected with the human B_2 receptor and aequorin cDNA the EC_{50} for T-kinin-evoked increase in luminescence was 741 ± 81nM (n=3), indicating a preference of this kinin for the rat B_2 receptor. The B_1 selective agonist des-Arg^{11}-T-kinin, up to 30μM, did not cause an increase in luminescence in cells expressing either the rat or human B_2 receptor.

3.3.2 BLADDER SMOOTH MUSCLE CELLS

The binding assay that had been successfully developed for the cloned B_1 receptor was modified by removing EDTA and adding sucrose to the binding buffer in order to detect binding of [^{3}H]-des-Arg^{10}-kallidin in rat bladder smooth muscle cells. The radioligand [^{3}H]-des-Arg^{10}-kallidin showed saturable binding in bladder smooth muscle cells with a K_D value of 0.10 ± 0.03nM and a B_{max} of 37 ± 3 fmol/mg (n=4) (Figure 3-20). A two-site model was also used to fit the data, however this did not give a statistically better fit (the Chi^2 value for a 1 site fit was 38 and for a 2 site fit it was 39). No K_D values for [^{3}H]-des-Arg^{10}-kallidin binding in other types of rat cells or tissue that express the B_1 receptor naturally have been reported. However, the affinity of [^{3}H]-des-Arg^{10}-kallidin for the rat bladder smooth muscle cell B_1 receptor was approximately 10 fold higher than that for the cloned rat receptor (See Section 3.3.1).

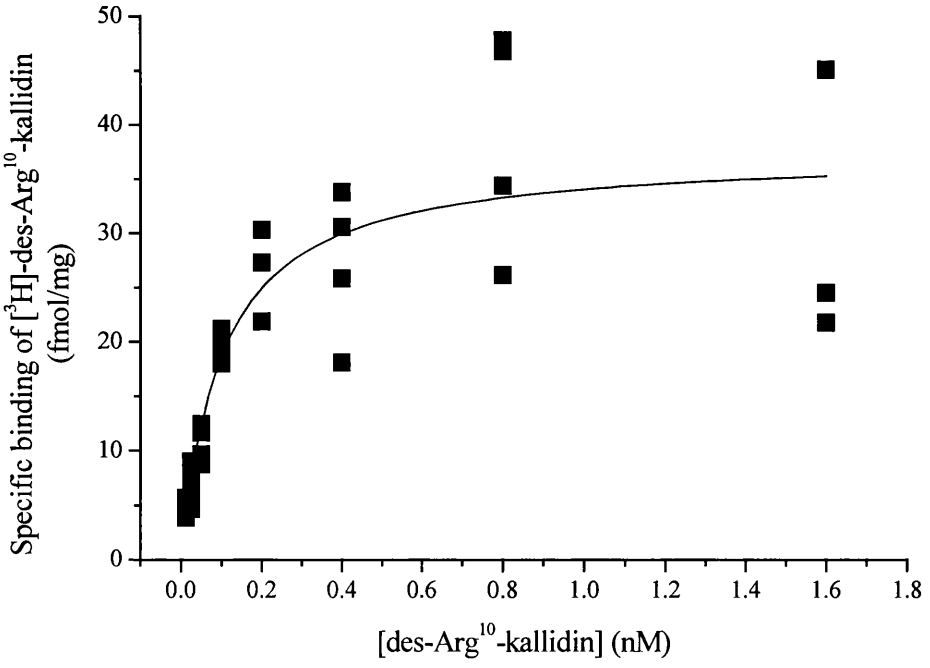

Figure 3-20 Binding of [^{3}H]-des-Arg^{10}-kallidin to bladder smooth muscle cells.
The data represent the specific binding at the concentrations shown of [^{3}H]-des-Arg^{10}-kallidin and are the combined results from 4 independent experiments.

The ability of a range of B_1 and B_2 compounds to displace the specific binding of 0.15nM [^{3}H]-des-Arg^{10}-kallidin in bladder smooth muscle cells was investigated (Table 3-4). The values were compared with those obtained in the Cos-7 membranes.

Compound	K_I (nM) Cos-7 Rat B_1 membranes	K_I (nM) Bladder cells
des-Arg^{10}-kallidin	1.6 ± 0.2	0.40± 0.15
B9858	3.0 ± 1.0	
des-Arg^9-bradykinin	15 ± 5	1.4 ± 0.4
[Leu^8]-des-Arg^9-bradykinin	31 ± 9	
des-Arg^{10}-HOE140	35 ± 7	9.3 ± 2.2
Des-Arg^{11}-T-kinin	46 ± 14	0.20 ± 0.07
Sar-[D-Phe^8]-des-Arg^9-bradykinin	59 ± 19	
kallidin	285 ± 38	
HOE140	573 ± 169	71 ± 19
bradykinin	6199 ± 920	72 ± 19
T-kinin	8282 ± 1839	296 ± 71
FR173657	> 30000	

Table 3-4 Comparison of K_I values in membranes prepared from Cos-7 cells transfected with rat B_1 cDNA with the K_I values obtained in bladder smooth muscle cells.
The data represent the K_I values, calculated from the IC_{50} values (the concentration giving 50% displacement of [^{3}H]-des-Arg^{10}-kallidin binding) using the Cheng-Prusoff equation (K_I = $IC_{50}/(1+ ([RL]/K_D))$ where [RL] is the concentration of radioligand, and are the means and s.e.m.s of 3-5 independent experiments.

All of the K_I values obtained in membranes prepared from Cos-7 cells transfected with the rat B_1 receptor were higher than in the bladder smooth muscle cells. The reason for this discrepancy is unclear but could be due to differences in post-translational modifications in Cos-7 cells. Previous reports have suggested that maturation of receptors may not be complete in transiently transfected cells and that the level of glycosylation can affect receptor affinity (Innamorati et al., 1996, Kusui et al., 1994). Despite these differences, the rank order of potency of the compounds was similar, the main exception being the potency of des-Arg^{11}-T-kinin, which was the equipotent to des-Arg^{10}-kallidin in

the bladder cells but less potent than des-Arg^{10}-kallidin at the cloned rat B_1 receptor expressed in Cos-7 cells.

In bladder smooth muscle cells all the compounds tested were all apparently more potent at displacing [^{3}H]-des-Arg^{10}-kallidin binding compared to their activity in the ^{45}Ca efflux assay (Table 3-1). Differences between binding and functional data have previously been reported for the B_2 receptor (Paquet et al., 1999), although not for the B_1 receptor (Schneck et al., 1994, Galizzi et al., 1994). In the ^{45}Ca efflux assay the EC_{50} values for the B_1 agonists, des-Arg^9-bradykinin (37nM), des-Arg^{10}-kallidin (39nM) and des-Arg^{11}-T-kinin (45nM) were all very similar. In contrast in the binding assay there was a greater variation in the K_I values for these compounds with des-Arg^{11}-T-kinin approximately 7 fold more potent than des-Arg^9-bradykinin.

3.4 REGULATION OF KININ RECEPTORS-FUNCTIONAL STUDIES

The ability of a variety of factors, including cytokines, cAMP elevating agents and serum, to affect the responsiveness of bladder smooth muscle cells to B_1 and B_2 agonists was investigated by measuring their ability to affect agonist-evoked ^{45}Ca efflux. The cells were incubated with ^{45}Ca for 4h at 37°C along with the factor under test, if a longer incubation was required it was added to the cells prior to starting the loading procedure for the time required. Following treatment, the ^{45}Ca efflux evoked by the B_1 agonist des-Arg^9-bradykinin (in the presence of 30nM HOE140 to prevent activation of B_2 receptors) or the B_2 agonist bradykinin, at a concentration that selectively activated B_2 receptors, was measured.

The responses to the kinin agonists were smaller in magnitude after 1 day in culture compared to 7-9 days (see Figure 3-3). The ability of IL-1ß, which increases the expression of the B_1 receptor in a variety of cell types (Galizzi et al., 1994, Phagoo et al., 1997), to potentiate the response to des-Arg^9-bradykinin examined in 1 day old cells was investigated. Agents that elevate cAMP have been reported to increase the level of B_2 receptors in smooth muscle cells (Yang et al., 1994, Schmidlin et al., 1998). Although there are no reports of the effect of cAMP on B_1 mediated responses or receptor levels, the possibility that cAMP could regulate the responsiveness to kinins in bladder smooth muscle cells was investigated. Neither dbcAMP, a membrane permeant analogue of cAMP, or IL-1ß augmented the response to des-Arg^9-bradykinin in these 1 day old cells. (Figure 3-21).

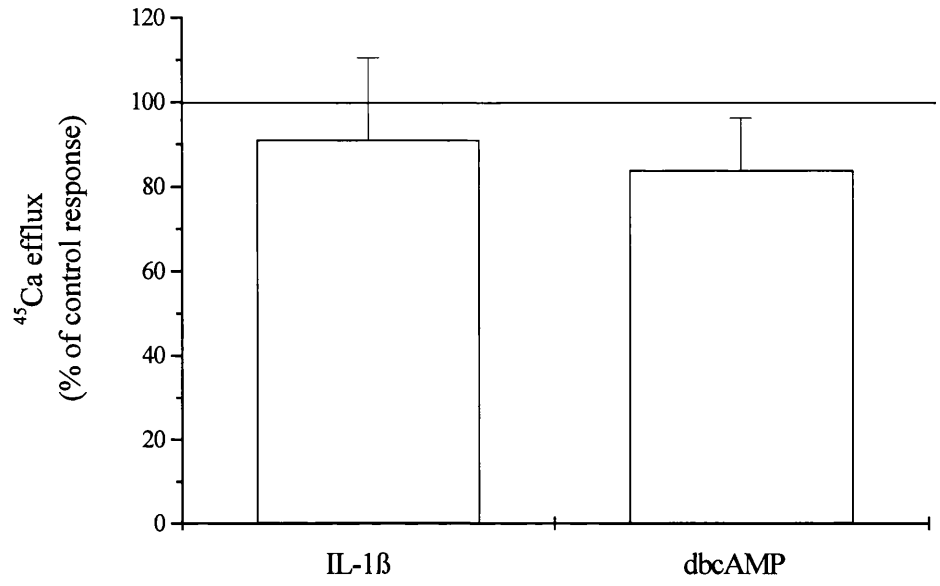

Figure 3-21 IL-1ß and dbcAMP did not increase the response to des-Arg^9-bradykinin in one day old cells.
The data represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin (in the presence of 30nM HOE140) in 1 day old cells following treatment for 4h with 100IU/ml IL-1ß or 1mM dbcAMP. The results are expressed as a percentage of the response in untreated cells and are the means and s.e.m.s of 3 experiments.

In a previous study, in which an attempt had been made to demonstrate the presence of the B_1 receptor on cultured sensory neurones, IL-1ß treatment did not induce expression of the B_1 receptor (Davis et al., 1996). In order to explore the possible reasons for the lack of effect of the cytokine, the presence of receptors for IL-1ß were investigated by determining whether there were any specific binding sites for [^{125}I]-IL-1ß on sensory neurones. In these experiments no specific binding was found, suggesting that sensory neurones do not express the receptor for IL-1ß. In contrast, it was possible to demonstrate binding sites for [^{125}I]-IL-1ß in the human lung fibroblast cell line IMR-90, in which IL-1ß induces a strong upregulation of the B_1 receptor (Phagoo et al., 1999). Using a similar assay, clear evidence of specific binding for [^{125}I]-IL-1ß in 8 day old bladder smooth muscle cells was found, as well as in IMR-90 cells, which were

included as a positive control (Figure 3-22), suggesting that bladder smooth muscle cells do express IL-1ß receptors.

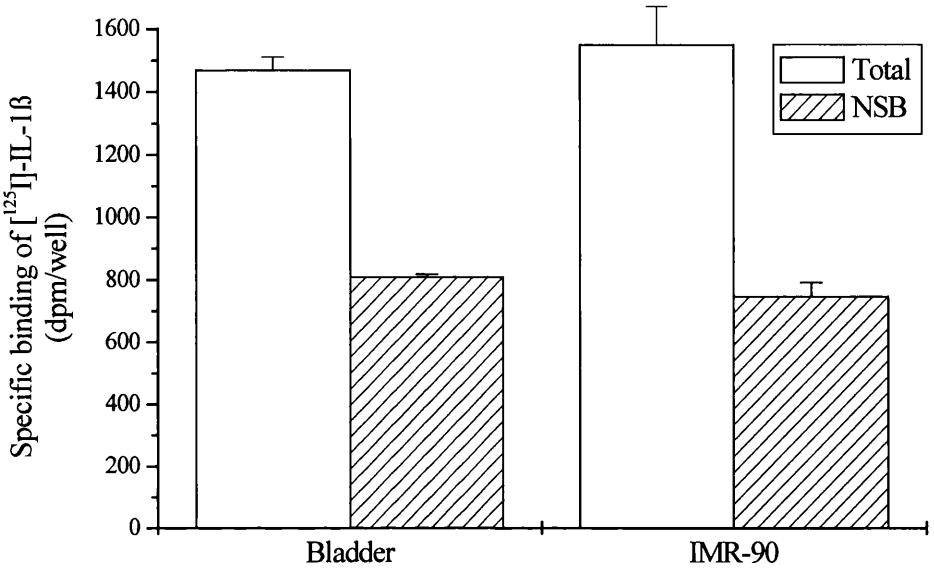

Figure 3-22 Binding of [^{125}I]-IL-1ß to bladder smooth muscle cells and IMR-90 cells.
The data represent the specific binding of [^{125}I]-IL-1ß (0.2nM) and are the means and s.e.m.s of one of two experiments, performed in triplicate, which gave similar results .

It was decided, therefore, to determine whether IL-1ß and dbcAMP could cause upregulation of the response to des-Arg^{9}-bradykinin in 7-9 day old bladder smooth muscle cells.

Bladder smooth muscle cells were normally maintained in the presence of 10% FCS. It was thought that factors in the serum might affect the expression of kinin receptors, so the effect of serum starvation on the responses to kinin agonists was investigated. In cells from which serum had been removed for 24h, the ^{45}Ca efflux evoked by des-Arg^9-bradykinin was reduced, although the response to bradykinin was unaffected (Figure 3-23)

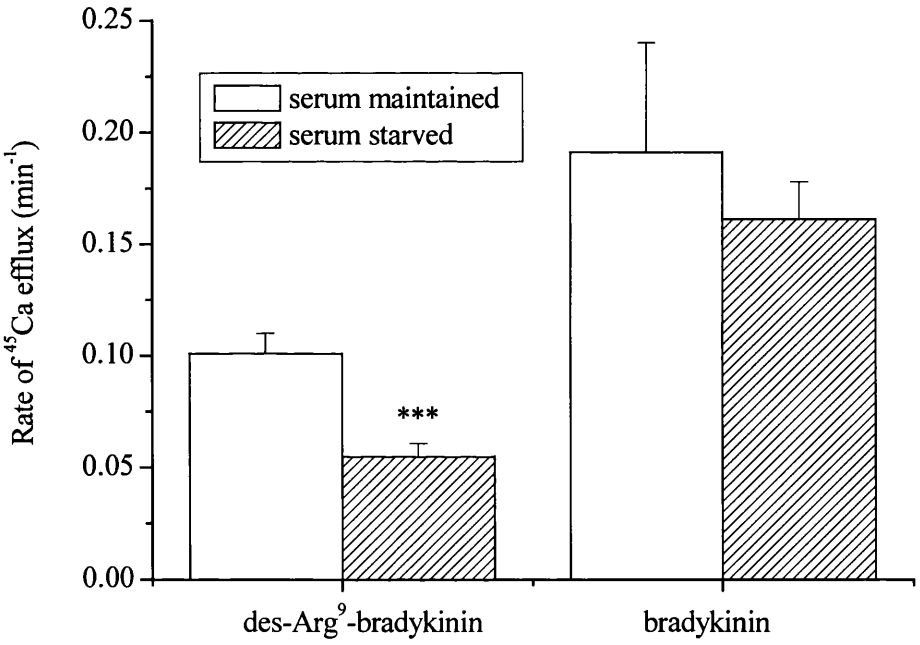

Figure 3-23 Effect of serum starvation on the response to des-Arg^9-bradykinin and bradykinin.
The data represent the increase in the rate of ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin in the presence of 30nM HOE140, or 100nM bradykinin, in cells grown in the presence or absence of serum for 24h. The data are the means and s.e.m.s of 25 (des-Arg^9-bradykinin) or 3 (bradykinin) experiments. *** $p < 0.001$ compared to serum present, unpaired Student's t-test on the evoked response.

As the response to des-Arg^9-bradykinin was reduced in the absence of serum, it was predicted that modulation of the response would be easier to detect in serum-starved cells. However, both IL-1ß and dbcAMP increased des-Arg^9-bradykinin-evoked ^{45}Ca efflux in serum-starved and serum-maintained cells.

The extent of upregulation was similar in the serum-starved and the serum-maintained cells. (Figure 3-24).

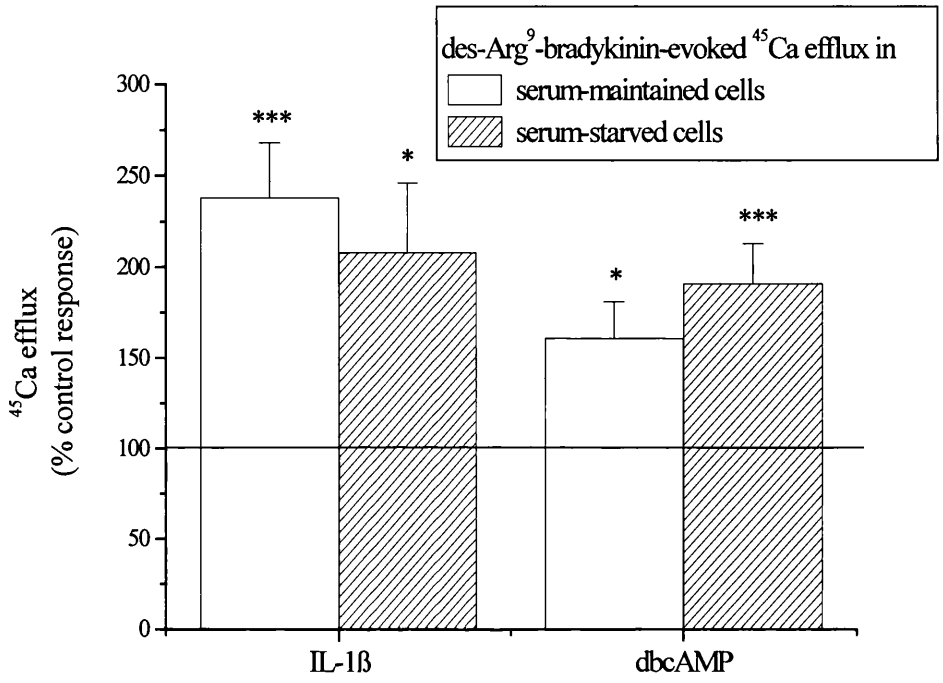

Figure 3-24 Effect of IL-1ß and dbcAMP on the response to des-Arg^9-bradykinin in serum-maintained and serum-starved cells.
The cells were maintained in the presence of serum or starved of serum for 20h before treatment with 100IU/ml of IL-1ß or 1mM dbcAMP for 4h. The results represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells (serum-maintained or serum-starved as appropriate). The data are the means and s.e.m.s of 6-15 experiments. * $p < 0.05$ compared to control, *** - $p < 0.001$ compared to control, paired Student's t-test on raw data.

In light of the results shown in Figure 3-24 and as serum-starved cells were difficult to work with as they did not adhere so firmly to the tissue culture plastic, all upregulation experiments were subsequently carried out on cells maintained in the presence of serum.

3.4.1 CHARACTERISATION OF THE EFFECTS OF CYTOKINES

The time-course of the effect of IL-1ß was investigated (Figure 3-25). The effect of IL-1ß was significant ($p < 0.05$) at 2h, peaked at 4h and was still significantly above control at 24h. IL-1ß (100IU/ml) treatment did not have any

effect on the basal rate of ^{45}Ca efflux which was 0.015 ± 0.001min^{-1} (n=6) in control cells and 0.017 ± 0.002 min^{-1} (n=6) in cells treated for 4h. The addition of IL-1ß involved changing the media on the cells. In order to control for any effect of adding fresh media to the cells on des-Arg^9-bradykinin-evoked ^{45}Ca efflux, the media was changed on control cells at the same time-points and the des-Arg^9-bradykinin-evoked ^{45}Ca efflux in IL-1ß treated cells was calculated as a percentage of the response in the time-matched control cells. However, analysis of the responses in the control cells showed that there was no statistically significant difference ($p > 0.5$, ANOVA) following addition of media at any of the time-points.

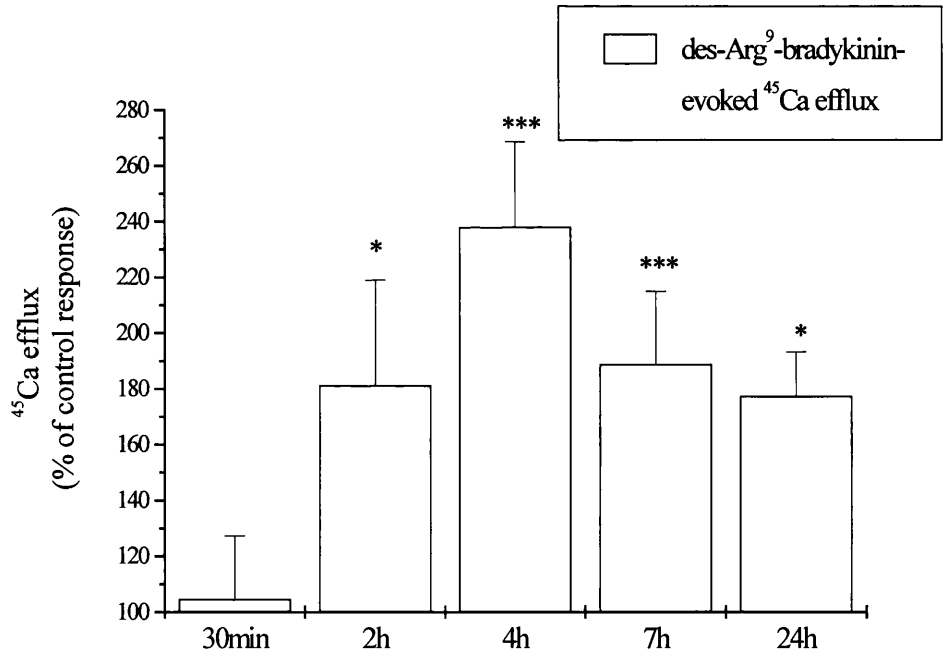

Figure 3-25 Time-course of the effect of IL-1ß treatment on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The results represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, following treatment for the times shown with 100IU/ml IL-1ß, as a percentage of the response in time-matched control cells. The data are the means and s.e.m.s of 3-5 independent experiments. * $p < 0.05$, *** $p < 0.001$ compared to matched control, paired Student's t-test on raw data.

Picking a time at which the effect of IL-1ß on B_1 responsiveness appeared to be maximally effective (4h) the effect of this cytokine on the concentration-response relationship for des-Arg^9-bradykinin was investigated. Treatment with 100IU/ml IL-1ß for 4h resulted in an increase in the maximum response evoked by des-Arg^9-bradykinin to 235 ± 34% (n=3) of that in control cells. The EC_{50} value was unaffected by IL-1ß treatment (11 ± 2nM in control cells and 16 ± 3nM in treated cells) (Figure 3-26).

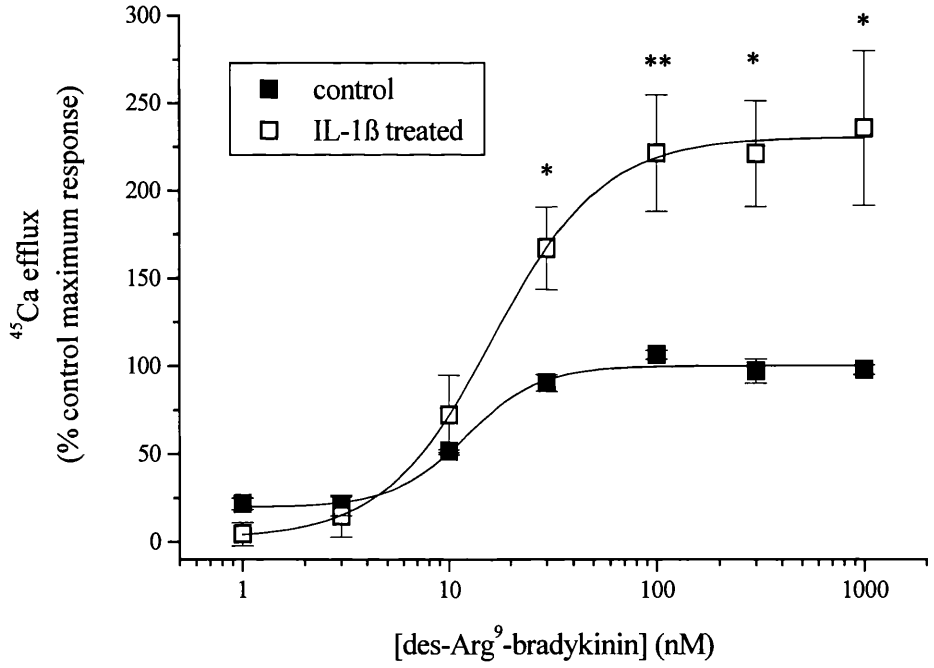

Figure 3-26 Effect of IL-1ß treatment on the concentration-response curve for des-Arg^9-bradykinin.
The results represent the increase in the rate of ^{45}Ca efflux evoked by a range of concentrations of des-Arg^9-bradykinin, in the presence of 30nM HOE140, following treatment for 4h with 100IU/ml of IL-1ß, as a percentage of the maximum response in control cells. The data are the means and s.e.m.s of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to control, paired Student's t-test on raw data. Slope factors (p): 2.4 (control), 1.6 (IL-1ß treated). EC_{50} values obtained from mean graph: 12nM (control), 17nM (IL-1ß treated).

To determine if the effect of IL-1ß on B_1 responsiveness involved the synthesis of new protein, the effect of the protein synthesis inhibitor cycloheximide was investigated. In this series of experiments cycloheximide caused a significant ($p < 0.05$) increase in the basal rate of efflux ($0.017 \pm 0.001 min^{-1}$ (n=4) in control cells and $0.025 \pm 0.002 min^{-1}$ (n=4) in treated cells), which resulted in an apparent decrease in the des-Arg^9-bradykinin-evoked ^{45}Ca efflux in cycloheximide treated cells (Figure 3-27). Despite this, it was clear that the IL-1ß-induced upregulation of the response to des-Arg^9-bradykinin was inhibited by cycloheximide.

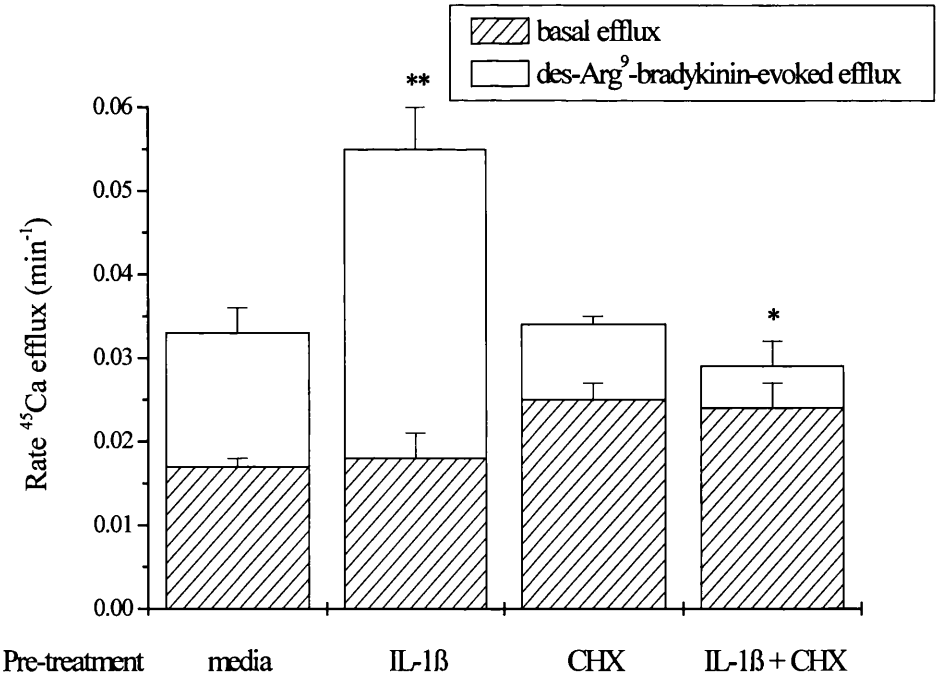

Figure 3-27 Effect of IL-1ß and cycloheximide on basal and des-Arg^9-bradykinin-evoked ^{45}Ca efflux in bladder smooth muscle cells.
The cells were incubated for 4h with 100IU/ml of IL-1ß, 70µM cycloheximide (CHX) or media alone. The data represent the basal rate of ^{45}Ca efflux and the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin in the presence of 30nM HOE140. The data are the means and s.e.m.s of 4 experiments. * $p < 0.05$, ** $p < 0.01$ compared to media alone, ANOVA followed by Dunnett's test on the evoked response.

In smooth muscle preparations such as the rabbit aorta it has been reported that the increase in response to des-Arg^9-bradykinin evoked by IL-1ß can be inhibited by steroids such as dexamethasone (Deblois et al., 1988). Incubation of bladder smooth muscle cells with dexamethasone (1μM for 4h) inhibited the effect of IL-1ß (Figure 3-28). Dexamethasone did not affect the basal rate of efflux (0.017 ± 0.001min^{-1} (n=3) in control cells and 0.018 ± 0.001min^{-1}(n=3) in treated cells) and did not significantly ($p > 0.05$) affect the des-Arg^9-bradykinin-evoked response in control cells.

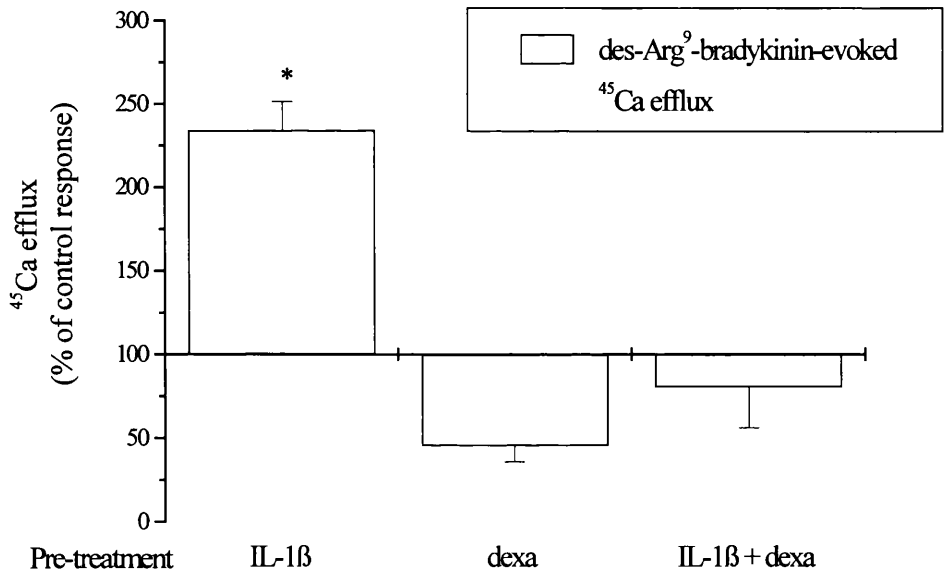

Figure 3-28 Inhibition by dexamethasone of the effect of IL-1ß on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were incubated with 100IU/ml IL-1ß, 1μM dexamethasone (dexa) or a combination of both for 4h. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data are the means and s.e.m.s of 3 experiments. * $p < 0.05$ compared to control, ANOVA followed by Dunnett's test on raw data.

Some studies have suggested a possible role for cyclooxygenase products in the up-regulation of B_1 receptor mediated effects in the bladder. For example in the isolated mouse bladder indomethacin (45μM) added 30min prior to LPS inhibited the increase in des-Arg^9-bradykinin-mediated contractions evoked by LPS (Busser et al., 1998). Incubation of bladder smooth muscle cells with indomethacin (45μM) for 30min prior to, and during a 4h treatment with IL-1ß did not inhibit the effect of IL-Iß (Figure 3-29). Indomethacin had no effect on the basal rate of efflux (0.013 ± 0.0004min^{-1} (n=3) in control cells and 0.017 ± 0.004min^{-1} (n=3) in treated cells.

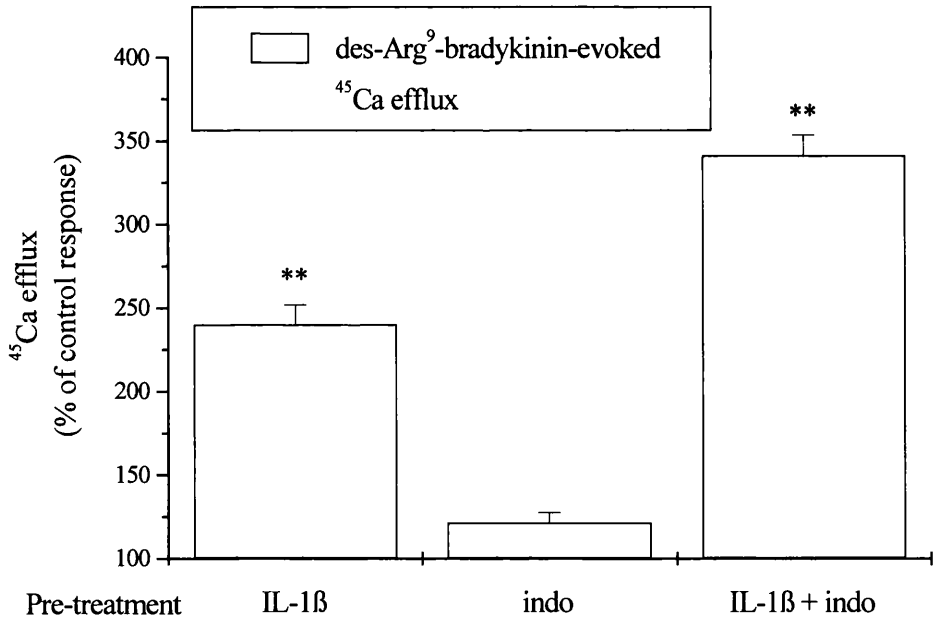

Figure 3-29 The increase in responsiveness to des-Arg^9-bradykinin evoked by IL-1ß was not inhibited by indomethacin.
The cells were pre-treated for 30min with 45μM indomethacin and then incubated with 100IU/ml IL-1ß, indomethacin (indo) or a combination of both for 4h. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data are the means and s.e.m.s of 3 experiments. ** $p < 0.01$, compared to control, ANOVA followed by Dunnett's test on raw data.

Interleukin-1 receptor antagonist (IL-1ra) is a naturally occurring protein with approximately 26% homology to IL-1ß. It binds to the IL-1 receptor and has

been shown to block the effects of IL-1ß, including the effect of IL-1ß on increased B_1 receptor expression (Phagoo et al., 1999). Treatment of cells with IL-1ra (50ng/ml for 4.5h) on its own resulted in a small increase in the des-Arg^9-bradykinin-evoked ^{45}Ca efflux. This suggests that IL-1ra may have some weak activity at the interleukin-1 receptor. In the presence of IL-1ra, IL-1ß did not cause a significant increase ($p > 0.05$) in des-Arg^9-bradykinin-evoked efflux above the level in cells treated with IL-1ra alone (Figure 3-30). In addition des-Arg^9-bradykinin-evoked efflux was significantly less ($p < 0.05$) in cells treated with IL-1ra plus IL-1ß, compared to cells treated with IL-1ß, implying that the effect of IL-1ß was mediated via the IL-1ß receptor. There was no effect of IL-1ra on the basal efflux ($0.015 \pm 0.001 min^{-1}$ (n=5) in control cells and $0.016 \pm 0.0003 min^{-1}$ (n=5) in treated cells).

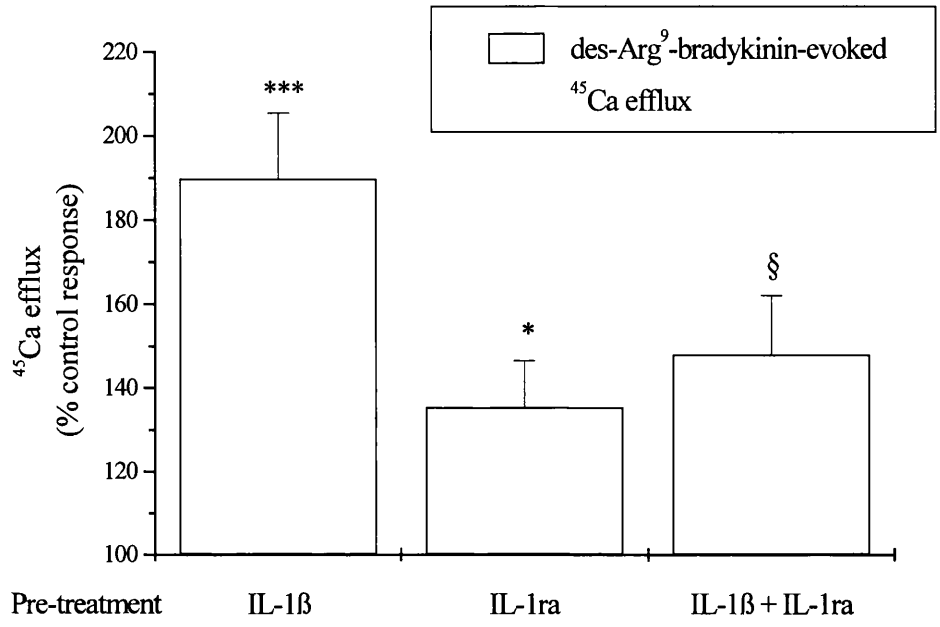

Figure 3-30 Inhibition by IL-1ra of the effect of IL-1ß on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were preincubated with IL-1ra (50ng/ml) for 30min before, and during, treatment with IL-1ß (100IU/ml for 4h). The data represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in untreated cells. The data are the means and s.e.m.s of 5 independent experiments. * $p < 0.05$, *** - $p < 0.001$ compared to control, § - $p < 0.05$ compared to IL-1ß treated, ANOVA followed by Bonferroni test on raw data.

One of the most important signalling events activated by IL-1ß is the activation of NF-κB, which in some studies has been shown to be involved in IL-1ß-induced upregulation of the B_1 receptor (Schanstra et al., 1998). The inhibitor PDTC can be used to investigate the role of NF-κB, however, treatment of bladder smooth muscle cells with this compound resulted in the death of the cells. Similar findings have been made in vascular smooth muscle cells where treatment with PDTC was reported to cause apoptosis (Tsai et al., 1996).

IL-1ß has also been reported to activate protein kinases including PKA and PKC (Shirakawa et al., 1988, Muñoz et al., 1990, Limatola et al., 1997). The effect of the broad spectrum kinase inhibitor staurosporine on the increase in response to des-Arg^9-bradykinin evoked by IL-1ß was investigated. Staurosporine had no

effect on the basal efflux rate which was 0.017 ± 0.001 min^{-1} (n=5) in control cells and 0.017 ± 0.002 min^{-1} (n=5) in treated cells, and did not cause a significant reduction (p> 0.05, ANOVA followed by Bonferroni test) in the control response to des-Arg^9-bradykinin. In the presence of staurosporine, IL-1ß was able to induce an increase in the des-Arg^9-bradykinin-evoked ^{45}Ca efflux rate (Figure 3-31) suggesting that the upregulation evoked by IL-1ß was not mediated by a staurosporine-sensitive kinase.

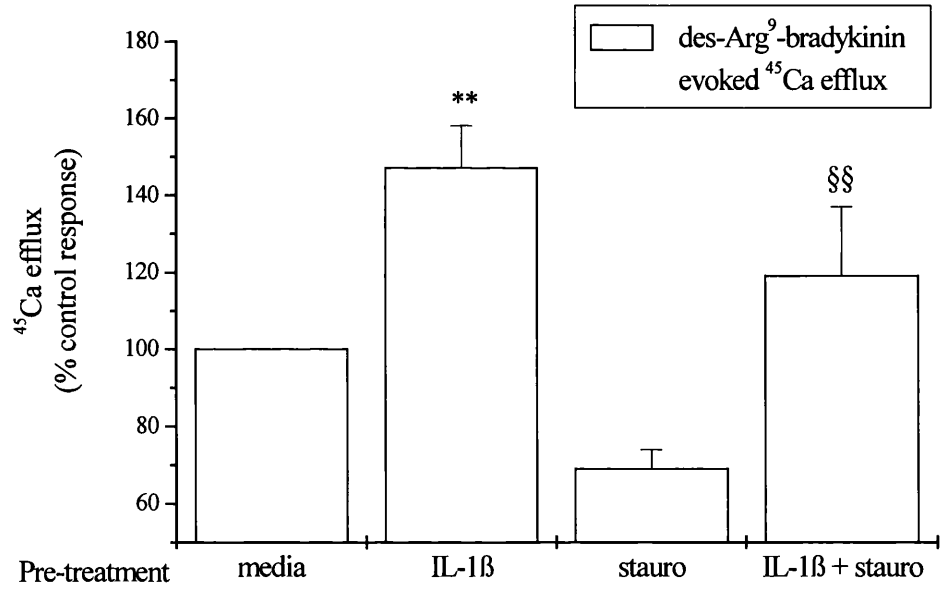

Figure 3-31 Treatment with staurosporine did not affect the ability of IL-1ß to increase the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were pre-treated for 30min with staurosporine (100nM) before, and during, incubation with IL-1ß (100IU/ml) for 4h. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data are the means and s.e.m.s of 5 experiments. ** $p < 0.01$ compared to control, §§ $p < 0.01$ compared to staurosporine alone, ANOVA followed by Bonferroni test on raw data.

As well as IL-1ß, LPS and other cytokines, such as TNFα, have been shown to increase the response of various cell types to B_1 receptor agonists (Galizzi et al.,

1994, Phagoo et al., 1997). Treatment of bladder smooth muscle cells for 4h with rat TNFα (10ng/ml) or LPS (10μg/ml) resulted in an increase in the ^{45}Ca efflux evoked by des-Arg^{9}-bradykinin (Figure 3-32). Neither LPS or TNFα affected the basal efflux rate (0.016 ± 0.001 min^{-1} (n=10) in control cells, 0.018 ± 0.001 min^{-1} (n=6) in TNFα treated cells and 0.018 ± 0.001 min^{-1} (n=7) in LPS treated cells).

3.4.2 EFFECT OF CYTOKINES ON B_2 MEDIATED RESPONSES

In addition to investigating the effect of cytokines and LPS on B_1-mediated responses, the effect of these agents on B_2 mediated responses was also investigated. The B_2 receptor is generally considered to be constitutively expressed, however, it has been reported that IL-1ß can increase expression of the B_2 receptor in some cell types, for example in human bronchial smooth muscle cells IL-1ß (10IU/ml for 3h) induced an increase in B_2 receptor density (Schmidlin et al., 1998). Using similar conditions to those used by Schmidlin et al., (100IU/ml for 4h) no IL-1ß-induced increases in B_2 mediated responses were observed in bladder smooth muscle cells. In addition, TNFα and LPS, under conditions that increased B_1 mediated responses, had no effect on bradykinin-evoked ^{45}Ca efflux (Figure 3-32).

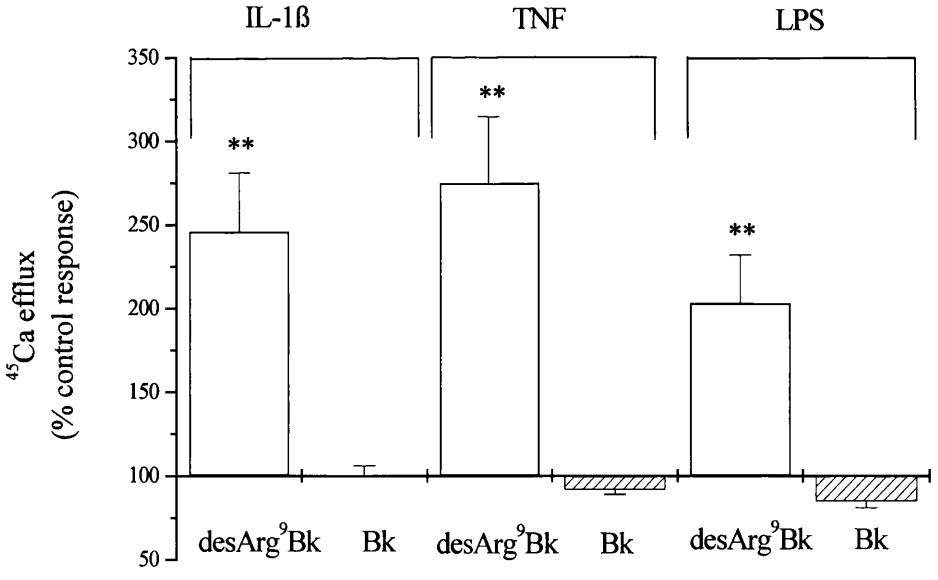

Figure 3-32 Effect of IL-1ß, TNFα and LPS on the ^{45}Ca efflux evoked by des-Arg^9-bradykinin and bradykinin in bladder smooth muscle cells.
Bladder smooth muscle cells were treated for 4h with 100IU/ml IL-1ß, 10ng/ml TNFα or 10µg/ml LPS. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, or 100nM bradykinin as a percentage of the response to each agonist in control cells. The data are the means and s.e.m.s of 3 independent experiments. ** $p < 0.01$, compared to control, ANOVA followed by Dunnett's test on raw data.

3.4.3 CHARACTERISATION OF THE EFFECT OF cAMP

Treatment of bladder smooth muscle cells with 1mM dbcAMP augmented the increase in ^{45}Ca efflux rate evoked by des-Arg^9-bradykinin (Figure 3-33). DbcAMP treatment caused a small but significant decrease in the basal efflux rate (0.045 ± 0.002 min^{-1} (n=24) in control cells and 0.037 ± 0.002 min^{-1} (n=24) in dbcAMP treated cells). This was too small, however, to account for the increase in des-Arg^9-bradykinin-evoked ^{45}Ca efflux rate caused by dbcAMP treatment (Figure 3-33).

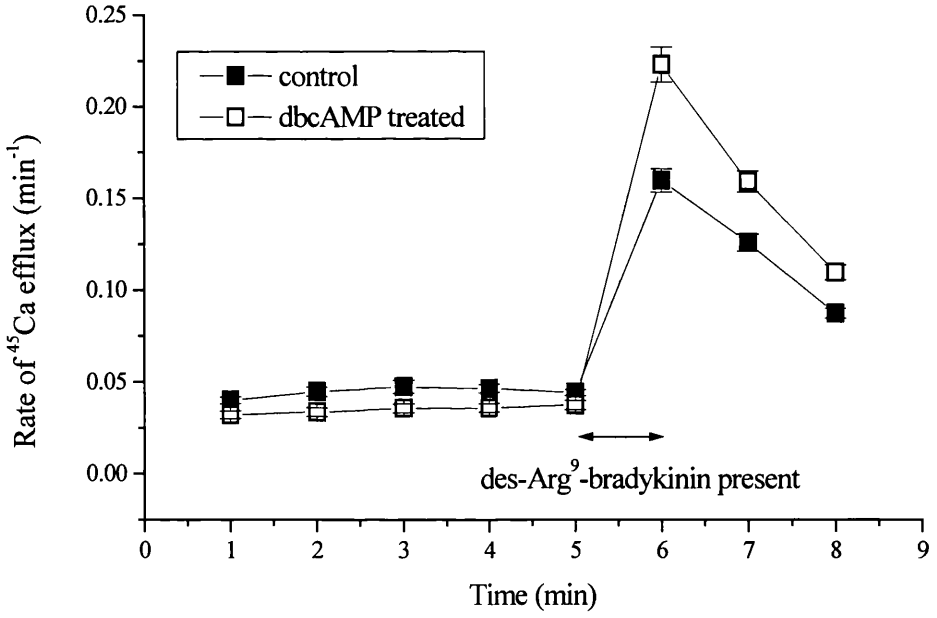

Figure 3-33 Effect of dbcAMP on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The results represent the rate of ^{45}Ca efflux before and during application of 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, in bladder smooth muscle cells following treatment with 1mM dbcAMP for 4h. The arrow ↔ indicates the period (1min) when des-Arg^9-bradykinin was applied to the cells. The data are the means and s.e.m.s of 24 experiments.

The potentiating effect of dbcAMP on the des-Arg^9-bradykinin-evoked response was significant ($p < 0.05$) after 4h of treatment, peaked around 8h and was still significantly ($p < 0.05$) above the control level at 24h (Figure 3-34).

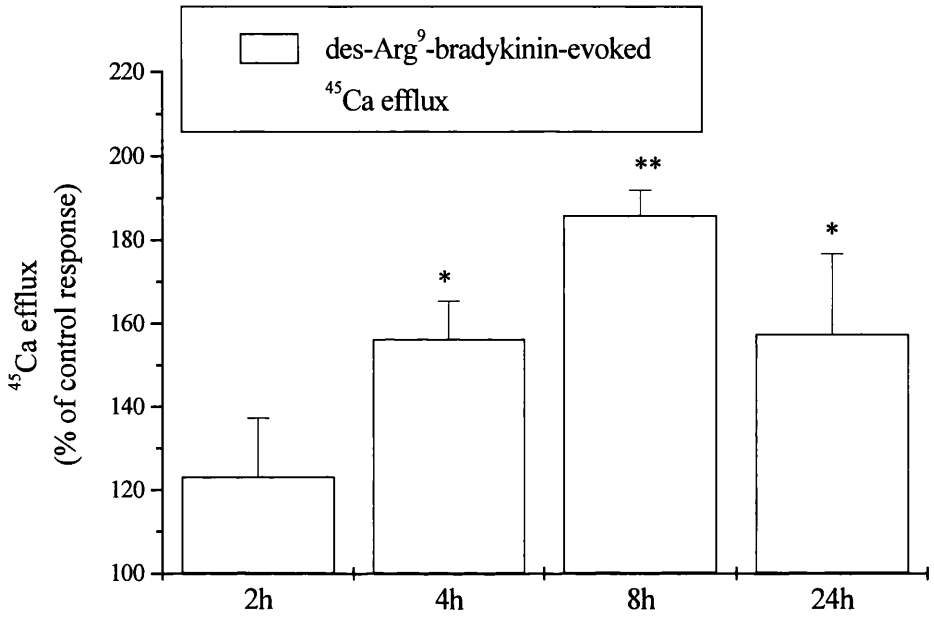

Figure 3-34 Time-course of the effect of dbcAMP on the response to des-Arg^9-bradykinin. The results represent the increase in the rate of ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, in bladder smooth muscle cells following treatment with 1mM dbcAMP for the times shown, as a percentage of the response in control cells. The data are the means and s.e.m.s of 3 experiments. * $p < 0.05$, ** $p < 0.01$ compared to control, ANOVA followed by Dunnett's test on raw data.

The effect of dbcAMP treatment on the concentration-response relationship for des-Arg^9-bradykinin was investigated using a 4h treatment with dbcAMP, a time which consistently gave upregulation. Following treatment with dbcAMP for 4h the maximum response evoked by des-Arg^9-bradykinin was increased to 163 ± 16% of the maximum response in untreated cells but there was no effect on the EC_{50} value (48 ± 9nM in control cells, 41 ± 12nM in treated cells) (Figure 3-35).

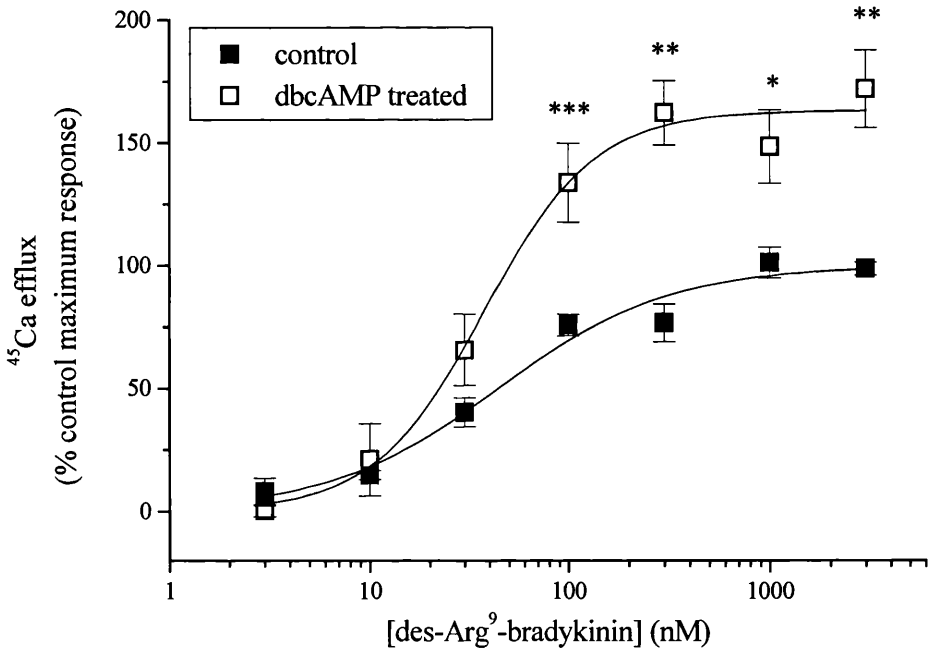

Figure 3-35 Effect of dbcAMP treatment on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The data represent the increase in the rate of ^{45}Ca efflux evoked by a range of concentrations of des-Arg^9-bradykinin, in the presence of 30nM HOE140, following treatment for 4h with 1mM dbcAMP. The data are expressed as a percentage of the maximum response in untreated cells and are the means and s.e.m.s of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control, paired Student's t-test on raw data. Slope factors (p): 1.0 (control), 1.6 (dbcAMP treated). EC_{50} values obtained from mean graph: 44nM (control), 38nM (dbcAMP).

The possibility that the increase in responsiveness to des-Arg^9-bradykinin evoked by dbcAMP might involve new protein synthesis was investigated using the protein synthesis inhibitor cycloheximide. Incubation of bladder smooth muscle cells with cycloheximide (70μM for 4h) prevented the dbcAMP-induced potentiation of the response to des-Arg^9-bradykinin. In this series of experiments cycloheximide had no significant effect ($p > 0.1$) on the basal efflux rate (0.043 ± 0.009 min^{-1} (n=3) in control cells and 0.059 ± 0.005 min^{-1} (n=3) in cycloheximide treated cells). In control cells cycloheximide had no effect on the response to des-Arg^9-bradykinin (Figure 3-36).

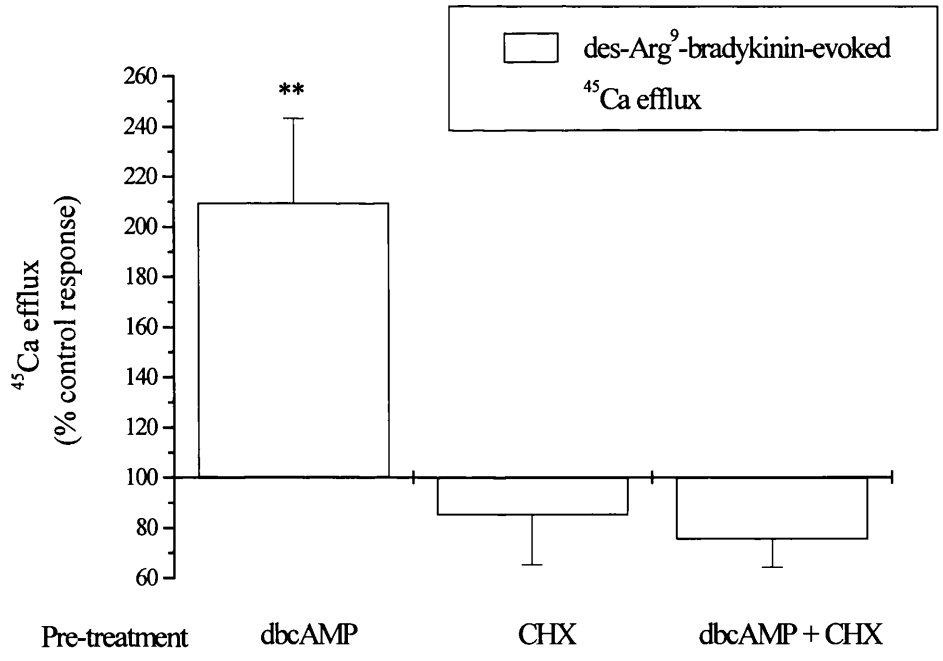

Figure 3-36 Inhibition by cycloheximide of the effect of dbcAMP treatment on the response to des-Arg^9-bradykinin.
The cells were incubated for 4h with 1mM dbcAMP, 70μM cycloheximide (CHX) or a combination of both. The data represent the increase in the rate ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data represent the means and s.e.m.s of 3 experiments. ** $p < 0.01$ compared to control, ANOVA followed by Dunnett's test on raw data.

The effect of dbcAMP was mimicked by forskolin, which activates adenylate cyclase directly (Figure 3-37). In contrast, treatment of bladder smooth muscle

cells with 1mM dibutyryl cyclic GMP (dbcGMP) for 4h had no effect on the response to des-Arg^9-bradykinin. Like dbcAMP, forskolin caused a small, significant ($p < 0.05$, paired Student's t-test) decrease in the basal rate of efflux (0.046 ± 0.004 min^{-1} (n=8) in control cells and 0.027 ± 0.008 min^{-1} (n=5) in forskolin treated cells), however dbcGMP had no effect on the basal efflux (0.051 ± 0.008 min^{-1} (n=4)). These results indicated that the effect of dbcAMP was not a general effect of cyclic nucleotides but was a specific effect of cAMP.

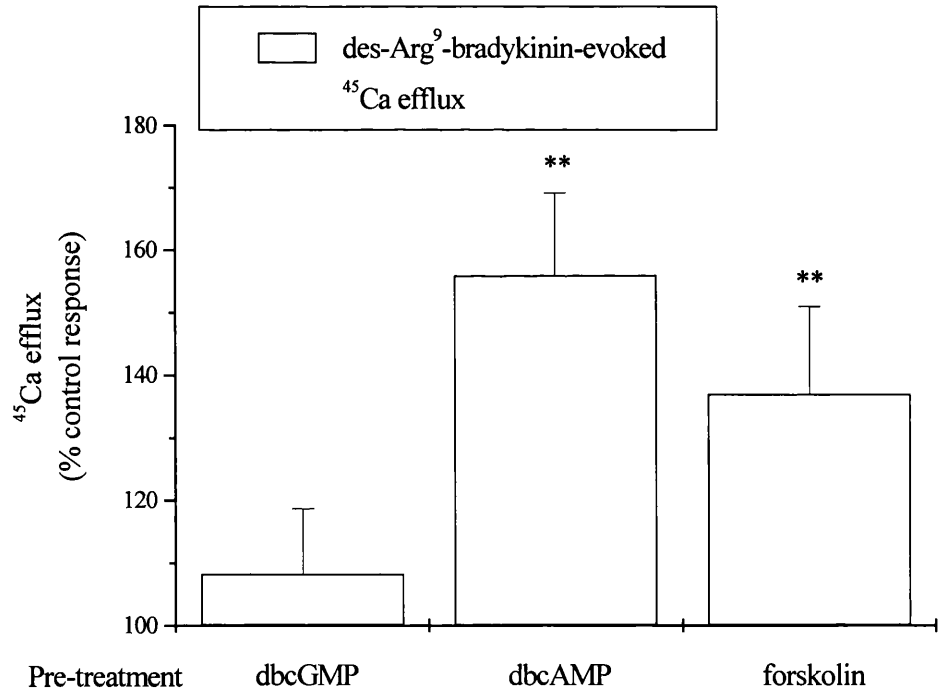

Figure 3-37 Effect of dbcAMP, dbcGMP and forskolin on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were incubated for 4h with 1mM dbcGMP, 1mM dbcAMP or 10μM forskolin. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data represent the means and s.e.m.s of 4-8 experiments. ** $p < 0.01$ compared to control, ANOVA followed by Dunnett's test on raw data.

Dexamethasone inhibited the increase in responsiveness to des-Arg^9-bradykinin evoked by IL-1ß (see Figure 3-28). In order to compare the potentiation induced by these modulators, the effect of dexamethasone on the cAMP-induced increase in des-Arg^9-bradykinin-evoked ^{45}Ca efflux was also investigated. Dexamethasone inhibited the effect of dbcAMP (Figure 3-38) but had no significant effect on the response to des-Arg^9-bradykinin in naïve cells and nor did it affect the basal efflux rate (0.045 ± 0.005 min^{-1} (n=6) in control cells and 0.053 ± 0.008 min^{-1} (n=3) in treated cells). This pattern was similar to the effect of dexamethasone on the IL-1ß-induced upregulation of the response to des-Arg^9-bradykinin (Figure 3-28).

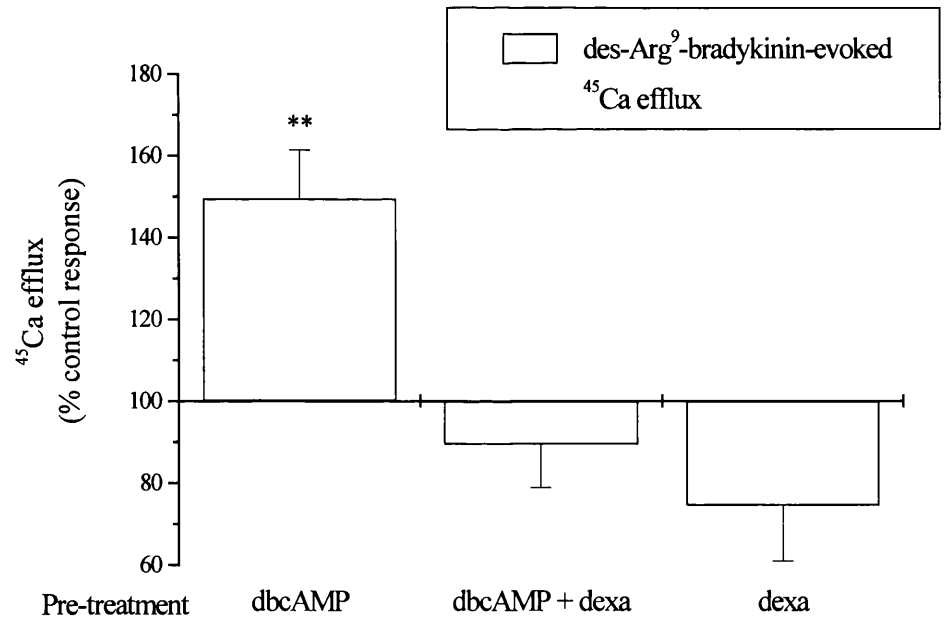

Figure 3-38 Inhibition by dexamethasone of the effect of dbcAMP on the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were incubated with 1mM dbcAMP, 1µM dexamethasone (dexa) or a combination of both for 4h. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells and are the means and s.e.m.s of 3-6 experiments. ** $p < 0.01$ compared to control, ANOVA followed by Dunnett's test on raw data.

The most likely mechanism for the action of dbcAMP would involve activation of PKA. In order to investigate this, the ability of staurosporine (a broad spectrum kinase inhibitor) to inhibit the effect of dbcAMP was investigated. In this series of experiments staurosporine significantly ($p < 0.01$, paired Student's t-test) reduced the basal efflux rate from 0.059 ± 0.001 min^{-1} (n=3) in control cells to $0.028 \pm 0.002 min^{-1}$ (n=3) in staurosporine treated cells. Staurosporine had no effect on des-Arg^9-bradykinin-evoked ^{45}Ca efflux in naïve cells but clearly blocked the ability of dbcAMP to potentiate the response to des-Arg^9-bradykinin (Figure 3-39). This contrasts with its lack of effect on the IL-1ß-mediated potentiation of the response to des-Arg^9-bradykinin (Figure 3-31).

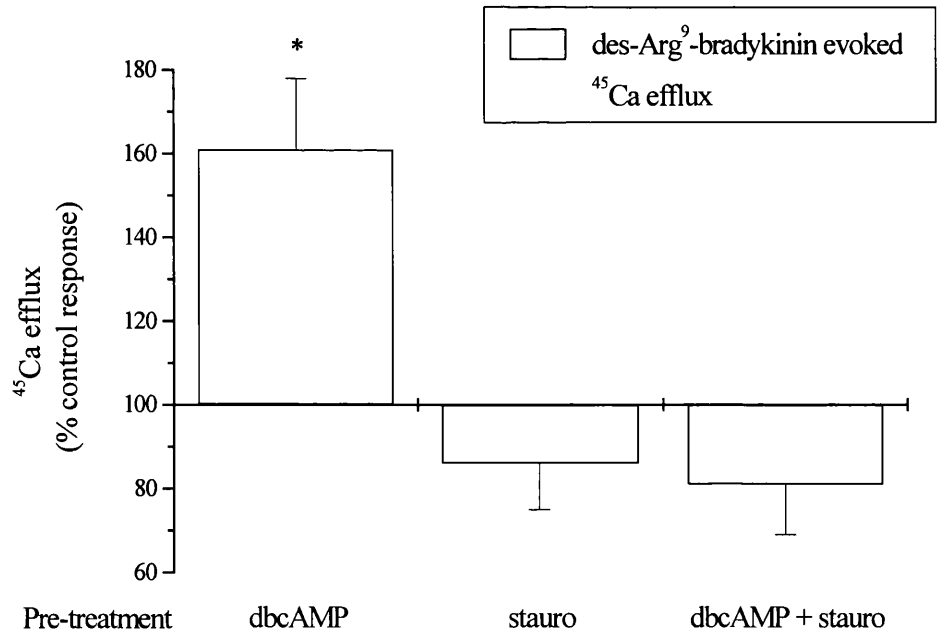

Figure 3-39 Treatment with staurosporine inhibited the ability of dbcAMP to increase the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were pre-treated for 30min with staurosporine (100nM) before, and during, incubation with 1mM dbcAMP for 4h. The data represent the increase in the rate of ^{45}Ca efflux evoked by 1µM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data are the means and s.e.m.s of 3 experiments. * $p < 0.05$ compared to control, ANOVA followed by Dunnett's test on raw data.

As many aspects of the effects of dbcAMP and IL-1ß on the response to des-Arg^9-bradykinin appeared to be similar, the possibility that the effect of dbcAMP was mediated indirectly by inducing the production of IL-1ß was investigated by determining whether the effect of dbcAMP could be inhibited by IL-1ra. IL-1ra did not affect the ability of dbcAMP to increase the response to des-Arg^9-bradykinin, indicating that dbcAMP was not acting via IL-1ß (Figure 3-40). In this series of experiments IL-1ra did not alter the response to des-Arg^9-bradykinin in naïve cells nor did it have any effect on the basal efflux rate (0.048 ± 0.006 min^{-1} (n=4) in control cells and 0.053 ± 0.008 min^{-1} (n=4) in IL-1ra treated cells).

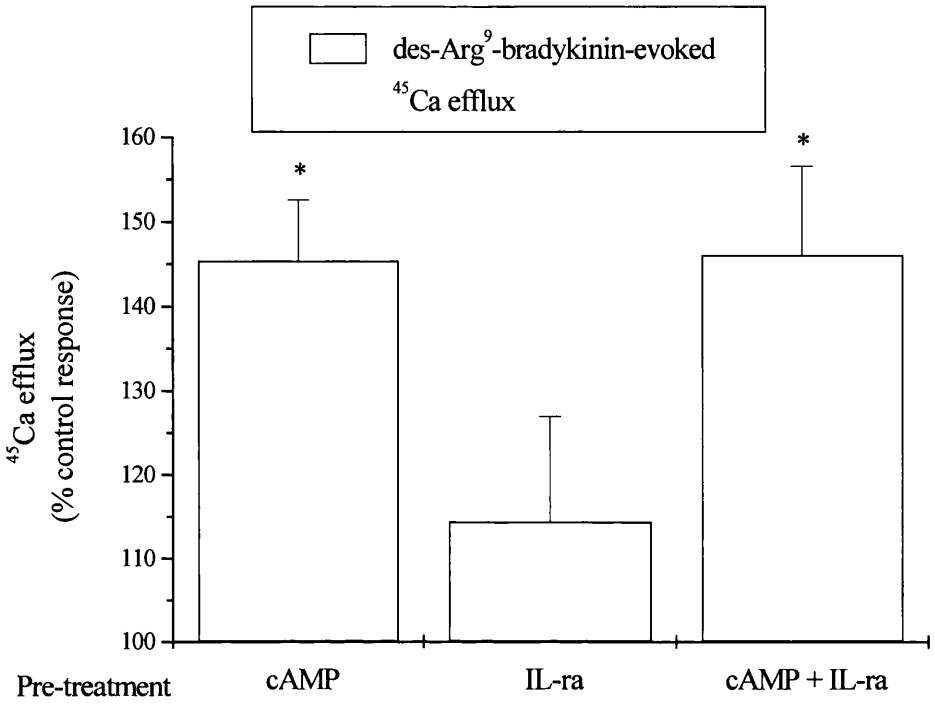

Figure 3-40 Treatment with IL-1ra did not affect the ability of dbcAMP to increase the response to des-Arg^9-bradykinin in bladder smooth muscle cells.
The cells were pre-treated for 30min with IL-1ra (50ng/ml) before, and during, incubation with 1mM dbcAMP for 4h. The data represent the increase in ^{45}Ca efflux evoked by 1μM des-Arg^9-bradykinin, in the presence of 30nM HOE140, as a percentage of the response in control cells. The data are the means and s.e.m.s of 5 experiments. * $p < 0.05$ compared to control, ANOVA followed by Dunnett's test on raw data.

3.4.4 EFFECT OF dbcAMP ON B_2 MEDIATED RESPONSES

It has previously been reported that a 24h treatment with dbcAMP (1mM) or forskolin (10μM) increased the expression the B_2 receptor in tracheal smooth muscle cells (Yang et al., 1994). However, in bladder smooth muscle cells, treatment with dbcAMP for 24h did not increase the response to 100nM bradykinin (Figure 3-41). Shorter incubations, that were effective in increasing B_1 mediated responses in bladder smooth muscle cells, also had no effect on the response to a range of concentrations of bradykinin.

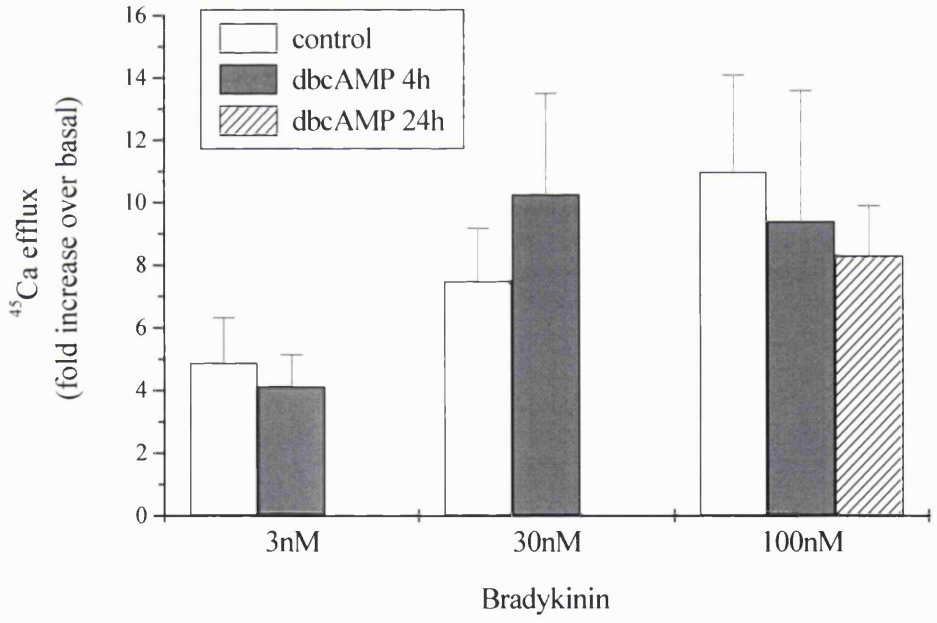

Figure 3-41 DbcAMP did not affect the response to the B_2 receptor agonist bradykinin in bladder smooth muscle cells.
The cells were treated with 1mM dbcAMP for 4h or 24h. The data represent the increase in ^{45}Ca efflux, expressed as fold increase over basal, evoked by the concentrations shown of bradykinin. The data are the means and s.e.m.s of 3-7 experiments.

3.5 REGULATION OF THE B_1 RECEPTOR – BINDING STUDIES

Having demonstrated that both IL-1ß and dbcAMP were able to potentiate the functional response evoked by des-Arg^9-bradykinin, receptor binding studies were performed to determine if these effects were mediated at the level of the receptor itself, or were distal to the B_1 receptor. The level of [^{3}H]-des-Arg^{10}-kallidin specific binding was investigated in bladder smooth muscle cells treated with IL-1ß or dbcAMP for a range of times. Treatment of bladder smooth muscle with IL-1ß (100IU/ml) resulted in an increase in the specific binding of 1.5nM [^{3}H]-des-Arg^{10}-kallidin (a saturating concentration). The increase was significant ($p < 0.05$) following 2h of treatment, peaked at 7h, but after 24h the effect was reduced and was not significantly ($p > 0.05$) above the control (Figure 3-42).

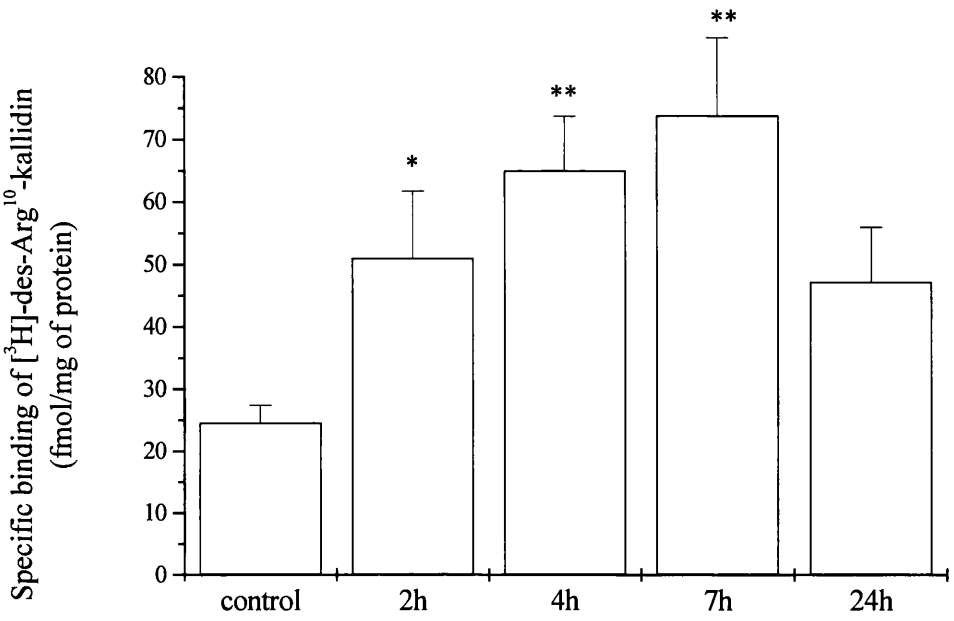

Figure 3-42 Effect of IL-1ß treatment on binding of [^{3}H]-des-Arg^{10}-kallidin in bladder smooth muscle cells.

The cells were incubated with 100IU/ml IL-1ß for the times shown. The results represent the specific binding (non-specific binding was defined in the presence of 3μM des-Arg^{10}-kallidin) and are the means and s.e.m.s of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ ANOVA followed by Dunnett's test.

The binding of 1.5nM [^{3}H]-des-Arg^{10}-kallidin was investigated in bladder smooth muscle cells treated with 1mM dbcAMP for a range of times. In contrast to IL-1ß there was no significant effect ($p > 0.05$, Dunnett's test) at any of the time-points studied (Figure 3-43). As the concentration of [^{3}H]-des-Arg^{10}-kallidin used in these experiments (1.5nM) was a saturating concentration (see Figure 3-20), the possibility that dbcAMP treatment would have affected the K_D cannot be ruled out. However, as there was no effect of dbcAMP on the EC_{50} value in the functional assay, in contrast to the clear increase of the maximum response, this possibility seems unlikely.

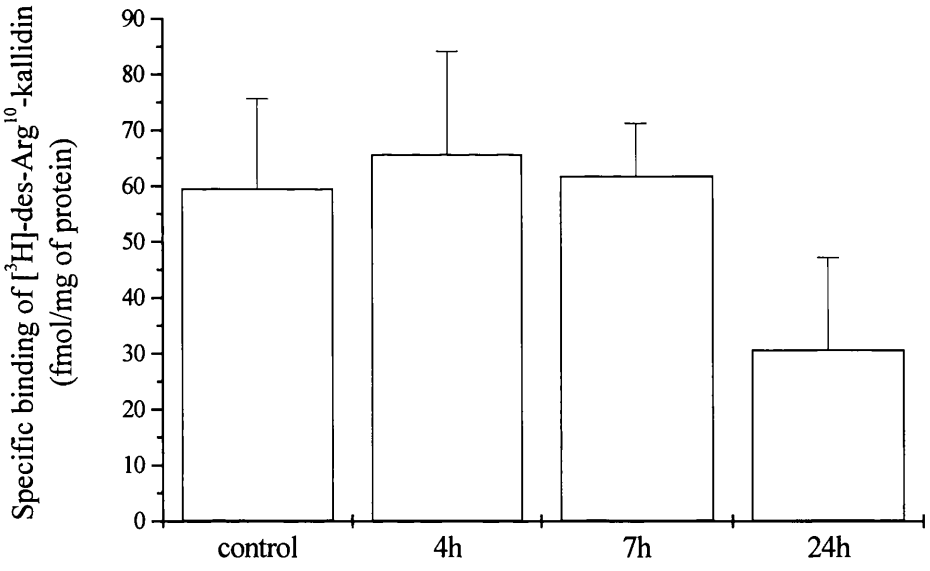

Figure 3-43 DbcAMP did not affect binding of [^{3}H]-des-Arg^{10}-kallidin in bladder smooth muscle cells.
The cells were incubated with 1mM dbcAMP for the times shown. The results represent the specific binding (non-specific binding was defined in the presence of 3µM des-Arg^{10}-kallidin) and are the means and s.e.m.s of 4 independent experiments.

3.6 REGULATION OF THE B_1 RECEPTOR – RT-PCR STUDIES

Having shown that IL-1ß, but not dbcAMP, increased the level of [^{3}H]-des-Arg^{10}-kallidin binding, RT-PCR was used to investigate if this pattern was reflected in the levels of B_1 receptor mRNA. Bladder smooth muscle cells were treated for 4h with 100IU/ml IL-1ß or 1mM dbcAMP, conditions that consistently gave upregulation of des-Arg^9-bradykinin-evoked ^{45}Ca efflux. Total RNA was prepared from the cells and a cDNA copy made using reverse transcriptase. The DNA was then subjected to PCR using B_1 specific primers, and primers for cyclophilin as a control. Samples were taken at the cycle numbers shown and the amount of DNA visualised on an agarose gel, Figure 3-44 shows a typical gel. At lower cycle numbers (up to 30), there was a clear increase in the amount of B_1 receptor PCR product in samples from IL-1ß treated cells, whereas there was no difference between the dbcAMP treated samples and control. As expected once the PCR reaction had reached saturation (about 35 cycles) there was no difference between the amount of PCR product in any of the samples. There was no effect of either IL-1ß or dbcAMP on the amount of cyclophilin PCR product at any of the cycle numbers investigated.

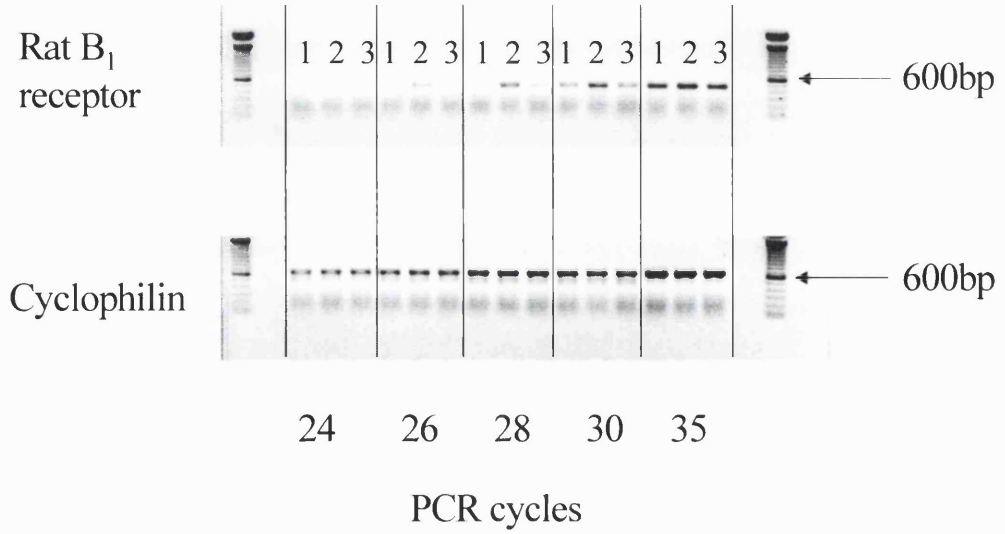

Figure 3-44 Effect of IL-1ß and dbcAMP on the level of B_1 receptor mRNA in bladder smooth muscle cells.
Total RNA was prepared from (1) untreated bladder smooth muscle cells, or cells treated with (2) 100IU/ml IL-1ß or (3) 1mM dbcAMP for 4h. A cDNA copy was synthesised using reverse transcriptase and then subjected to the number of cycles of PCR indicated using rat B_1 receptor and cyclophilin specific primers. The PCR products were visualised on a 1% agarose gel.

In order to quantify the amount of DNA in each sample, each band was measured by densitometry, and calculated as percentage of the cyclophilin band from the same treatment and cycle number (Figure 3-45).

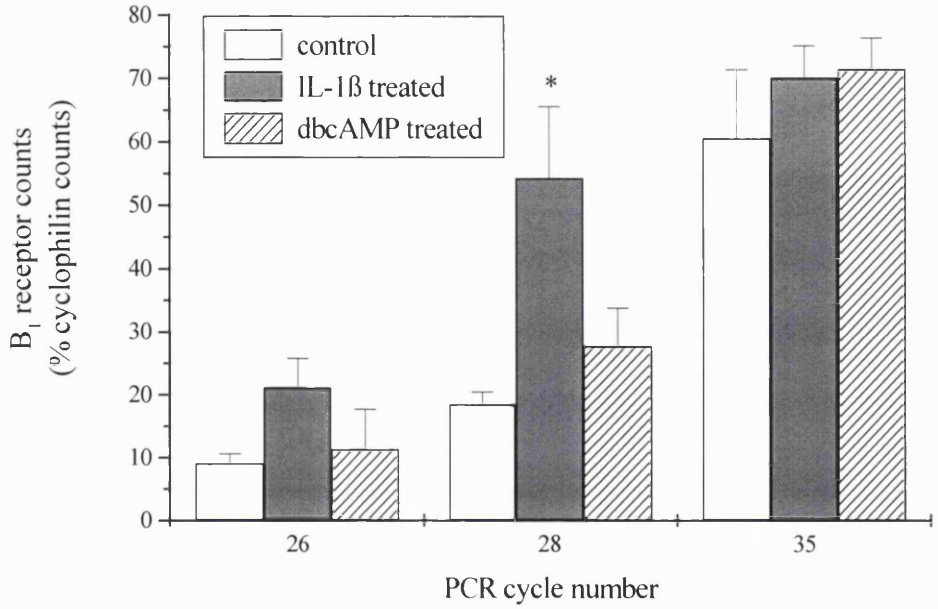

Figure 3-45 Effect of IL-1ß and dbcAMP on the level of B_1 receptor mRNA in bladder smooth muscle cells.
Total RNA was prepared from bladder smooth muscle cells treated with 100IU/ml IL-1ß or 1mM dbcAMP for 4h. A cDNA copy was synthesised using reverse transcriptase and then subjected to the number of cycles of PCR indicated using rat B_1 receptor and cyclophilin specific primers. The amount of DNA in the B_1 bands was calculated as a percentage of the amount in the cyclophilin bands, the data represent the means and s.e.m.s of 3 independent experiments. * $p < 0.05$ compared to control, ANOVA followed by Dunnett's test.

The results presented here demonstrate that both IL-1ß and dbcAMP induce increased responsiveness to the B_1 agonist des-Arg^9-bradykinin in bladder smooth muscle cells. For IL-1ß this effect appears to be the result of a direct effect on the level of B_1 receptor. However, the effect of dbcAMP, although dependent on new protein synthesis, appears to be downstream of the receptor. In contrast to the B_1 receptor, the B_2 receptor in bladder smooth muscle cells does not appear to be regulated by either IL-1ß or dbcAMP.

4 DISCUSSION

In this project kinin receptors and their regulation has been studied for the first time in rat bladder smooth muscle cells. The rat bladder was chosen as it has previously been reported that, following treatment with LPS, it expresses the B_1 receptor at very high levels compared to other tissues (Jones et al., 1999). In addition, studies of inflammatory conditions of the bladder, such as IC, have demonstrated a potential role for kinins. The cellular model developed in this study has allowed the receptors expressed on smooth muscle cells to be characterised in isolation from other cell types. In contrast to other cellular models used to study the B_1 receptor, such as rabbit aorta smooth muscle cells, bladder smooth muscle cells express both B_1 and B_2 receptors, and it appears that a single cell can express both kinin receptor subtypes. This has allowed a detailed comparison of the pharmacology and regulation of kinin receptors in the same cell type.

The results described clearly show that smooth muscle cells of bladder origin express both B_1 and B_2 kinin receptors and is, in fact, one of the few demonstrations of active B_1 receptors on primary cultures of cells from rat tissue. In addition to the pharmacological characterisation of the kinin receptors, the use of isolated cells has enabled the regulation of the receptors by a range of inflammatory mediators to be investigated. This will aid understanding of the behaviour of kinin receptors on bladder smooth muscle *in vivo* during inflammatory conditions of the bladder.

4.1 DEVELOPMENT OF THE CELLULAR MODEL

Methods for isolating smooth muscle cells usually involve culturing cells from explants of tissue or enzymatic digestion of the tissue. The approach taken in this study was to use enzymatic digestion, based on a method described by Levesque et al. (1993) for isolating rabbit aorta smooth muscle cells. The isolated cells showed the characteristic phenotype of smooth muscle cells in culture, changing from long, thin spindle shaped cells in the first couple of days following isolation, to a flatter, more spread out shape after 3-4 days in culture, a process described as dedifferentiation by Chamley-Campbell et al. (1979). The cells multiplied in culture and showed the "hill and valley" pattern typical of cultured smooth muscle cells. To confirm that the cells were of smooth muscle origin an antibody to the smooth muscle form of actin was used. Actin is a very widely expressed protein and it has six different isoforms, of which two are expressed in practically all cells. The other four forms are only expressed in muscle and are differentiation markers for muscle tissue. The α form is specific for smooth muscle and antibodies to this form of actin have been used widely to confirm the identity of primary cultures of smooth muscle cells (Baskin et al., 1993, Levesque et al., 1993). In this study approximately 90% of the cells cultured from the rat bladder stained positively with the anti-α smooth muscle actin, confirming that the majority of the cells were of smooth muscle origin. The identity of the remaining non-muscle cells was not determined, although it is likely that fibroblasts would be present. Antibodies to fibronectin can be used to determine the presence of fibroblasts, however, as bladder smooth muscle cells have been shown to produce fibronectin (Baskin et al., 1993) this was not considered to be a sufficiently selective marker for bladder fibroblasts. In fact,

in one study where fibroblasts from the bladder were isolated the lack of staining with anti-α smooth muscle actin was used as an indication of the presence of fibroblasts (Coplen et al., 1994). The potential significance of the 10% of cells that did not stain for α smooth muscle actin is not known. However, fibroblasts do express kinin receptors (Phagoo et al., 1996) and could therefore, if present, make a small contribution to the agonist-evoked responses. It is also possible that the unidentified cells may release mediators that affect the expression of kinin receptors. For example, IL-1ß has been reported to induce prostaglandin release from fibroblasts (Lerner and Modeer, 1991), however, because of the percentage of other cell types is low, any effects would be relatively small.

4.2 PHARMACOLOGY

As rat bladder smooth muscle cells have not previously been used for the study of kinin receptors it was necessary to define thoroughly which receptors were expressed and whether their pharmacology was consistent with that reported for kinin receptors in other rat systems. Although both B_1 and B_2 receptors are thought to usually couple to PIC, it was not known if this was the case in bladder smooth muscle cells. Following activation of PIC there is an increase in $[Ca^{2+}]_i$, this leads to an increase in the rate of efflux of calcium from the cell. If the cells have been previously equilibrated with ^{45}Ca, then this increase in the rate of efflux can be detected. Both B_1 and B_2 agonists evoked an increase in the rate of ^{45}Ca efflux, indicating that they were both coupled to PIC. This assay was then used for a detailed pharmacological characterisation of the responses to kinin agonists. In addition, for B_1 receptors, a binding assay was developed using the B_1-specific ligand [^{3}H]-des-Arg^{10}-kallidin to detect the presence of B_1 receptor protein.

4.2.1 CALCIUM EFFLUX STUDIES

Both B_1 agonists such as des-Arg^9-bradykinin and des-Arg^{10}-kallidin and B_2 agonists such as bradykinin and kallidin evoked ^{45}Ca efflux in bladder smooth muscle cells. The EC_{50} values for bradykinin (2.9nM) and kallidin (3.2nM) - evoked ^{45}Ca efflux were very similar and comparable with values reported for bradykinin-evoked responses in other primary cultures of rat cells. For example, the EC_{50} value for bradykinin-induced $Ins(1,4,5)P_3$ formation in arterial smooth muscle cells was 5nM (Dixon et al., 1994), and in cultured dorsal root ganglion cells it was 7.6nM (Harvey and Burgess, 1996). There is currently no comparable data available for B_1 agonists in cultured rat smooth muscle cells,

however, the EC_{50} value for the des-Arg^9-bradykinin-evoked increase in $[Ca^{2+}]_i$ in rat mesangial cells was 560nM (Bascands et al., 1993), which is somewhat higher than the EC_{50} value for des-Arg^9-bradykinin (37nM) in rat bladder smooth muscle cells. This value is, however, within the same range as the values reported for des-Arg^9-bradykinin-evoked contractions in rat smooth muscle preparations. For example, the pD_2 value in the ileum was 8.3 (Meini et al., 1996), in portal vein rings the EC_{50} value was 46nM (Campos and Calixto, 1994) and in strips of bladder smooth muscle the EC_{50} value was 58nM (Meini et al., 1998).

As the rat kinin receptors would not normally be exposed to kallidin and des-Arg^{10}-kallidin it might be thought that these peptides would be less potent than bradykinin and des-Arg^9-bradykinin. However, the potencies of bradykinin and kallidin were very similar, and the potencies of the B_1 agonists, des-Arg^9-bradykinin and des-Arg^{10}-kallidin, were very close to each other. This is consistent with other studies where the activity of kallidin and bradykinin have been compared and found to be very similar, for example in rat vascular smooth muscle cells (Yang et al., 1999), and also in rat superior cervical ganglia (Seabrook et al., 1995).

The data with the agonists indicated that it was likely that the cells expressed both B_1 and B_2 receptors, however, further studies with a range of B_1 and B_2 selective antagonists were used to confirm this. The compounds selected for this purpose were: HOE140, a peptide B_2 antagonist; FR173657, a non-peptide B_2 antagonist; des-Arg^{10}-HOE140, [Leu^8]-des-Arg^9-bradykinin and B9858, all peptide B_1 antagonists. In these experiments bradykinin and des-Arg^9-

bradykinin were used at concentrations close to their EC_{50} values. The rank order of potencies for the antagonists against bradykinin was typical of B_2 receptor pharmacology: HOE140 > FR173657 > B9858 = des-Arg^{10}-HOE140 >> [Leu^8]-des-Arg^9-bradykinin. Against des-Arg^9-bradykinin the rank order of potency was B9858 = [Leu^8]-des-Arg^9-bradykinin > des-Arg^{10}-HOE140 > HOE140 > FR173657, consistent with known B_1 receptor pharmacology. These data are in agreement with the hypothesis that rat bladder smooth muscle cells express both B_1 and B_2 kinin receptors.

HOE140 has been reported to be a competitive antagonist in some studies and a non-competitive antagonist in other studies, depending on the species and tissue (Marceau et al., 1994, Félétou et al., 1995). In the presence of HOE140 the concentration-response curve for bradykinin in bladder smooth muscle cells was shifted to the right with a reduction in the maximum response, suggesting that in these cells there was a non-competitive component to its activity. This contrasted with data obtained in the human neuroblastoma cell line SK-N-SH, in which, in our hands and using the same assay conditions as bladder smooth muscle cells, HOE140 showed clear competitive behaviour (data not shown).

Although bradykinin is considered to be a B_2 agonist and des-Arg^9-bradykinin a B_1 agonist, neither is completely selective. In initial experiments to determine the concentration-response relationship for des-Arg^9-bradykinin it was not possible to determine an EC_{50} value because a maximum response could not be reached. By repeating the experiment in the presence of HOE140, a maximal response was reached and the EC_{50} value could be determined. This suggested that, at high concentrations, des-Arg^9-bradykinin was able to activate B_2

receptors. This was confirmed in experiments in which the ability of des-Arg^9-bradykinin to evoke ^{45}Ca efflux in the presence of [Leu^8]-des-Arg^9-bradykinin was investigated. At concentrations below 1μM, des-Arg^9-bradykinin-evoked ^{45}Ca efflux was completely blocked by 100nM [Leu^8]-des-Arg^9-bradykinin and HOE140 had no effect on the response. However, above 1μM, des-Arg^9-bradykinin was able to evoke ^{45}Ca efflux in the presence of 100nM [Leu^8]-des-Arg^9-bradykinin and the additional response was completely blocked by HOE140 confirming that it was mediated by B_2 receptors. In contrast, des-Arg^{10}-kallidin behaved differently, showing very little activity at the B_2 receptor. Although this was the only example of a difference in the pharmacology of bradykinin-derived peptides and kallidin-derived peptides in bladder smooth muscle cells, it could reflect the fact that kallidin is not formed in the rat. It might be expected that des-Arg^{10}-kallidin would show more activity than des-Arg^9-bradykinin at the human B_2 receptor, however, in experiments using CHO cells transfected with the human B_2 receptor both des-Arg^{10}-kallidin and des-Arg^9-bradykinin were completely inactive (data not shown).

In a parallel series of experiments the ability of bradykinin to activate B_1 receptors was investigated. In order to do this it was necessary to try to block the activity of bradykinin at the B_2 receptor using HOE140. However, even at a high concentrations of HOE140 (1μM), there still appeared to be a B_2-mediated component to the response. Although the ^{45}Ca efflux evoked by bradykinin (3μM or 30μM) could be partially inhibited by100nM [Leu^8]-des-Arg^9-bradykinim, a concentration that completely inhibited the response to des-Arg^{10}-kallidin (10μM), a component of the response was still unaffected. This suggests that, although high concentrations of bradykinin were able to activate

B_1 receptors, it was not possible to block the activity of bradykinin at the B_2 receptor completely with HOE140, making it impossible to determine an EC_{50} value for bradykinin at the B_1 receptor.

Both T-kinin, which is unique to the rat, and its putative breakdown product, des-Arg^{11}-T-kinin, evoked ^{45}Ca efflux in bladder smooth muscle cells. The potency of T-kinin-evoked ^{45}Ca efflux was similar to that of bradykinin and kallidin and the potency of des-Arg^{11}-T-kinin was close to that of des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin. T-kinin-evoked ^{45}Ca efflux was inhibited by HOE140 and not by [Leu^{8}]-des-Arg^{9}-bradykinin confirming that it was acting at the B_2 receptor. The ^{45}Ca efflux evoked by des-Arg^{11}-T-kinin was inhibited by [Leu^{8}]-des-Arg^{9}-bradykinin and not by HOE140 indicating that it was selective for the B_1 receptor. In contrast to des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin, both of which gave substantially smaller responses than bradykinin and kallidin respectively, the response to des-Arg^{11}-T-kinin was equivalent in magnitude to that of T-kinin. This could suggest that des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin are only partial agonists at the rat B_1 receptor whereas des-Arg^{11}-T-kinin is a full agonist. This possibility could be confirmed by investigating whether des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin can reduce the response of des-Arg^{11}-T-kinin. It would, therefore, be very interesting to determine whether des-Arg^{11}-T-kinin was a partial agonist relative to des-Arg^{10}-kallidin at the human B_1 receptor.

Although des-Arg^{11}-T-kinin is a potent agonist at the rat B_1 receptor, however, it is not known whether it has a role *in vivo*. Like all kinins, T-kinin is subject to degradation by a variety of enzymes. Studies *in vitro* have demonstrated that it

can be converted to bradykinin by an aminopeptidase (Vieira et al., 1994), is sensitive to carboxypeptidases, but is resistant to ACE (Passaglio and Vieira, 1996). The extent to which T-kinin is metabolised by carboxypeptidase *in vivo* is not known, however, it is likely that some des-Arg^{11}-T-kinin would be formed. T-kinin is formed from the inactive precursor T-kininogen by the action of T-kininogenase. The production of T-kininogen is stimulated by inflammatory mediators such as IL-1ß, LPS and cAMP (Takano et al., 1995, Yayama et al., 2000) and it is the major kininogen found in rat plasma in inflammatory states (Barlas et al., 1985a). Given the large amount of T-kininogen present during inflammation, it is therefore possible that des-Arg^{11}-T-kinin would be present in high concentrations, although this has not been investigated.

4.2.2 BINDING STUDIES

The native rat B_1 receptor is poorly characterised and there is only one report of specific binding of a B_1-specific ligand in primary cultures of cells derived from rat tissue. Specific binding of [^{3}H]-des-Arg^9-bradykinin was reported in cultured kidney mesangial cells, however, the binding was very low and has never been repeated (Bascands et al., 1993). No binding of B_1-specific ligands in rat cell lines has been reported and no specific binding of [^{3}H]-des-Arg^{10}-kallidin was found in a range of rat cell lines of smooth muscle and fibroblast origin (data not shown). Initial attempts to show binding of [^{3}H]-des-Arg^{10}-kallidin in rat bladder smooth muscle cells were unsuccessful. However, following the cloning of the rat B_1 receptor, from the bladder, a binding assay was developed using membranes prepared from Cos-7 cells transfected with the rat B_1 receptor cDNA, see below and Jones et al. (1999). With a few alterations

(removing EDTA from the binding buffer and adding sucrose) it was possible to use this assay to measure B_1 receptors in bladder smooth muscle cells.

4.2.2.1 CLONED RECEPTOR

Membranes prepared from Cos-7 cells transfected with the rat B_1 cDNA showed saturable binding of the B_1-specific ligand [^{3}H]-des-Arg^{10}-kallidin with a B_{max} of 276fmol/mg of protein and a K_D value of 1nM. The B_{max} was approximately 10 fold higher than that obtained in bladder smooth muscle cells (37fmol/mg). This is typical of transiently transfected cells where expression of the receptor is under control of a strong promoter, in this case the cytomegalovirus promoter. The K_D value (1nM) in the Cos-7 membranes was similar to that reported by Ni et al. (1998a) in membranes prepared from HEK293 cells transfected with the rat B_1 receptor cDNA using an alternative B_1 ligand [^{125}I]-Sar-Tyr-εAhx-Lys-des-Arg^9-bradykinin (1.9nM). A series of displacement experiments with a range of compounds were carried out to investigate the pharmacology of the cloned B_1 receptor from rat and as expected all the B_1 compounds were more potent than the B_2 compounds. Des-Arg^{10}-kallidin was the most potent B_1 compound, which is surprising as the rat B_1 receptor would not be expected to come into contact with kallidin-derived peptides.

The activities of T-kinin and des-Arg^{11}-T-kinin at the rat B_1 receptor were compared to their activities at the human B_1 receptor using membranes prepared from Cos-7 cells transfected with the human B_1 receptor cDNA. Des-Arg^{11}-T-kinin was 30 times more potent at the rat B_1 receptor than the human. In contrast, des-Arg^{10}-kallidin, which would not be formed in the rat, was approximately 10 fold more potent at the human B_1 receptor compared to the rat

(Jones et al., 1999). As expected for a B_2 agonist T-kinin had very little activity at either the human and rat B_1 receptors. The rat selectivity of T-kinin was investigated in a functional assay. Using cells transfected with the calcium-sensitive protein aequorin and the rat or human B_2 receptors it was possible to demonstrate that T-kinin was 20 fold more potent at the rat receptor than at the human.

4.2.2.2 B_1 BINDING IN BLADDER SMOOTH MUSCLE CELLS

In rat bladder smooth muscle cells [^{3}H]-des-Arg^{10}-kallidin showed saturable binding and appeared to bind to a single site with a K_D of 0.1nM and a B_{max} of 37fmol/mg of protein. The affinity of [^{3}H]-des-Arg^{10}-kallidin for the native B_1 receptor in bladder smooth muscle cells was, therefore, approximately 10 fold higher than its affinity for the cloned rat B_1 receptor (1nM) and the possible reasons for this are discussed below. The pharmacology of the native B_1 receptor was investigated further by examining the ability of a range of compounds to displace the binding of [^{3}H]-des-Arg^{10}-kallidin from bladder smooth muscle cells. Although all the B_2 compounds were capable of displacing the binding of [^{3}H]-des-Arg^{10}-kallidin, the B_1 compounds were significantly ($p < 0.05$, unpaired Student's t-test) more potent than the B_2 ligands. There was a greater spread in the potencies of the B_1 agonists in the binding assay compared to the ^{45}Ca efflux assay. For example the K_I value for des-Arg^9-bradykinin (1.4nM) was significantly greater ($p < 0.05$, unpaired Student's t-test) than the K_I values for des-Arg^{10}-kallidin (0.4nM) and des-Arg^{11}-T-kinin (0.2nM) whereas their EC_{50} values in the ^{45}Ca efflux assay were all very similar.

All the kinin compounds tested in the binding assay were apparently more potent in the bladder smooth muscle cells than in membranes prepared from Cos-7 cells expressing the rat B_1 receptor, although the rank order of potency was similar. The reasons for this difference in K_I values (and for the difference in the K_D value for des-Arg^{10}-kallidin) are unclear but it may be related to the maturation of the receptor protein. It has been reported for the V2 vasopressin receptor that full glycosylation does not occur in transiently transfected cells (Innamorati et al., 1996). The effect of glycosylation on a receptor depends on the receptor type. For example, the affinity of gastrin-releasing peptide for its receptor is reduced if the receptor is deglycosylated, however, deglycosylation of the closely related neuromedin B receptor does not affect the affinity of neuromedin B binding (Kusui et al., 1994). Both the B_1 and B_2 receptors have three sites for glycosylation and it has been shown that the affinity of the B_2 receptor for bradykinin is unaffected by deglycosylation (Yaqoob et al., 1995). Although inhibitors of glycosylation, such as tunicamycin, inhibit full expression of the B_1 receptor (Sardi et al., 1999) the effect of deglycosylation on the affinity of B_1 ligands for the receptor has not been investigated. Another possible factor that may affect the affinities of the compounds for the receptor is the coupling to the G-protein. As Cos-7 cells are derived from monkey, it may be that the coupling between the rat receptor and G-protein is not optimal in these cells. It is possible that transfecting the rat receptor into a rat cell line would overcome both the problems with the glycosylation and the G-protein coupling and lead to affinities closer to the native receptor. Differences between native and cloned B_1 receptors have not been reported for other species, for example the K_I values for the cloned receptor from rabbit, expressed in a cell

line from a different species, Cos-7, agree well with the K_I values in rabbit aorta smooth muscle cells (MacNeil et al., 1995, Schneck et al., 1994). Also, the K_I values for the human receptor expressed in a same species cell line, HEK293, compare well with the values at the native receptor in IMR-90 cells (Bastian et al., 1997).

All the compounds tested were more potent in the binding assay than in the ^{45}Ca efflux assay. There are several potential explanations for this difference, however, the most likely is the composition of the buffer. The binding assay was carried out in a low salt TES-based buffer, but for the ^{45}Ca efflux assay it was necessary to use a physiological buffer with a much higher salt concentration. It has long been known that sodium ions reduce the affinity of a range of ligands for G-protein coupled receptors. For example, the affinity of bradykinin for the rat B_2 receptor was reduced in the presence of physiological concentrations of sodium (Snell et al., 1990) and in a study with the human B_2 receptor the affinities of a range of kinins were reduced in a physiological buffer compared to a TES-based binding buffer (Paquet et al., 1999). In the α_2-adrenergic receptor it was found that mutation of a single aspartate to asparagine abolished the allosteric regulation of ligand binding by sodium ions. The residue was found to be conserved in all G-protein coupled receptor sequences investigated suggesting that this is a universal mechanism of regulation (Horstman et al., 1990).

The effect of sodium on the B_1 receptor has not been widely investigated, however, in a study in rabbit aorta smooth muscle cells a good correlation between binding of [^{3}H]-des-Arg^{10}-kallidin and B_1-induced PGI_2 production was

found, and interestingly a high salt physiological buffer was used in the binding assay. Schneck et al. (1994) compared binding and B_1-induced increases in $Ins(1,4,5)P_3$ production in rabbit aorta smooth muscle cells. They also found a good correlation between the binding and functional data, but in this case they had in fact used a low salt buffer for their binding assay. It is possible that carrying out the binding studies in bladder smooth muscle cells in high salt physiological buffer would have given K_I values closer to the EC_{50} and IC_{50} values obtained in the functional assay. Unfortunately it was not possible to test this hypothesis because, during optimisation of the binding assay for the cloned receptor, it was found that the specific binding in the presence of physiological buffer was too low to provide a useful signal for characterisation. This may be an indication that the affinity of des-Arg^{10}-kallidin for the B_1 receptor is reduced in physiological buffer. In addition, in HEK293 cells transfected with the human B_1 receptor, which have a very high level of expression, the affinity of des-Arg^{10}-kallidin was reduced from approximately 1nM in TES-based binding buffer to 2-10nM in physiological buffer (data not shown).

Another possible explanation for the differences between the binding and functional assays is the actual concentration of the ligand that is seen by the receptor. The binding assay was carried out at 4°C in the presence of a range of protease inhibitors. This may mean that degradation of the ligand was greatly reduced and HPLC analysis of the medium from the binding assay did in fact indicate that there was no apparent degradation of [^{3}H]-des-Arg^{10}-kallidin under the conditions of the binding assay. The ^{45}Ca efflux assay was carried out at 37°C and no inhibitors were present. Although the compounds were present only for a very short time (1-4 min) degradation can occur very quickly. For

example, in the presence of ACE at 37°C bradykinin has a half-life of only 5-10min (Passaglio and Vieira, 1996). It is therefore possible that the final concentration of kinin was reduced in the ^{45}Ca efflux assay. It would be possible to investigate this further be carrying out the functional assay in the presence of the peptidase inhibitors. However, it had already been demonstrated that, in functional assays in Cos-7 cells transfected with rat or human B_1 receptors and aequorin, the potency of the B_1 agonists des-Arg^9-bradykinin and des-Arg^{10}-kallidin was not increased by the presence of the ACE inhibitor enalapril (data not shown).

In conclusion, therefore it appears that the most likely explanation for the difference in activity between the binding and functional assays is the lack of sodium in the binding assay

4.2.3 SUMMARY OF PHARMACOLOGY

In summary, the results of the pharmacological profiling of the kinin receptors in bladder smooth muscle cells was consistent with the cells expressing both B_1 and B_2 receptors and with both receptors being coupled to PIC. This meant that primary cultures of rat bladder smooth muscle cells were a suitable cellular model for studying the regulation of B_1 and B_2 receptors expressed in the same cell, therefore increasing the understanding of their role in inflammatory conditions of the bladder.

4.3 REGULATION OF KININ RECEPTORS

In the Introduction evidence was presented that indicates that kinins appear to be involved in inflammatory conditions of the bladder, with a possible role for both B_1 and B_2 receptors. In most studies of the effects of kinin antagonists in animal models of bladder inflammation B_2 receptor antagonists but not B_1 antagonists have been effective at reducing the symptoms of inflammation (Maggi et al., 1993, Ahluwalia et al., 1994, Ahluwalia et al., 1998). However, in a study of turpentine-induced hyper-reflexia, a B_1 antagonist was ineffective 2h post-turpentine instillation but by 5h it was capable of reducing the hyper-reflexia induced by this inflammatory insult (Jaggar et al., 1998). In addition there is evidence that the B_1 receptor is induced by inflammatory stimuli such as CYP (Belichard et al., 1999) or turpentine (Roslan et al., 1995), but the expression of the B_2 receptor is unaffected (Belichard et al., 1999). In order to determine how the expression of kinin receptors on bladder smooth muscle cells might be altered during inflammation, the effect of a variety of inflammatory mediators on agonist-evoked ^{45}Ca efflux, binding of [^{3}H]-des-Arg^{10}-kallidin and B_1 receptor mRNA was investigated. The mediators chosen were the pro-inflammatory cytokines, IL-1ß and TNFα, the bacterial toxin, LPS and cAMP, which may be increased in bladder smooth muscle cells if prostanoids are elevated.

4.3.1 THE B_1 RECEPTOR

In bladder smooth muscle cells IL-1ß, TNFα and LPS all caused an increase in des-Arg^9-bradykinin-evoked ^{45}Ca efflux. These mediators have all been reported to potentiate B_1-mediated responses in other cell types (Galizzi et al.,

1994, Phagoo et al., 2000). In addition, cAMP, which has not previously been shown to affect B_1-mediated responses, increased des-Arg^9-bradykinin-evoked ^{45}Ca efflux. In order to determine if cAMP had a similar mechanism of action to IL-1ß, or if it represented a novel mechanism of B_1 receptor regulation, the effects of IL-1ß and cAMP were compared and contrasted in an extensive series of experiments.

The time-course of the IL-1ß-induced increase in des-Arg^9-bradykinin-evoked ^{45}Ca efflux in bladder smooth muscle cells was similar to that reported for the IL-1ß-induced increase in [^{3}H]-des-Arg^{10}-kallidin binding in the human fibroblast cell lines WI38 (Phagoo et al., 2000) and IMR-90 (Zhou et al., 1998). In these cells the effect of IL-1ß was detectable at around 2h, peaked at around 4h and although, still above control, was reduced at 24h. Although IL-1ß has been reported to increase B_1-mediated responses and [^{3}H]-des-Arg^{10}-kallidin binding in cultured rabbit smooth muscle cells (Levesque et al., 1993, Galizzi et al., 1994) the time-course of this effect was not investigated. In smooth muscle preparations, such as the rabbit aorta, the IL-1ß-mediated increase in des-Arg^9-bradykinin-evoked contractions has been widely reported (Deblois et al., 1988, Deblois et al., 1991, Levesque et al., 1995). However, in these studies only one or two times, typically 3h and 6h of IL-1ß treatment were used and the effect of IL-1ß treatment for 24h was not investigated. In an *in vitro* preparation of the rat bladder IL-1ß treatment increased the spontaneous development of a contractile response to des-Arg^9-bradykinin over a period of 7h, however, again the effect of longer treatments were not investigated (Roslan et al., 1995). It is, therefore, difficult to say whether the time-course of the effect of IL-1ß in bladder smooth muscle cells is typical of smooth muscle preparations.

It is unclear why the effect of IL-1ß was reduced following 24h of treatment. In the experiments reported in this thesis the cells received fresh IL-1ß at the beginning of the 4h loading period with ^{45}Ca. This means that degradation of the IL-1ß is unlikely to be the reason for the reduction in its effect, however, it is not known what would have happened to the response if the cells had not received fresh IL-1ß at this point. Measurement of the levels of IL-1ß in the media over the incubation period using an enzyme-linked immunosorbent assay would help to clarify this point. The reduction in the IL-1ß-mediated upregulation towards basal levels by 24h may suggest that the expression of the B_1 receptor can be down-regulated. This could be a result of the presence of negative regulatory elements in the 5' region of the B_1 receptor gene (Yang, X. et al., 1998). Mediators that might induce down-regulation of the B_1 receptor have not been investigated widely, however, a recent report suggested that treatment with IL-6 lead to a reduction in the level of B_1 receptor mRNA in cardiac myocytes (Yayama et al., 2000). Interestingly, in rat aortic smooth muscle cells IL-1ß stimulated IL-6 production (Beasley, 1997), and this could also be occurring in rat bladder smooth muscle cells. Another possible explanation for the decreased effect of IL-1ß following 24h of treatment could be that the IL-1ß receptor is down-regulated by prolonged exposure to its ligand. This hypothesis could be tested by investigating the effect of exposure to IL-1ß on the specific binding of [^{125}I]-IL-1ß, however, there are no reports of such a phenomenon occurring in other preparations or cell lines.

The pattern of a gradual increase in B_1 expression up to a maximum response, followed by a decrease to near basal levels is typical of the induction of B_1 mediated responses or receptor levels in a variety of models of inflammatory

pain, although the time-course varies dramatically with the model. For example, in the rat paw, des-Arg^9-bradykinin-evoked oedema *in vivo* was enhanced by treatment with IL-1ß and TNFα. The effects of these cytokines were evident at 15min and peaked at 60min or 30min respectively and were reduced but still above control levels at 360min (Campos et al., 1998). In CYP-treated rats the expression of the B_1 receptor in the bladder increased with time up until 48h and then was reduced by 168h post CYP treatment, but was still above control. This pattern corresponds to the time-course of the observed inflammatory effects following CYP treatment (Belichard et al., 1999). At the other extreme, systemic treatment of mice with *Mycobacterium bovis* bacillus calmette-guérin (BCG), a known stimulant of the immune response, potentiated B_1 receptor-induced nociception and oedema formation in the formalin test in mice. The effect of BCG on the B_1-mediated response was maximal at 30-45 days post injection and still elevated at 60 days (de Campos et al., 1998).

IL-1ß treatment caused an increase in the maximum ^{45}Ca efflux evoked by des-Arg^9-bradykinin but had no effect on the EC_{50} value. This is similar to the results of studies in which other cell types were treated with IL-1ß, TNFα or EGF and [^{3}H]-des-Arg^{10}-kallidin binding measured. In rabbit vascular smooth muscle cells IL-1ß and EGF treatment resulted in an increase in the B_{max}, with no effect on the K_D (Galizzi et al., 1994, Schneck et al., 1994) and in human fibroblasts the same effect was observed following IL-1ß and TNFα treatment (Phagoo et al., 2000). In studies where a functional response has been measured following IL-1ß treatment the effect is often more complicated. For example, in the rabbit aorta, treatment with IL-1ß for 6h caused an increase in the maximal contraction evoked by des-Arg^9-bradykinin and also caused a decrease in the

EC_{50} value (Deblois et al., 1988). A similar effect was reported for the mouse bladder treated with LPS for 3h where there was an increase in the maximum contraction evoked by des-Arg^9-bradykinin and a leftward shift of the concentration response curve (Busser et al., 1998). In contrast, in the human umbilical vein, IL-1ß treatment for 75min followed by a 5h incubation resulted in a decrease in the EC_{50} for des-Arg^9-bradykinin-evoked contractions with no effect on the maximum response (Sardi et al., 1998). In the latter example, the lack of effect on the maximum response could be due to the presence of spare receptors, i.e. not all receptors have to be occupied to elicit a maximal response. Consequently an increase in the number of receptors will result in a leftward shift of the concentration-response curve with no effect on the maximum response.

No effects of dbcAMP on B_1-mediated responses have previously been reported. The time-course of its effect was similar to that of IL-1ß although the peak response was seen slightly later at 8h. As for IL-1ß, the effect was reduced following 24h of treatment. The effect of dbcAMP on the concentration-response curve for des-Arg^9-bradykinin was also similar to the effect of IL-1ß, with an increase in the maximum response but no effect on the EC_{50} value. As treatment with IL-1ß and dbcAMP resulted in an increase in the maximum response, this could reflect an increase in receptor number. In experiments in which protein synthesis was blocked with cycloheximide the upregulation caused by IL-1ß and dbcAMP was blocked. This is consistent with the inhibition of IL-1ß-induced increases in B_1-mediated responses by cycloheximide that has been reported in other cell types and tissues (Audet et al., 1994, Phagoo et al., 2000).

The glucocorticosteroid dexamethasone also inhibited both IL-1ß and dbcAMP-induced upregulation of des-Arg^9-bradykinin-evoked ^{45}Ca efflux. Glucocorticosteroids are potent anti-inflammatory agents (Barnes and Adcock, 1993) which exert their effects by binding to a cytoplasmic receptor which then translocates to the nucleus. The receptor complex can then exert its effects in two different ways:

1. It can interact with a glucocorticoid response element (GRE) within the promoter regions in various genes where its influence can be positive or negative. However, there are no reports of a GRE in the promoter region of the B_1 receptor.

2. Alternatively the complex can interfere with the binding of various transcription factors to DNA. This has been shown for the transcription factors AP-1 and NF-κB (McEwan et al., 1997) and for CREB (Akerblom et al., 1988), all of which have response elements in the B_1 receptor gene.

Although dexamethasone inhibited both IL-1ß and dbcAMP-induced upregulation of the response to des-Arg^9-bradykinin there was no effect on the control response to des-Arg^9-bradykinin. This is consistent with the fact that there is no GRE in the promoter region of the B_1 receptor, and is an indication that the effects of IL-1ß and dbcAMP involve synthesis of a new protein under the control of one or more transcription factors. Dexamethasone has been shown to inhibit the upregulation of the response to des-Arg^9-bradykinin in a variety of models. For example, in the rabbit aorta dexamethasone inhibited both the spontaneous and IL-1ß-stimulated increase in des-Arg^9-bradykinin-evoked contraction (Deblois et al., 1988). In the CYP model of bladder

inflammation dexamethasone inhibited the increase in des-Arg^9-bradykinin-evoked contractions (Lecci et al., 1999a).

The inhibition of the stimulatory effects of IL-1ß and dbcAMP by cycloheximide and dexamethasone suggests that both IL-1ß and dbcAMP stimulate synthesis of a new protein. However, this does not indicate whether the same protein is upregulated by IL-1ß and dbcAMP or whether this protein is the B_1 receptor. Following the cloning of the rat B_1 receptor gene it has been demonstrated that the promoter region contains sequences for binding of a variety of transcription factors, including NF-κB, AP-1 and CREB (Ni et al., 1998a, Jones et al., 1999). This suggested that it was possible that IL-1ß and dbcAMP had a direct effect on B_1 receptor mRNA synthesis by activating one or more transcription factors. In order to determine if this was occurring, the effect of IL-1ß and dbcAMP on mRNA for the B_1 receptor was investigated using RT-PCR. An increase in B_1 receptor mRNA following IL-1ß treatment was observed. In contrast, dbcAMP did not affect the levels of B_1 receptor mRNA. Although only one time-point was used in the RT-PCR experiments, the time chosen (4h) was one which consistently gave upregulation in the ^{45}Ca efflux assay but was before the peak time for upregulation. Assuming that there would be a similar delay between upregulation of the message and the receptor, as seen in WI-38 cells (Phagoo et al., 2000), then if B_1 receptor mRNA upregulation was responsible for the increased response to B_1 agonists, it would have been elevated at this time.

The increase in B_1 receptor mRNA induced by IL-1ß could have resulted from an increased level of transcription or from enhanced stability of the mRNA. It

would be possible to investigate whether there is an increased level of transcription using nuclear run-on experiments (Greenberg & Ziff, 1984). In these experiments the nuclei are isolated and any transcripts that have been initiated are elongated using ^{32}P-labelled nucleotides. The product formed is then hybridised to a DNA plasmid containing the gene of interest.

Alternatively the effect of IL-1ß on the stability of the mRNA could be investigated by treating the cells with IL-1ß and then stopping further transcription with an inhibitor such as actinomycin D. Samples would then be taken at various times and the amount of B_1 receptor mRNA measured. As no mRNA would be synthesised in the presence of actinomycin D, the level of mRNA would fall over time. If IL-1ß induced stabilisation then this rate of decline would be slower. In one study using IMR-90 cells the increase in B_1 receptor mRNA induced by IL-1ß was solely due to increased transcription (Schanstra et al., 1998) and in another it was due to both increased transcription and stabilisation (Zhou et al., 1998). However, in the human lung fibroblast line HEL299 the increase in B_1 receptor mRNA following IL-1ß or TNFα treatment was due to post-transcriptional mechanisms alone (Haddad et al., 2000). Increased stabilisation of mRNA is likely to be caused by inhibition of the synthesis of a highly labile protein which itself causes the rapid breakdown of the B_1 receptor mRNA. Evidence for this comes from the observation that cycloheximide causes an increase in the stability of B_1 receptor mRNA (Zhou et al., 1998, Haddad et al., 2000).

Consistent with the effect of IL-1ß on the level of B_1 receptor mRNA there was an increase in binding of [^{3}H]-des-Arg^{10}-kallidin binding following IL-1ß

treatment. The time-course of this effect was similar to that seen in the ^{45}Ca efflux assay, although the effect of IL-1ß was not significant at 24h. However, the size of the IL-1ß effect at this time-point was very similar in both assays, so this may reflect greater experimental variability in the binding assay. Treatment with dbcAMP had no effect on the binding of [^{3}H]-des-Arg^{10}-kallidin, which is consistent with the lack of effect on the level of B_1 receptor mRNA.

4.3.1.1 MECHANISM OF IL-1ß EFFECT

Although the mechanism by which IL-1ß stimulates an increase in B_1 receptor mRNA in bladder smooth muscle cells was not elucidated, its initial interaction with the IL-1ß receptor and the signal transduction pathways activated would be the same. IL-1ß exerts its effects by acting on the type I interleukin-1 receptor (IL-1R_1), the closely related type II interleukin-1 receptor can also bind IL-1ß but does not transduce a signal. IL-1ß is unusual among cytokines in that there exists a related protein (IL-1ra) which binds to the same receptor but does not exert any effects. In bladder smooth muscle cells IL-1ra blocked the effect of IL-1ß on B_1-induced ^{45}Ca efflux indicating that IL-1ß is acting via its receptor. In contrast IL-1ra did not inhibit the dbcAMP-induced increase in B_1-mediated responses. In a few experiments IL-1ra itself caused a small increase in des-Arg^{9}-bradykinin-evoked ^{45}Ca efflux. Although there have been reports of IL-1ra acting as an agonist (Montuschi et al., 1995, Mitchell et al., 1993), it is possible that commercial preparations of IL-1ra could be contaminated with endotoxin, which has been shown to cause B_1 receptor upregulation, and that this could be the reason for the stimulation.

Downstream of its receptor, IL-1ß has been reported to activate a myriad of signalling pathways some of which are summarised in Figure 4-1, for reviews see Bankers-Fulbright et al., (1996) and Auron (1998).

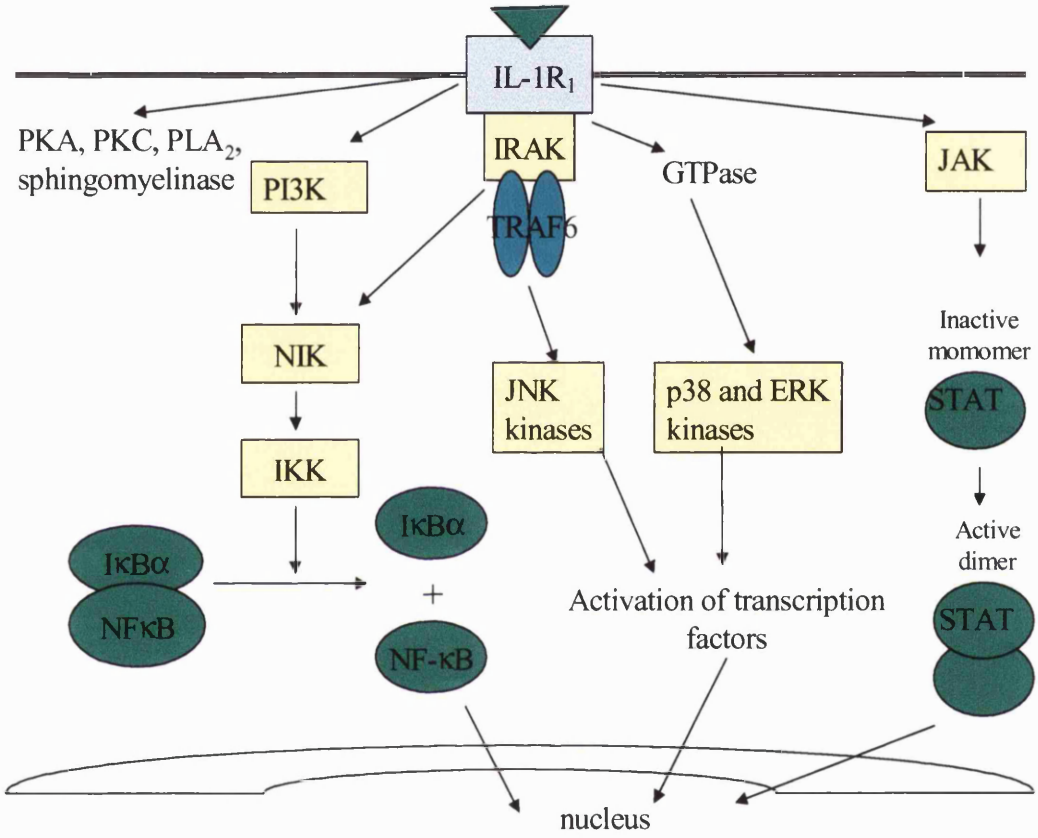

Figure 4-1 Outline of major signalling pathways activated by IL-1ß.

One of the earliest events in IL-1ß signal transduction is activation of a kinase known as interleukin-1 receptor associated kinase (IRAK). IRAK can interact with TNF receptor associated factor 6 (TRAF6) and NF-κB inducing kinase (NIK) to activate IκB kinase (IKK). This results in the phosphorylation of IκB, and its subsequent release from the NF-κB-IκB complex, releasing active NF-κB. Once released active NF-κB can translocate to the nucleus and induce transcription by binding to defined promoter elements, one of which is found in the 5'-flanking region of the B_1 receptor gene. IRAK is a serine/threonine

kinase and is susceptible to inhibition by staurosporine (Martin et al., 1994). However, the lack of effect of staurosporine on IL-1ß-induced upregulation in bladder smooth muscle cells suggests that another pathway is involved. It has also been suggested IL-1ß can activate the NF-κB pathway through phosphatidylinositol-3-kinase (PI3K) (Reddy et al., 1997, Marmiroli et al., 1998). The possible role of PI3K in the IL-1ß effects in bladder smooth muscle cells could be investigated using the inhibitor wortmannin (Ui et al., 1995).

Activation of NF-κB is one of the most important downstream events in IL-1ß signalling and several investigators have suggested a role for NF-κB in IL-1ß induction of B_1 receptor mRNA (Ni et al., 1998b, Schanstra et al., 1998), however not all studies agree and Zhou et al (1998) suggested that IL-1ß-induced activation of tyrosine kinases rather than NF-κB activation was involved. Janus kinases (JAK) are tyrosine kinases that have been reported to be involved in IL-1ß signalling (Ihle, 1995). JAKs can phosphorylate members of the signal transducing and activators of transcription (STAT) family of transcription factors leading to dimerisation and translocation to the nucleus (Tsukada et al., 1996). In order to investigate the role of NF-κB in IL-1ß-induced upregulation of B_1 responses in bladder smooth muscle cells the inhibitor PDTC was used. However, these experiments were unsuccessful probably due to its propensity to cause apoptosis in smooth muscle cells (Tsai et al., 1996). Further experiments with another NF-κB inhibitor such as sodium salicylate would be useful in determining the role of NF-κB in IL-1ß-induced upregulation of the B_1 receptor.

Among the signalling pathways reported to be activated by IL-1ß are various protein kinases including some isoforms of PKC and PKA (Shirakawa et al., 1988, Muñoz et al., 1990, Limatola et al., 1997). Again, however, the lack of inhibition by staurosporine on the effect of IL-1ß in bladder smooth muscle cells suggests that these kinases are not involved. This is consistent with previous observations where neither PKC nor PKA were shown to have a role in IL-1ß-induced upregulation of the B_1 receptor (Zhou et al., 1998). The lack of effect of staurosporine against IL-1ß is in direct contrast to its inhibition of cAMP-induced upregulation of B_1 mediated responses.

IL-1ß has been shown to stimulate production of prostanoids in a variety of cell types (Croxtall et al., 1996, Galizzi et al., 1994) and in human bronchial smooth muscle cells IL-1ß-induced cyclooxygenase-2 expression is involved in the upregulation of the B_2 receptor (Schmidlin et al., 2000). The role of cyclooxygenases in the regulation of the B_1 receptor is unclear. In the mouse bladder, LPS-stimulated increases in des-Arg^9-bradykinin-evoked contractions were inhibited by indomethacin (Busser et al., 1998) but in the rabbit aorta indomethacin was ineffective (Levesque et al., 1993). In rat bladder smooth muscle cells indomethacin did not inhibit IL-1ß-induced upregulation of B_1-evoked ^{45}Ca efflux, suggesting that prostanoids are not involved.

TNFα and LPS also increased des-Arg^9-bradykinin-evoked ^{45}Ca efflux. Time constraints did not allow these effects to be characterised as extensively as the effect of IL-1ß. However, it is likely that they also increased B_1 receptor mRNA, as reported by Phagoo et al. (2000) in human lung fibroblasts. TNFα

acts on a membrane receptor, subsequently activating a range of signalling pathways with many elements in common with IL-1ß signalling.

LPS signal transduction is more complex than that for IL-1ß and TNFα. The 55-kd glycoprotein CD14 binds LPS with high affinity and is involved in mediating LPS responses, which include activation of NF-κB and p38 MAP kinases. Binding of LPS to CD14 requires the serum factor, LPS-binding protein, which delivers LPS to CD14 expressing cells such as monocytes and macrophages (Kirschning et al., 1998). In cells which do not express CD14, such as endothelial cells and smooth muscle cells, soluble CD14, found in serum, is thought to perform the same function (Loppnow et al., 1995). As CD14 lacks a cytoplasmic domain, it does not elicit intracellular signalling events directly. Recently it has been found that a member of the Toll-like receptor (TLR) family, TLR2, is a signalling receptor that is activated by LPS in a response that depends on LPS-binding protein and CD14 (Yang, R-B. et al., 1998).

4.3.1.2 MECHANISM OF CYCLIC AMP EFFECT

The effect of cAMP on the des-Arg^9-bradykinin-evoked response was mimicked by treating the cells with forskolin, a direct activator of adenylate cyclase, and the response to des-Arg^9-bradykinin was unaffected by treatment with dbcGMP. These observations suggest that the effect of dbcAMP was not a non-specific effect, for example due to release of dibutyrate. The most likely mechanism of action for dbcAMP would be through activation of PKA. This was supported by the ability of staurosporine to inhibit the effect of dbcAMP. However, as staurosporine is not selective for PKA, activation of other kinases such as PKC

cannot be ruled out and it would be interesting to test other more specific kinase inhibitors. For example, an effect of PKC could be ruled out using an inhibitor such as bisindolylmaleimide. Compounds that inhibit PKA are available, for example H89 which binds to the ATP site of the kinase (Engh et al., 1996). H89, however, is not very potent or selective and often found to be ineffective (Boundy et al., 1998, Holen et al., 1996). There are also inhibitors of PKA that are based on the structure of cAMP and which bind to the regulatory site. However, there are problems with these as well, as they can break down during storage to form activators of PKA. Results from experiments where these inhibitors were used suggested that this was happening. Wiptide (Cheng et al., 1986) is a potent and selective inhibitor of PKA, however, it is not cell permeable and needs to be injected into the cell, making it impractical for these kinds of experiments.

The gene for IL-1ß has a CRE (Chandra et al., 1995) and so it was possible that dbcAMP-induced production of IL-1ß was involved in the effect of dbcAMP on des-Arg^9-bradykinin-evoked ^{45}Ca efflux. However, this is unlikely as an increase in both B_1 receptor mRNA and [^{3}H]-des-Arg^{10}-kallidin binding would have been observed if the effect of dbcAMP was mediated by IL-1ß. In addition, treatment of bladder smooth muscle cells with IL-1ra did not block the effect of dbcAMP, suggesting that dbcAMP was not working by stimulating IL-1ß production.

Given that there was no effect of cAMP on either the level of B_1 receptor mRNA or protein it is possible that its effect is downstream of the receptor. There are several points at which the effect could be mediated:

1. The coupling between the receptor and the G-protein could be enhanced.

2. The degree of activation of PIC could be modified.

3. The sensitivity of the Ins(1,4,5)P_3 receptor could be increased.

4. There could be a change in the pattern of calcium release and efflux.

As the signalling pathways for bradykinin and des-Arg^9-bradykinin in bladder smooth muscle cells are likely to be very similar, any effect of downstream of the receptor would be expected to affect the responses to both agonists. However, as dbcAMP treatment had no effect on the response to bradykinin (see Section 4.3.2) this would suggest that the effect of cAMP is on a part of the B_1 receptor signalling pathway that is distinct from that of the B_2 receptor.

One possible area of difference is the G-proteins to which the B_1 and B_2 receptors are coupled. Although it is likely that both the B_1 and B_2 receptors are coupled to G proteins of the G_q class in bladder smooth muscle cells, the precise subtype is not known. The use of agonist-stimulated [^{35}S]-GTPγS binding followed by immunoprecipitation with specific G-protein antibodies would make it possible to determine whether B_1 and B_2 receptors couple to different G-proteins and then to determine if the coupling was affected differentially by treatment with cAMP.

Another possible difference between B_1 and B_2 signalling is the pattern of release of intracellular calcium. The observation that dbcAMP treatment, in contrast to IL-1ß, reduced the basal rate of ^{45}Ca efflux, is an indication that the two regulators of B_1 activity had different effects on calcium homeostasis. It is

possible that the effect of dbcAMP on des-Arg^9-bradykinin-evoked responses may be due to an effect on calcium signalling. Preliminary experiments with bladder smooth muscle cells using a calcium imaging system showed that both bradykinin and des-Arg^9-bradykinin evoked a rise in $[Ca^{2+}]_i$, in many cases in the same cell. In addition, following application of des-Arg^9-bradykinin, but not bradykinin, oscillations in $[Ca^{2+}]_i$ were observed. Similar results were obtained in a study carried out by Mathis et al. (1996). Using rabbit mesenteric artery smooth muscle cells the authors showed that there were distinct differences between B_2 and B_1 mediated changes in $[Ca^{2+}]_i$. Following stimulation with a B_2 agonist, the $[Ca^{2+}]_i$ rose rapidly and then decayed back to basal levels. In contrast, following stimulation with des-Arg^9-bradykinin there was a sustained rise in $[Ca^{2+}]_i$, which in many cases showed oscillatory behaviour. Differences in the pattern of calcium release following stimulation with B_1 or B_2 agonists have also been observed in bovine pulmonary artery endothelial cells (Smith et al., 1995). More studies would be required to investigate the effect of cAMP on the pattern of calcium release evoked by the B_1 and B_2 agonists in bladder smooth muscle cells.

If a sustained increase in $[Ca^{2+}]_i$ is observed in a cell in response to an agonist, this is the result of entry of extracellular calcium, triggered by the emptying of intracellular calcium stores. This process is known as capacitative calcium entry (CCE). Mathis et al. (1996) showed that the sustained phase of the response to des-Arg^9-bradykinin in rabbit mesenteric artery smooth muscle cells was dependent on extracellular calcium. They suggested that one possibility for the lack of a sustained phase in response to bradykinin could be that the B_2 receptor desensitised very rapidly, resulting in a very short lived response. In contrast, in

rabbit mesenteric artery smooth muscle cells the B_1 receptor did not desensitise and therefore a more prolonged response was observed. Desensitisation of the responses to B_1 and B_2 agonists in the bladder smooth muscle cells was not investigated in detail, although in the cells illustrated in Figure 3-16 and Figure 3-17 it appears that the response evoked by a second application of either bradykinin or des-Arg^9-bradykinin was smaller than the first. More experiments would be needed to confirm if this pattern was typical. However, if it is the case that the B_1 receptor in bladder smooth muscle cells desensitises, then a possible mechanism for the action of dbcAMP is that it slows the desensitisation of the B_1 receptor, resulting in a more prolonged response.

It has been reported that cAMP can increase CCE in a variety of cell types (Petersen and Berridge, 1995, Song et al., 1998, Wu et al., 1999), however, these tend to be more immediate effects, occurring within a few minutes of application of dbcAMP during thapsigargin-induced CCE. Although an effect of dbcAMP on $[Ca^{2+}]_i$ homeostasis cannot be ruled out, the time-course of its effect on des-Arg^9-bradykinin-induced ^{45}Ca efflux was slower and required protein synthesis. It is therefore possible that cAMP can induce expression of a protein that can modulate CCE in a long term manner. The mechanism by which emptying of calcium stores triggers influx of calcium is not fully understood but may involve an, as yet, unidentified diffusible messenger (Putney and McKay, 1999). Once triggered, calcium is thought to enter the cell through channels of the transient receptor potential (TRP) family. There are at least six mammalian TRP forms and it is not known which ones are responsible for CCE, although TRP1, TRP4 and TRP5 are possible candidates (Putney and McKay, 1999). The regulation of

TRP channel expression has not been investigated, and it is possible that cAMP could regulate the expression of one or more members of this family.

Mathis et al. (1996) showed that des-Arg^9-bradykinin, but not bradykinin, evoked calcium oscillations in rabbit mesenteric artery smooth muscle cells. Similar results were obtained in the current study. The mechanism of calcium oscillations is not fully understood but is thought to involve calcium-induced calcium release (CICR). The Ins(1,4,5)P_3 receptor is sensitive to calcium and at a certain calcium concentrations there is rapid release of calcium, however, high concentrations of calcium inhibit further release. The $[Ca^{2+}]_i$ then falls as calcium is pumped back into the sarcoplasmic reticulum by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and out of the cell by the plasma membrane Ca^{2+}-ATPase (PMCA). Once the $[Ca^{2+}]_i$ has fallen back to a certain level, if Ins(1,4,5)P_3 is still present then calcium will be released again from the stores. The frequency of oscillations can be increased by over-expression of SERCA (Camacho and Lechleiter, 1993) and it is feasible that an increase in oscillation would result in an increase in Ca efflux. Interestingly, it has been shown that differentiation of HL60 myeloid cells with cAMP increases expression of one sub-type of SERCA (Launay et al., 1999).

4.3.2 THE B_2 RECEPTOR

The effect of treatment with IL-1ß, TNFα, LPS and dbcAMP on bradykinin-evoked ^{45}Ca efflux in bladder smooth muscle cells was investigated, however, none of these treatments affected the response to bradykinin. The absence of an effect of cytokines on B_2-mediated responses in bladder smooth muscle cells is consistent with studies which have shown that inflammatory treatments do not increase bradykinin-evoked contractions of the bladder (Busser et al., 1998,

Belichard et al., 1999). However, cytokines have been reported to alter B_2-mediated responses and receptor levels in airway smooth muscle cells, and it was thought B_2 receptors in bladder smooth muscle cells might also be regulated by cytokines. In human tracheal smooth muscle cells treatment with TNFα and IL-1ß increased the bradykinin-evoked rise in $[Ca^{2+}]_i$ (Amrani et al., 1997). The effects of the cytokines on the B_2 mediated responses required a minimum of 6h exposure, and this may explain the lack of effect in the current study, where a 4h treatment was used. However, in human bronchial smooth muscle cells treatment a 3h treatment with IL-1ß lead to a significant increase in bradykinin-evoked responses and B_2 receptor mRNA (Schmidlin et al., 1998). The lack of an effect of cytokines in bladder smooth muscle cells suggests that the B_2 receptor is only regulated by cytokines in certain tissues.

Schmidlin et al. (1998) demonstrated that the effect of IL-1ß on B_2-mediated responses and receptor levels was dependent on prostaglandin production and a subsequent rise in cAMP. A cAMP-dependent rise in bradykinin-induced responses and B_2 receptor density has been reported in canine tracheal smooth muscle cells following treatment with dbcAMP or forskolin for 24h (Yang et al., 1994). Neither a 4h or 24h treatment with dbcAMP had any effect on bradykinin-evoked ^{45}Ca efflux in bladder smooth muscle cells. This may suggest that, like cytokines, the B_2 receptor is only regulated by cAMP in certain tissues. Another possibility is that dbcAMP may have increased expression of the B_2 receptor, but this did not lead to an increase in bradykinin-evoked ^{45}Ca efflux. This has been reported in mesangial cells (Castaño et al., 1998) and glomerular podocytes (Costenbader et al., 1997) treated with PGE_2 or cAMP, where an increase in $[^3H]$-bradykinin binding sites was observed but the

bradykinin-evoked Ins(1,4,5)P_3 release and increase in $[Ca^{2+}]_i$ was unaffected suggesting that the newly formed receptor was not coupled.

4.4 RELEVANCE TO BLADDER INFLAMMATION

In this study it has been shown that B_1 receptor expression and responses in bladder smooth muscle cells were increased by treatment with the cytokines IL-1ß and TNFα, and the bacterial toxin LPS. In addition, B_1-evoked functional responses were increased following treatment with dbcAMP. In contrast, B_2-mediated responses were unaffected by any of these inflammatory mediators. These data are consistent with previous reports that have shown that LPS and IL-1ß increase B_1-evoked contractions in the bladder *in vitro* (Busser et al., 1998, Roslan et al., 1995). However, there are no reports of any effects of dbcAMP treatment on B_1 receptor-evoked contractions of the isolated bladder. Such experiments would be useful in determining the importance of the effect of dbcAMP found in the present study.

If cytokines or cAMP levels are increased in the bladder during inflammatory conditions then these findings have potentially important implications for the level of B_1 receptor activity. The B_1 receptor is induced in the bladder following CYP-induced inflammation (Belichard et al., 1999) and it has been shown that antibodies to TNFα and IL-1ß reduce the effects of CYP, as measured by bladder weight and plasma protein extravasation (Gomes et al., 1995). The current results suggest that IL-1ß and/or TNFα may be the mediators that increase B_1 expression during CYP-induced inflammation, however, to confirm this it would be necessary to determine whether IL-1ra or an antibody to TNFα could prevent the CYP-induced upregulation of the B_1 receptor. The

physiological relevance of the induction of the B_1 receptor in this condition is unclear. The ability of B_1 antagonists to inhibit the symptoms of CYP-induced cystitis such as the decrease in bladder volume, increase in micturition frequency and plasma extravasation has not been investigated, although it has been shown that a B_1 antagonist does not inhibit the increase in bladder tone associated with CYP-induced inflammation (Lecci et al., 1999a). However, increased responsiveness of bladder smooth muscle cells to des-Arg^9-bradykinin would lead to an increase in locally evoked contractions and, possibly, prostanoid production. These could both lead to an increase in reflex-evoked contractions. More studies with B_1 antagonists in the CYP model would indicate whether there is a B_1 receptor-mediated component to the hyper-reflexia, which has been reported for turpentine-induced inflammation, and whether inhibiting the B_1 receptor would a useful means to counteract some of the side-effects of CYP therapy.

The possible role of cytokines in IC is unclear. In a study in which the presence of cytokines in intact sections of bladder epithelia was determined using antibodies, IL-1ß was detectable in epithelial cells from IC patients but absent from sections from healthy bladder. In addition, in bladder sections from some patients with IC, detectable levels of IL-6, IL-4 and IFNγ were found in non-epithelial cells but not in sections from healthy bladders (Hang et al., 1998). No effects of IL-4 on B_1 receptor expression have been reported, however, IFNγ has been reported to increase B_1 expression in T-lymphocytes (Prat et al., 1999) and IL-6 has been reported to decrease B_1 expression in rat cardiac myocytes (Yayama et al., 2000). Studies in which the levels of cytokines in the urine were measured have shown that levels of IL-1ß and TNFα were not elevated in IC

patients (Peters et al., 1999, Felsen et al., 1994, Martins et al., 1994). However, in one of these studies (Martins et al., 1994) where the IL-1ß content of the urine from control, IC and bacterial cystitis patients was investigated, a significant increase was shown in the bacterial cystitis patients. Although there are no reports of the effects of kinins in bacterial cystitis this suggests it would be worth investigating the presence of B_1 receptors in this condition. Although there are no reports of IL-1ß in the urine of IC patients other cytokines, including IL-2, IL-6 and IL-8 have been reported to be elevated (Peters et al., 1999), and all of these cytokines have been reported to affect the expression of the B_1 receptor. IL-2 increased the development of the contractile response to des-Arg^9-bradykinin in the rabbit aorta (Deblois et al., 1988) and IL-8 has been reported to increase the expression of the B_1 receptor in human lung fibroblasts (Bastian et al., 1998). Therefore, the increased levels of the B_1 receptor that have been reported in bladder biopsies from IC patients may have been induced by other cytokines as well as IL-1ß. More studies in which the level of B_1 expression in IC patients is investigated and its role in mediating the inflammation, pain and hyper-reflexia associated with IC are needed, but it is likely that the B_1 receptor would be a useful therapeutic target for this disease.

The increased responsiveness of bladder smooth muscle cells to des-Arg^9-bradykinin following treatment with dbcAMP represents a novel mechanism by which the B_1 receptor can be regulated. The importance of this effect *in vivo* is not known, however, activation of the DP, IP, EP_2 and EP_4 classes of prostanoid receptor leads to an increase in cAMP. The bladder has been shown to release PGE_2 and $PGF_{2\alpha}$ in response to a variety of peptides including bradykinin, substance P and neurokinin A (Saban et al., 1997), and levels of prostanoids

have been shown to be increased in inflammatory conditions of the bladder (Farkas et al., 1980, Saban et al., 1994). This suggests that prostanoids released during bladder inflammation could increase the level of B_1 receptor activity. Consistent with this it has been shown that indomethacin inhibits the increased expression of the B_1 receptor in some models of bladder inflammation (Marceau et al., 1980, Busser et al., 1998). Another way in which prostanoids may play a role in the inflamed bladder is via their ability, acting via cAMP, to potentiate the effects of bradykinin on sensory neurones (Smith et al., 2000). The activation of the B_1 receptor on smooth muscle cells would be likely to lead to further prostanoid production, this could then further enhance both B_1-mediated responses and sensitisation of sensory neurones to bradykinin.

The B_2 receptors on bladder smooth muscle cells do not appear to be regulated by the same factors that alter the level of B_1 receptor expression or activity. This is consistent with studies that have shown that the contractile response of the bladder to bradykinin is unchanged following CYP-induced inflammation. However, B_2 antagonists are effective at reducing the symptoms of CYP-induced inflammation. i.e. the decrease in bladder volume, the increase in micturition frequency and plasma protein extravasation (Maggi et al., 1993, Ahluwalia et al., 1994). This suggests that kinins are produced during CYP-induced inflammation and act on the constitutively expressed B_2 receptor on smooth muscle cells and on the sensitised B_2 receptors present on sensory neurones. Evidence for this has come from experiments demonstrating that capsaicin pre-treatment, which suppresses the function of a subpopulation of primary afferents, abolished the effects of CYP. In contrast to CYP, xylene-

induced hyper-reflexia, which is not sensitive to capsaicin treatment, was not inhibited by a B_2 antagonist (Maggi et al., 1993).

5 CONCLUSION AND FUTURE STUDIES

This study has clearly demonstrated that smooth muscle cells derived from the rat bladder express both B_1 and B_2 kinin receptors and that the level of B_1 receptor activity is subject to regulation by mediators that would be elevated in inflammatory conditions. In contrast, the B_2 receptor does not appear to be regulated in the same way. This is consistent with studies that have shown that the level of the B_1 receptor in the inflamed bladder is increased, but that the B_2 receptor is not affected. A proposed scheme of the potential interactions between cytokines, prostanoids and kinins in the bladder is described below and shown in Figure 5-1.

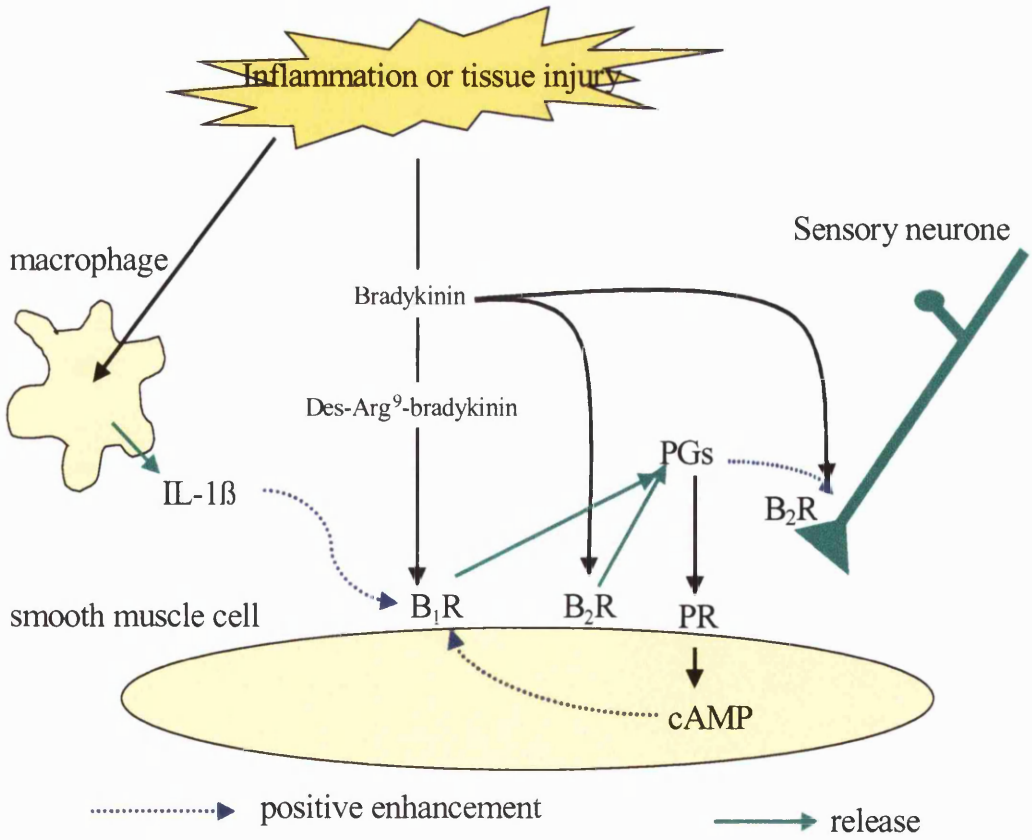

Figure 5-1 Interaction of IL-1ß, prostanoids and kinins in the inflamed bladder
PR - prostanoid receptor, PGs - prostanoids

The onset of inflammation leads to production of both B_1 and B_2 agonists. The B_2 agonists can activate the B_2 receptor on sensory neurones and stimulate the constitutively expressed B_2 receptor on other cell types, such as smooth muscle cells, leading to production of prostanoids. The release of prostanoids results in the sensitisation of sensory neurones, to both bradykinin and other stimuli, leading to pain. As inflammation progresses the expression of the B_1 receptor is increased by cytokines released from macrophages, leading to enhanced prostanoid production. As well as sensitising sensory neurones the prostanoids may also increase the response of smooth muscle cells to B_1 agonists. The response of the B_2 receptor on smooth muscle cells is unaffected by cytokines and prostanoids, and therefore as inflammation progresses the importance of the B_1 receptor increases.

This scheme is consistent with current knowledge on the role of kinins in the bladder, however, there is much that is still unclear and remains to be investigated. Although it has been shown that the bladder can produce prostanoids in response to kinin agonists this had not so far been demonstrated in bladder smooth muscle cells. A role for prostanoids in the regulation of B_1 receptor activity is proposed on the basis of the effect of dbcAMP. However, it is not known which prostanoid receptors are expressed by bladder smooth muscle cells and whether activation will lead to cAMP production. The use of the cellular model described in this thesis to measure prostanoid and cAMP production could help to answer some of these questions.

The effects of B_1 antagonists in models of bladder inflammation have not been extensively tested and more studies are needed before the importance of the B_1

receptor in inflammatory conditions of the bladder can be determined. The recent development of non-peptide B_1 antagonists will aid in these studies. Clearly many mediators are involved in bladder inflammation, however, B_1 antagonists have the potential to reduce the effects of these mediators and break the cycle of pain and inflammation, and could therefore be of therapeutic benefit.

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