CHARACTERISATION OF THE KININ

~~THE BRADYKININ~~ RECEPTOR IN THE HUMAN NASAL AIRWAY: AND ITS ROLE ~~ITS CHARACTERISTICS AND ROLE~~ IN ALLERGIC RHINITIS

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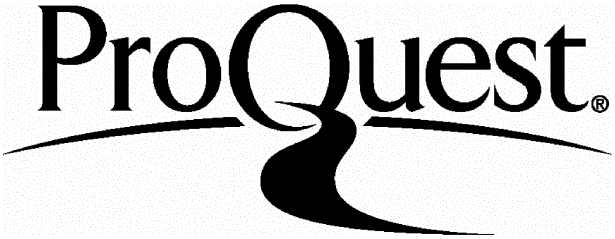

ABSTRACT

This thesis presents an investigation into the characteristics of the bradykinin receptor in the human nasal airway. There is evidence that bradykinin is a mediator of allergic rhinitis; an allergen-induced inflammatory disorder of the nasal airway. Intranasal administration of bradykinin induces some of the symptoms of allergic rhinitis. The effect of a variety of bradykinin receptor antagonists on the actions of bradykinin in the human nasal airway *in vivo* is investigated. This characterisation of the human nasal bradykinin receptor is extended by performing radioligand binding experiments using *ex vivo* human nasal tissue. Using these two different experimental approaches, the nasal bradykinin receptor appears to be of the B_2 type.

Using atopic human subjects, the effect of the bradykinin receptor antagonist, icatibant, on experimentally-induced seasonal and perennial allergic rhinitis provides data strongly implicating bradykinin in the pathophysiology of perennial allergic rhinitis.

The role of nitric oxide in the nasal response to bradykinin, histamine and antigen is also investigated by inhibiting nitric oxide generation in the human nasal cavity. Bradykinin has been shown in other systems to release histamine from mast cells. The effect of histamine H_1 receptor antagonists on the nasal actions of bradykinin is investigated, as is the effect of bradykinin on histamine release from *ex vivo* human nasal tissue. From these experiments both nitric oxide and histamine appear to mediate, at least in part, the response of the human nasal cavity to bradykinin challenge. Nitric oxide also appears to be a mediator of experimentally-induced allergic rhinitis.

Platelet activating factor (PAF) is a mediator of inflammation which appears to be generated in allergic rhinitis. If PAF is administered to a normal, non-atopic, human nasal cavity it increases the responsiveness of the airway to histamine and bradykinin. The final chapter of this thesis implicates a role for kinin generation in PAF-induced human nasal hyperresponsiveness.

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CHAPTER 1
INTRODUCTION

1.1 Introduction

The central mediator of nasal allergy was originally considered to be histamine. However, drugs which inhibit the actions of histamine fail to alleviate all of the symptoms of the nasal allergic reaction, suggesting that the actions of histamine alone do not fully account for the pathophysiology of nasal allergy. It is now apparent that a number of other inflammatory mediators are released into the nasal cavity during the allergic response, but their relative importance is not yet fully elucidated. This study investigates the contribution of one inflammatory mediator, bradykinin, to the inflammation which occurs in the nasal allergic disease, allergic rhinitis. More specifically, this study will focus on the characteristics of the bradykinin receptor in the human nasal airway, as this receptor provides a target for future clinical treatments of allergic rhinitis.

1.2 The human nose

1.2.1 Anatomy of the human nasal airway

A septum divides the human nasal airway into left and right nasal cavities. Where these cavities open anteriorly onto the face is referred to as the anterior nares or nostrils. Posteriorly, the region where the cavities open out into the nasopharynx is known as the posterior nares. The human nose is often described as having two parts. The external nose is the region projecting from the face and consists of alar cartilage covered in skin. This cartilage maintains the patency of the nostrils when air is entering the nasal airway. Continuous with, and posterior to, the external nose is the internal nasal cavity. This part of the nose lies within the bones of the head. The part of the nose where the external nose meets the internal nasal cavity is called the nasal valve. On the lateral wall of the internal nasal cavity there are three horizontal bony projections which in the clinical literature are referred to as the superior, middle and inferior turbinate bones. The anatomical literature

calls these bones conchae, but throughout this thesis the clinical name of turbinates will be used. The inferior turbinate bone is the largest of the three and is often the point at which the nasal cavity is at its most narrow. The turbinates bones projecting into the nasal cavity greatly increases the surface area of the human nasal mucosa. Inferior to each of the turbinates there are meati which lead into ducts. These ducts link the nasal cavity to the sinuses of the head and the conjunctiva of the eye. Inflammation of the nasal mucosa can lead to blockage of these ducts with pathological consequences.

Blood is supplied to the human nasal mucosa by branches of the external and internal carotid arteries. The sensory innervation of the nasal mucosa is provided by branches of the trigeminal nerve. Nasal parasympathetic innervation is supplied by the facial nerve and sympathetic innervation by nerves from the carotid plexus who have their cell bodies in the superior cervical ganglion.

1.2.2. Functions of the human nasal airway

The nose has a number of important physiological functions. It filters, warms and humidifies the inspired air so delivering the air to the lungs in the optimal state. The glandular and vascular elements of the human nasal mucosa warm and humidify the air so that when it reaches the nasopharynx it is at a temperature of 31^0C and 95% saturated with water. The shape of the internal nasal cavity produces turbulence in the air flowing through the cavity which causes the particles of solid material contained in the air to be deposited on the mucous blanket which covers the nasal mucosa.

The roof of the internal nasal cavity has a specialised olfactory epithelium which contains branches of the olfactory nerve. This region is responsible for the sense of smell. A number of neural reflexes are activated by stimulation of the nasal airway. Chemical, thermal or physical stimulation of the nasal mucosa can cause sneezing; physical exertion leads to vasoconstriction of the blood vessels in the nasal mucosa; an increase or decrease of temperature in the nasal airway leads to vasodilatation or vasoconstriction, respectively

and pressure applied to the axilla causes a decrease in the blockage of the contralateral nasal cavity (Burrows & Eccles, 1985).

1.2.3. Histology of the human nasal airway

On the surface of the human nasal mucosa there is a fluid bilayer. The outer layer consists of a mucous blanket which traps foreign particles including bacteria and dust. This mucous layer is constantly being swallowed and replaced by new glandular secretions. The inner layer of fluid consists of an aqueous solution in which the cilia of the nasal epithelium beat, transporting the mucous layer backwards into the nasopharynx. This aqueous inner layer contains most of the proteins secreted by the serous submucosal glands, these secretions having an antimicrobial function.

The epithelium consists of cilliated columnar epithelial cells, noncilliated columnar epithelial cells, goblet cells and basal cells (Kaliner, 1994). The goblet cells secrete mucous glycoproteins onto the epithelial surface. Beneath the epithelium is a submucosa containing mucus, seromucous and serous glands. While the mucous glands secrete mucous glycoproteins, the serous glands secrete lysozyme, lactoferrin, neutral endopeptidase, leucoprotease inhibitors and IgA (Kaliner, 1994)

A variety of inflammatory cell types are contained in the nasal submucosa of normal and atopic humans. These will be reviewed later.

The nose is a highly vascular tissue with the blood vessels supplying the epithelium and glands containing large pores in their basement membranes analogous to the fenestrations present in the renal glomerulus. The fenestrae in the blood vessel basement membranes allow rapid extravasation of plasma through the vessel walls which presumably aids the rapid humidifying of the inspired air. Cavernous sinusoids are present between the capillaries and venules, particularly in the submucosa covering the middle and inferior turbinate bones. Normally these sinusoids are in a contracted state under the control of the

sympathetic nervous system, but if sympathetic tone is reduced then vasodilatation of the blood vessels supplying the sinusoids causes the sinusoids to become engorged with blood. This leads to swelling of the mucosa and blockage of the nasal airway. In 80% of the population an alternate and cyclic blocking of the right and left nasal cavities can be detected with the blockage of the cavity changing side approximately every 4 hours. This cyclic blockage is due to the sinusoids in the nasal mucosa filling with blood under the control of the sympathetic nervous system.

Vasodilators, such as some of the inflammatory mediators to be reviewed later, can produce an increase in nasal mucosal blood flow and fill the sinusoids with blood. This will produce swelling of the nasal mucosa and blockage of the nasal airway. The swelling of the mucosa may be enhanced by plasma extravasation from the blood vessels generating tissue oedema. Also, enhanced glandular secretion into the nasal cavity can also potentially lead to congestion of the nasal cavity.

The rate at which air flows through the nasal airway is determined by the difference in pressure between the anterior and posterior nares and the resistance to airflow through the cavity. The airflow through the nasal cavity (I) is described by Ohm's law:

$$\Delta P = I \bullet R$$

where ΔP represents the pressure difference across the nasal airway and R is the resistance to airflow through the nasal cavity.

The resistance to airflow through the nasal airway is dependent on the diameter of the nasal cavity. If the nasal airway is considered to be a tube of constant radius and the airflow through the nose is considered to be streamline, then the following equation describes the relationship between the radius of the nasal airway and the rate of airflow through it:

$$I = \frac{\pi \Delta P r^4}{8\eta l}$$

in which I is the rate of airflow, ΔP is the pressure difference, r is the radius of the nasal airway, l is the length of the nasal airway and η is the viscosity of the air. This equation is known as Poiseuille's law. Its relevance to this project is that small changes in the radius of the nasal airway, such as would occur with swelling of the mucosa covering the turbinate bones, will produce large changes in the airflow through the nasal cavity.

It should be noted, however, that airflow through the nasal cavity is not streamline. As air passes through the nose it hits a number of structures, for example the turbinate bones. This causes the air to change direction forming a turbulent airflow through the nose. Therefore, Poiseuille's law cannot be strictly applied, but small changes in the radius of the nasal cavity still produce large changes in airflow.

1.3 Allergic rhinitis

Allergic rhinitis is the single most common allergic disease; with approximately 15% of the population being affected. The symptoms of allergic rhinitis result from inflammation of the mucosa of the nasal cavity induced by an allergic response to inhaled allergen. Allergic rhinitis may be seasonal or perennial in nature; seasonal when the allergen exposure only occurs at certain times in the year and perennial when allergen exposure occurs all year round. The allergens for seasonal allergic rhinitis include tree pollen, grass pollen, moulds and certain spores. Seasonal allergic rhinitis is more commonly called hay fever. Patients presenting with perennial allergic rhinitis have positive nasal reactions to house-dust mite (*Dermatophagoides pteronyssinus*) and sometimes also react to animal danders and certain foods. Data presented in later chapters demonstrates that these two types of allergic rhinitis have differences in their pathophysiology and, therefore, for all the studies reviewed in this introduction, the allergen used will be clearly defined.

Allergic rhinitis is considered to be an example of a type I hypersensitivity reaction. There are two distinct stages in the development of this type of disease.

1) Sensitisation. Allergen inhaled in the nasal cavity is presented to the immune system where it is recognised as being foreign and in susceptible individuals the immunoglobulin IgE is synthesised and becomes fixed to cells.

2) In the second stage the same allergen encounters cells, for example mast cells, which have bound to receptors on their surface the allergen-specific IgE. When allergen crosslinks the bound IgE, inflammatory mediators such as histamine are released and the symptoms of inflammation are produced.

This study investigates the final stage in this process, the role of inflammatory mediators in allergic rhinitis. In particular, studies will focus on the involvement of the mediators known as kinins in allergic inflammation of the human nasal airway.

1.3.1 Symptoms of allergic rhinitis

The symptoms of allergic rhinitis are pruritus (itching) of the nose, sneezing, rhinorrhoea ('runny nose') and nasal congestion. Pruritus and sneezing result from sensory nerve stimulation and nasal congestion is a result of swelling of the nasal mucosa due to vasodilatation of the mucosal blood vessels and tissue oedema. Excess glandular secretion into the nasal cavity can potentially cause further congestion. Rhinorrhoea is a result of glandular secretion and extravasation of plasma into the nasal cavity. Plasma extravasation is due to an increase in the permeability of the nasal microcirculation. If grass pollen antigen is introduced into only one nostril then there is increased glandular secretion and plasma extravasation into the challenged nasal cavity. However, the contralateral unchallenged nostril also produces glandular secretions but there is no

plasma extravasation. This contralateral glandular secretion can be inhibited by atropine indicating that the response is mediated by cholinergic neurones (Raphael *et al.*, 1991). Therefore, following administration of grass pollen antigen via one nostril into the nasal airway of an allergic individual there is increased plasma extravasation into the ipsilateral nasal airway, but increased glandular secretion both ipsilateral and contralateral to the antigen challenge. The initial response to antigen being administered to an atopic nasal cavity is known as the early response. However, it is common for a late response to occur 4-12 hours after the initial challenge. This late response is characterised by an influx of inflammatory cells into the nasal mucosa and prolonged nasal congestion and nasal hyperresponsiveness to antigen challenge (Naclerio *et al.*, 1985).

A number of cell types and inflammatory mediators have been investigated with respect to the pathophysiology of allergic rhinitis and the remainder of this introduction will review their contributions. However, before introducing the cells and mediators, the techniques used to investigate their roles in allergic rhinitis will be briefly reviewed.

1.4 Methods for studying the role of inflammatory mediators and cells in the human nasal airway

For the study of the pharmacology of the human nasal airway, a variety of different and complementary techniques are available. They include:

- application of drugs and naturally occurring substances into the nasal airway.
- the assessment of the patency of the nasal airway.
- lavage of the nasal airway to collect released substances.
- collection of nasal secretion.
- assessment of subjective appreciation of nasal symptoms such as blockage, pain, rhinorrohea etc.
- brushing the nasal airway to collect superficial cells.
- biopsy of nasal tissue or use of nasal tissue removed during routine surgery.

Application of substances to the nasal airway can be achieved by instilling a solution of the substance into the nasal airway, spraying an aerosol into the nasal cavity or placing a paper disc containing the substance into the airway at a suitable point such as the inferior turbinate bone.

There are several methods for the assessment of the patency of the nasal airway: rhinomanometry (Kern, 1973); acoustic rhinometry (Jackson *et al*, 1977); nasal peak flow (Holmstrom *et al*, 1990) and body plethysmography (Nolte & Luder-Luhr, 1973).

Rhinomanometry measures the airflow through the nasal cavity and the pressure difference across the nasal airway. By applying Ohm's Law, the resistance to airflow through the nasal cavity can be calculated. Acoustic rhinometry measures the patency of the nasal airway and thus produces an anatomical measure of nasal congestion. Austin & Foreman (1994b) demonstrated that measurements of nasal patency using acoustic rhinometry reflect resistance to airflow measured by rhinomanometry. Acoustic rhinometry has the advantage that it requires minimal subject training.

Nasal lavage has been performed in a variety of ways. In one method (Hilding, 1972) a modified Foley catheter is used to produce a seal between the posterior nasal cavity and the pharynx. With the head extended, saline can then be inserted into and recovered from the nasal cavity via the catheter. In other methods, an aerosol is sprayed into the nasal cavity and then recovered either by asking the subject to expel it on to a plastic dish (Linder *et al*, 1988) or by suction into a trap (Druce *et al*, 1985).

In the method of Naclerio *et al* (1983) saline is introduced into the nostril and after 10s, the subject flexes the head and pours the saline from the nasal cavity into a collecting vessel. The method gives recoveries of 80% to 90% and is non-invasive. It does, however, require some subject training to avoid swallowing of the saline.

The technique of nasal lavage allows the substances released into the nasal cavity to be quantified. The albumin content of the nasal lavage is commonly used as a measure of the vascular permeability of the nasal blood vessels. The concentration of inflammatory mediators, such as histamine, bradykinin and leukotrienes, in the lavage fluid can be measured before and after antigen is administered into the nasal cavity. Increased concentration of an inflammatory mediator after antigen challenge indicates that the mediator is generated during the pathophysiology of allergic rhinitis. The ability of the nasal lavage fluid to release methanol from N-α-*p*-tosyl-L-arginine methyl esterase (referred to as TAME esterase activity) is a measure of the amount of the kinin forming enzymes, kallikreins, and mast cell tryptase released into the nasal cavity.

Nasal secretion can be measured by placing preweighed absorbent material into the nasal cavity and reweighing it after a fixed time (Holmberg *et al*, 1990). The subject may also be asked to blow secretion onto a preweighed absorbent material. Alternatively, mucus glycoprotein and serous secretions can be measured in nasal lavage fluid (Greiff *et al*, 1993).

Symptoms such as pain, itching, running, blockage, sneezing can be recorded using a visual analogue scale (Aitken, 1969).

Cells and tissue from the nasal cavity can be obtained by brushing the nasal cavity (Pipkorn *et al*, 1988), by nasal biopsy, or by acquiring the tissue removed from turbinectomy. Brushing and biopsy can give information about histological changes in the nasal cavity but tissue from turbinectomy specimens is sufficient for preparing membrane preparations for ligand binding studies, for preparing explants which can be used to measure mucus secretion (Mullol *et al*, 1993) or for the recovery of nasal cells by tissue dispersion.

1.5 Inflammatory cells in the human nasal mucosa and their relevance to allergic rhinitis

1.5.1 Mast cells and basophils

In the human nasal mucosa, mast cells are located in close proximity to the nerves and blood vessels and occasionally in the epithelium (Kaliner, 1994). Most nasal mast cells are found in the superficial 200μm of the mucosa. In normal subjects, there are about 7000 mast cells/mm^3 in the sub-epithelium tissue but only 50/mm^3 in the epithelium (Kaliner, 1994). Immunohistochemical studies have shown an increase in the numbers of mast cells in the epithelium of subjects with seasonal and perennial allergic rhinitis compared with non-atopic control subjects (Bentley *et al.*, 1992). The nasal epithelial mast cells stain tryptase positive, chymase negative (Kaliner, 1994). The sub-epithelial human nasal mast cells, which constitute the majority of the mast cells in the nasal cavity, stain positive for tryptase and chymase (Kaliner, 1994). Only one study has shown an increase in the number of sub-epithelial mast cells in subjects with seasonal allergic rhinitis (Viegas *et al.*, 1987); other studies found no difference in sub-epithelial mast cell numbers when non-allergic and allergic rhinitis subjects were compared (Enerback *et al.*, 1986). The epithelial mast cells are in an activated state in symptomatic allergic rhinitis. This is demonstrated by ultrastructural evidence of degranulation using electron microscopy (Enerback *et al.*, 1986), and by raised levels of the mast cell products, histamine and tryptase, in the nasal cavity, following nasal administration of grass pollen antigen (Walden *et al.*, 1988). Mast cells are the only cells in the human nasal mucosa which express surface IgE (Igarashi *et al.*, 1993).

Human nasal mast cells release a variety of preformed and newly synthesised mediators including histamine, prostaglandin D_2 (PGD_2), leukotriene C_4 (LTC_4) and tryptase (Howarth, 1995). In addition, these mast cells also contain preformed cytokines in particular interleukin-4 (IL-4), IL-5, IL-6 and tumour necrosis factor-alpha (TNF-α) (Bradding *et al.*, 1993; Bradding *et al.*, 1995a). The human nasal mast cells staining positive for tryptase and chymase contain the majority of the total mast cell IL-4, whereas

IL-5 and IL-6 are restricted almost exclusively to the tryptase positive, chymase negative cells (Bradding *et al.*, 1995b). The IL-4 content of the human nasal mast cells is greater in biopsies from subjects with allergic rhinitis than normal subjects. Staining for IL-4 indicates that this cytokine is secreted by the mast cells during seasonal and perennial allergic rhinitis (Bradding *et al.*, 1993).

Following nasal grass pollen antigen challenge in subjects with seasonal allergic rhinitis, the number of basophils in the nasal mucosa increases (Bascom *et al.*, 1988). When activated, basophils release histamine and LTC_4, but not PGD_2. In the late response of allergic rhinitis, histamine is released into the nasal cavity but there is no release of PGD_2 (Naclerio *et al.*, 1985). As basophil numbers increase following antigen challenge, and as there is no PGD_2 production, the histamine release in the late response may be the result of basophil activation. Further evidence for basophil activation in the late response is provided by the use of corticosteroids (Bascom *et al.*, 1988). Experiments *in vitro* show that corticosteroids inhibit mediator release from basophils (Schleimer *et al.*, 1981), but not mast cells (Schleimer *et al.*, 1983). The histamine release in the late response, but not the early response, is inhibited by treatment with corticosteroids indicating basophil mediator release in the late response of allergic rhinitis. Circulating basophils also contain cytokines (Brunner *et al*; 1993), but the their relevance in allergic rhinitis is still unclear.

Therefore, the mast cell and basophil can release mediators which are capable of generating the symptoms of allergic rhinitis (for example histamine and LTC_4) and cytokines which can contribute to the recruitment and activation of cells in the allergic nasal mucosa.

1.5.2 Lymphocytes

Lymphocytes are one of the most abundant cell types in the human nasal mucosa (Igarashi *et al.*, 1993). An increase in lymphocyte number has been reported in subjects with seasonal and perennial allergic rhinitis (Calderon *et al.*, 1994). These cells are in an

activated state (increased IL-2 receptor expression) in subjects with perennial allergic rhinitis compared with non-atopic controls (Calderon *et al.*, 1994). The increase in lymphocyte number appears mainly to consist of CD4 positive cells which have the potential to produce a range of pro-inflammatory cytokines. Cytokine production from these cells appears to contribute to the recruitment of eosinophils into the nasal cavity (Durham *et al.*, 1992).

1.5.3 Eosinophils

An increased number of eosinophils is evident in biopsies of the nasal mucosa taken from subjects with allergic rhinitis (Bentley *et al.*, 1992). Moreover, the products of eosinophil activation, eosinophil cationic protein (ECP) and major basic protein (MBP) are increased in the nasal secretions following antigen challenge in subjects with perennial and seasonal allergic rhinitis (Linder *et al.*, 1987; Togias *et al.*, 1988; Bascom *et al.*, 1989). Therefore, in allergic rhinitis, eosinophils are recruited into the nasal mucosa where they have the potential to contribute to the nasal inflammatory response. Eosinophil activation causes the release of the toxic basic protein, major basic protein (MBP), which is capable of causing airway hyperresponsiveness (Gundel *et al.*, 1991). Recently, it has been reported that MBP-induced hyperresponiveness in the lower airway of the rat is dependent upon the generation of bradykinin (Coyle *et al.*, 1995): this will be discussed further in chapter 7. Eosinophils can also produce platelet-activating factor (PAF), which is a chemoattractant for eosinophils (Henocq & Vargaftig, 1986). PAF can cause eosinophil activation in the human nasal mucosa. This activation can be measured by increased levels of ECP in the nasal lavage fluid following challenge with PAF (Austin & Foreman, 1993): this eosinophil activation may be involved in PAF-induced nasal airway hyperesponsiveness (see chapter 7). Eosinophil activation causes the generation of arachidonic acid metabolites; in particular LTC_4. Cytokines enhance the production of LTC_4 from eosinophils (Howarth, 1995). Although LTC_4 levels are increased in the nasal fluid following antigen challenge in subjects with allergic rhinitis, the LTC_4 may originate from mast cells or basophils as well as eosinophils. Human nasal

eosinophils are also a source of cytokines such as IL-5 (Bradding *et al.*, 1993). Therefore, eosinophil cytokine production may further contribute to nasal inflammation.

Also present in the human nasal submucosa are platelets, neutrophils and Langerhan's cells. These cells may also contribute to nasal inflammation (Howarth, 1995).

1.6 Inflammatory mediators and their relevance to allergic rhinitis

1.6.1 Kinins

Historically, the first reports relating to the kinin system refer to a substance in rabbit and human urine that caused hypotension in anaesthetised dogs (Abelous & Bardier, 1909). As this substance was believed to be derived from the pancreas, it was named kallikrein from the Greek word for pancreas, *kallikreas*. Werle and colleagues (1937) incubated kallikrein with plasma and found a substance capable of contracting the guinea-pig ileum and causing hypotension: it was identified as the peptide kallidin. Snake venom incubated with plasma leads to the formation of a substance causing a slow, delayed contraction of the guinea-pig isolated ileum: this substance was named bradykinin from the Greek for movement, *kinin* and slow, *brady* (Rocha E Silva *et al.*, 1949).

The kinins, bradykinin and the closely related peptide kallidin are, respectively, nine and ten amino acid peptides formed by the action of enzymes called kallikreins on precursors called kininogens.

Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Kallidin
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Bradykinin

There are two types of kallikrein enzymes: plasma kallikrein which is present in the circulation and cleaves bradykinin from high-molecular weight kininogen and a kallikrein present in various tissues which cleaves lys-bradykinin (kallidin) from high- and low-molecular weight kininogens. Once formed, the kinins mediate most of their actions via

activation of two membrane receptor types termed B_1 and B_2. The actions of bradykinin and kallidin include vasodilatation, increased vascular permeability, stimulation of nerves, stimulation of epithelial cell ion transport and contraction of intestinal and uterine smooth muscle. The kinins are broken down by two families of enzymes, kininase I and II. Each factor involved in the generation of the kinins will now be reviewed in more detail.

1.6.1.1 The generation and breakdown of bradykinin

1.6.1.1.1 Kininogens

Three types of kininogen have been identified in mammals: high-molecular weight (HMW) kininogen which is present in the blood, low-molecular weight (LMW) kininogen in the blood and tissues and T-kininogen which is found exclusively in the rat (Habermann, 1963; Suzuki *et al.*, 1967; Jacobsen, 1966; Okamoto & Greenbaum, 1983). Both HMW-kininogen and LMW-kininogen are the product of a single gene in humans, their differing primary structures being produced by alternate splicing of the gene transcript (Kitamura *et al.*, 1985). The human kininogen gene contains 11 exons, nine of which, at the 5'-terminal, code for the mRNA producing the heavy chain common to both HMW and LMW kininogen (Kitamura *et al.*, 1985). Exon 10 contains the sequence producing the kinin-containing region of the kininogens together with a sequence which produces the HMW-kininogen light-chain. Exon 11 codes for the LMW kininogen light chain. The heavy chain, which has the same basic structure in both HMW and LMW kininogen, has the ability to inhibit cysteine proteases (Sueyoshi *et al.*, 1985). The light chain of HMW kininogen contains a domain rich in histidine, proline and lysine which binds to damaged endothelial surfaces, crystals and degraded cartilage products. Once plasma kallikrein releases bradykinin from the region between the light and heavy chains, the HMW kininogen light chain enhances clotting by activation of clotting factors (Kaplan, 1978). The light chain of HMW kininogen also contains the domain which binds plasma kallikrein or clotting factor XI (Sugo *et al.*, 1980). Both the heavy and light chains participate in the binding of HMW kininogen to endothelial cells (Jiang *et al.*, 1992;

Asakura *et al.*, 1992), platelets (Meloni *et al.*, 1992) and neutrophils (Wachtfogel *et al.*, 1994). The function of the light chain of LMW kininogen remains unclear.

1.6.1.1.2 Plasma kallikrein

Plasma kallikrein is the product of a single gene (Seidah *et al.*, 1989) and its mRNA is expressed exclusively in the liver. The inactive form, plasma prekallikrein, is secreted by hepatocytes and circulates in the plasma bound to high-molecular weight kininogen (Mandle *et al.*, 1976). Human plasma prekallikrein is a single-chain polypeptide of 619 amino acids that is converted to the active, plasma kallikrein by cleavage of an internal Arg-Ile bond (Chung *et al.*, 1986). Plasma kallikrein is composed of a heavy chain (371 amino acids) and a light chain (248 amino acids) held together by a disulphide bond. It is the light chain of plasma kallikrein which contains its catalytic activity: being homologous to trypsin proteases (Chung *et al.*, 1986). Plasma kallikrein hydrolyses Lys-Arg and Arg-Ser bonds in high-molecular weight kininogen to release bradykinin. Low molecular weight kininogen is a poor substrate for plasma kallikrein, bradykinin can be generated from this kininogen in the presence of neutrophil elastase (Sato & Nagasawa, 1988).

1.6.1.1.3 Tissue kallikrein

Three genes clustered on chromosome 19 have been identified as belonging to the human kallikrein gene family (Baker & Shine, 1985; Fukushima *et al.*, 1985; Schedlich *et al.*, 1987; Evans *et al.*, 1988), but more may exist as 19 genomic clones have been identified which hybridise with monkey kallikrein cDNA probes (Murray *et al.*, 1990). Tissue kallikrein is present in many human tissues, particularly the pancreas, kidney, salivary glands and the mucosa of the airways and gastrointestinal tract. Human urinary tissue kallikrein has been widely studied and appears to be synthesised bound to 17 amino acids which are cleaved off to form an inactive precursor. The inactive form of human tissue kallikrein is activated by cleaving seven amino acids from the NH_2-terminal (Takahashi

et al., 1986). Active tissue kallikrein consists of 238 amino acids with multiple glycosylation sites (Takahashi *et al.*, 1988; Lu *et al.*, 1989; Kellermann *et al.*, 1988), and this variable glycosylation may explain the heterogeneity in the observed molecular weights of tissue kallikrein (Fiedler & Hirschauer, 1981). Tissue kallikrein releases kallidin by hydrolysis of Met-Lys and Arg-Ser bonds in the high and low molecular weight kininogen molecules. The failure of tissue kallikrein to generate bradykinin results from the inability to accommodate the Lys-Arg-Pro sequence present in the kininogen molecule. Tissue kallikrein is, therefore, unable to cleave the Lys-Arg bond and produce bradykinin (Bhoola *et al.*, 1992).

1.6.1.1.4 Mechanisms of kinin formation

The formation of bradykinin in the plasma is dependent upon coagulation factor XII (Hageman factor). Hageman factor, in its unactivated state, is a single-chain globulin with a molecular weight of 80,000 (Cochrane & Wuepper, 1971). Upon contact with certain negatively charged surfaces, for example glass, silicates or heparin (Proud & Kaplan, 1988), Hageman factor becomes activated and converts plasma prekallikrein to kallikrein; and bradykinin is cleaved from HMW-kininogen. Activated Hageman factor also activates coagulation factor XI, which circulates bound to HMW-kininogen. Activated plasma kallikrein has a powerful positive feedback effect in that it further activates Hageman factor. Tissue kallikrein can be activated by a variety of proteolytic enzymes, for example trypsin, plasmin or plasma kallikrein (Proud & Kaplan, 1988). There have been reports that a number of other proteases generated in the inflammatory response may be able to liberate kinins from kininogen: these include mast cell tryptase (Proud *et al.*, 1987b), calpains (Higahiyama *et al.*, 1986), eosinophil cationic protein (Venge *et al.*, 1979) and proteases derived from the house-dust mite (Takahashi *et al.*, 1990). The mechanisms of bradykinin formation by plasma and tissue pathways is shown in figure 1.1

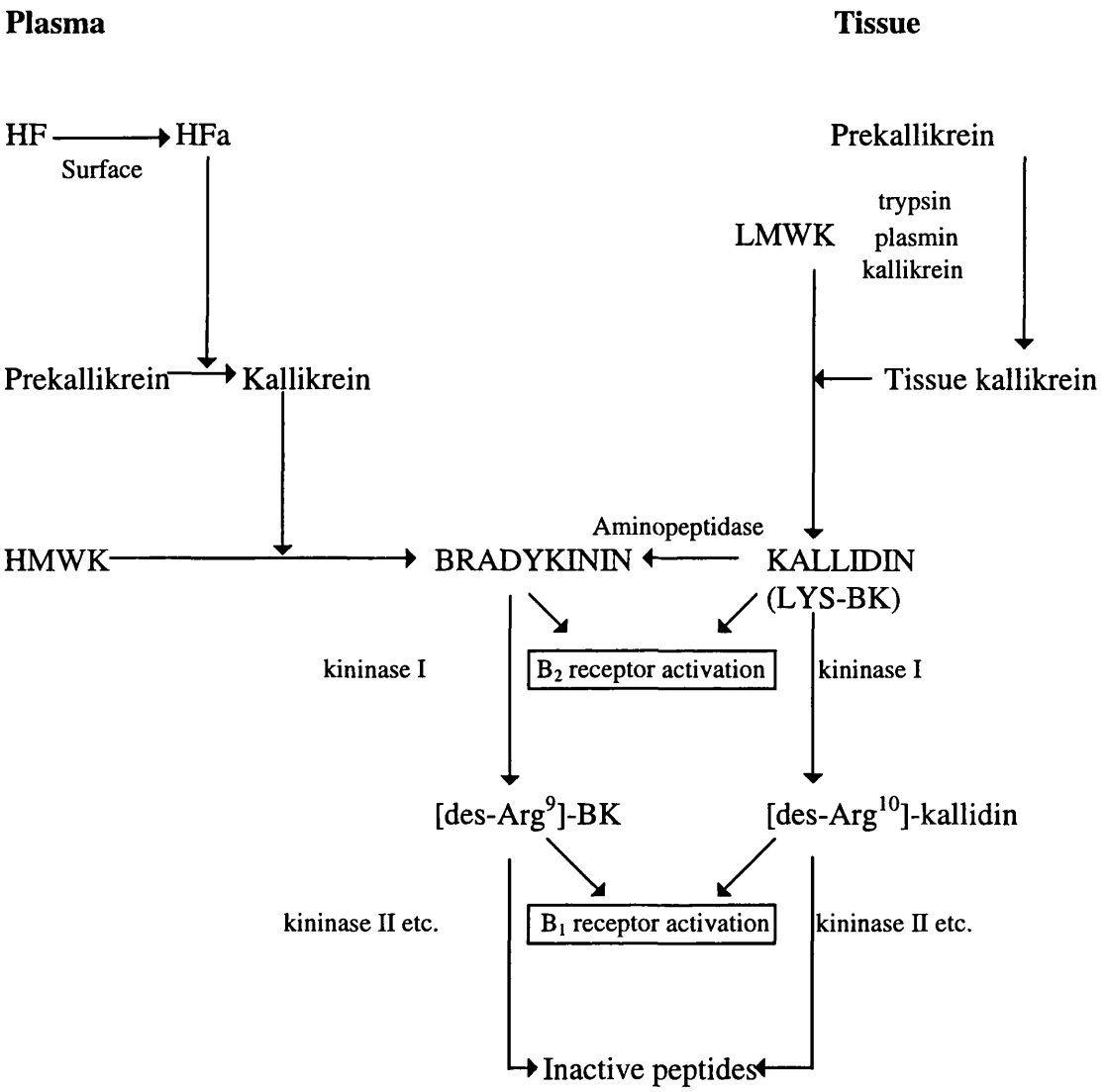

Figure 1.1. Mechanisms of kinin generation and breakdown. HF and Hfa = Hageman factor, unactivated and activated. HMWK and LMWK = high and low molecular weight kininogen, respectively.

1.6.1.1.5 Kininase I - carboxypeptidases

Kininase I is a family of enzymes consisting of carboxypeptidase N and M (Bhoola *et al.*, 1992). Carboxypeptidase N is synthesised in the liver and is the major kininase in human plasma (Zacest *et al.*, 1974), being attached to the vascular endothelium (Palmierti *et al.*, 1986). It removes the Arg^9 residue from the C-terminus of bradykinin forming [des-arg^9]-bradykinin, a kinin B_1 receptor agonist. Carboxypeptidase M is located in various sites including the human kidney, lung and fibroblasts (Bhoola *et al.*, 1992). Its mechanism of action in kinin inactivation is the same as carboxypeptidase N.

1.6.1.1.6 Kininase II - peptidases

The kininase II family consists of angiotensin-converting enzyme (ACE) and neutral endopeptidase. ACE is a single-chain transmembrane glycoprotein which binds to cell membranes (Mombouli & Vanhoutte, 1995). If exogenous bradykinin is administered to humans, it is mainly inactivated by endothelial ACE after one passage through the lungs (Bonner *et al.*, 1990). ACE hydrolyses two bonds in the bradykinin molecule. First it releases the Phe^8-Arg^9 fragment from the C-terminus, then ACE hydolyses the Ser^6-Pro^7 bond (Bhoola *et al.*, 1992). As well as being present in the lungs, ACE is present in many other sites, including the kidney, brain, placenta and intestines. Neutral endopeptidase has been shown to be present in various sites including the human kidney, intestines, fibroblasts and neutrophils (Bhoola *et al.*, 1992). Like ACE, neutral endopeptidase inactivates bradykinin by releasing the Phe^8-Arg^9 fragment from the C-terminus.

1.6.1.2 Bradykinin receptors and receptor antagonists

Bradykinin receptors were originally classified according to the relative agonist potencies, in isolated vascular smooth muscle preparations, of a series of structurally related peptide analogues of bradykinin (Regoli & Barabe, 1980). This led to the receptors being classed as B_1 and B_2.

1.6.1.2.1 Bradykinin B_1 receptor

Bradykinin B_1 receptors, classically found in the rabbit aorta or mesenteric vein, show the following order of agonist potency:

[des-Arg^{10}]-kallidin > [des-Arg^{9}]-bradykinin > kallidin > bradykinin > [Tyr(Me)^{8}]-bradykinin.

The cleavage of the C-terminal Arg^{9} residue from the native kinins is catalysed by the kininase I family of enzymes. [Des-Arg^{10}]-kallidin and [des-Arg^{9}]-bradykinin are about 10 times more potent B_1 receptor agonists than kallidin and bradykinin (Regoli & Barabe, 1980). The demonstration of this distinct order of agonist potency at the B_1 kinin receptor provided the first pharmacological criterion to demonstrate the receptors presence. The second and decisive criterion for defining B_1 receptor-mediated effects came with the development of selective B_1 receptor antagonists. The first antagonist was developed by replacing the Phe^{8} residue of [des-Arg^{9}]-bradykinin with a leucyl residue (Regoli *et al.*, 1977). A number of B_1 receptor antagonists have now been produced and their selectivity provides a critical means of characterising B_1 receptor-mediated effects.

The human B_1 kinin receptor is a 353 amino acid protein which has 36% sequence homology with the human B_2 receptor (Menke *et al.*, 1994). The cloned receptor possesses seven transmembrane domains, typical of G-protein-linked receptors. It also

contains three potential N-glycosylation sites (Menke *et al.*, 1994). Interestingly, cloned murine DNA coding for a functional bradykinin receptor exhibits both B_1 and B_2 pharmacological selectivity when expressed in xenopus oocytes (Mcintyre *et al.*, 1993). Cloned receptors from rat and human sources exhibited only B_2 receptor properties. However, Hess *et al.* (1994) replicated this experiment and found the cloned mouse DNA expressed the B_2 kinin receptor profile only.

The B_1 kinin receptor is expressed in only a few tissues but appears to be up-regulated in some forms of tissue injury and inflammation (Marceau, 1995).

1.6.1.2.2 Bradykinin B_2 receptor

The order of agonist potency at the bradykinin B_2 receptor is as follows:

kallidin ≥[Tyr(Me)^8]-bradykinin ≥ bradykinin > [des-Arg^{10}]-kallidin>

[des-Arg^9]-bradykinin.

This order of potency was first identified in isolated smooth muscle tissue preparations, such as the rabbit jugular vein or dog carotid artery (Regoli & Barabe, 1980). It now appears that most effects of bradykinin *in vivo*, such as inflammation, bronchoconstriction and hypotension, as well as in isolated tissues are mediated by the B_2 receptor.

Bradykinin B_2 receptor antagonists have been extensively developed and have revealed a lot of information about the physiological and pathological roles of bradykinin. The equilibrium dissociation constants for bradykinin B_2 receptor antagonists have been calculated from functional and binding studies in a number of tissues. For a selection of B_2 antagonists, these data are summarised in Table 1.1. It is interesting that bradykinin B_2 receptor antagonists appear more potent in binding studies than in functional studies (table 1.1). This has been termed the 'binding paradox' (Hall, 1992) and appears to be

related to the different ionic composition of the buffers used in binding and functional studies. Burch *et al.* (1994) reported that the binding of bradykinin and bradykinin analogues to the B_2 receptor is sensitive to the concentration of cations in the medium. Both the study by Burch *et al.* (1994) and a study by Ransom *et al.* (1992) demonstrate that if binding experiments are performed in a physiological buffer, for example Kreb's buffer, then bradykinin and bradykinin analogues have approximately 20 fold lower affinity for the B_2 receptor than in a medium of low ionic strength (e.g., N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES) medium). Therefore, although the rank order of affinities of the antagonist ligands in binding studies correlates well with the order of antagonist affinities calculated from functional studies, the absolute values for the equilibrium dissociation constants calculated by binding studies must be interpreted with caution.

The first B_2 antagonists were produced by substituting D-phenylalanine for the L-proline in position 7 of the bradykinin sequence (Stewart & Vavrek, 1985). Various [D-Phe^7]-bradykinin analogues have since been developed, but all have a relatively weak affinity for the B_2 bradykinin receptor and are substrates for enzymatic degradation by carboxypeptidase N (Proud *et al.*, 1987a). An example of a D-Phe substituted bradykinin B_2 receptor antagonist is NPC 567 ([D-Arg^0, Hyp^3, D-Phe^7]-bradykinin) which has a relatively low potency against B_2 receptor-mediated effects ($pA_2 \approx$ 5-6, reviewed in table 1.1). NPC 567 and other D-Phe substituted bradykinin B_2 receptor antagonists have been reported to be partial agonists in certain tissues (Regoli *et al.*, 1993). NPC 567 at low concentrations (10^{-7}-10^{-8} M) acts as an antagonist, but at higher concentrations (10^{-6} M) acts as a partial agonist with about half the efficacy of bradykinin. The agonist effects of NPC 567 can be blocked by the pure antagonist [D-Arg^0, Hyp^3, D-Phe^7, Leu^8]-bradykinin (Regoli *et al.*, 1993), demonstrating that the agonist activity of NPC 567 is receptor-mediated. The affinity for the B_2 receptor is increased and the agonistic activity is greatly reduced by replacing the phenylalanine with leucine at position 8 of the bradykinin primary structure, for example [D-Arg^0, Hyp^3, D-Phe^7, Leu^8]-bradykinin (Regoli *et al.*, 1990).

		Functional studies	Radioligand binding studies		
Ligand	Tissue	Equilibrium dissociation constant (nM)	Equilibrium dissociation constant (nM)	Type of binding experiment	Reference
NPC567	Human lung		265.82	Competition	Trifilieff *et al.*, 1994
	Guinea pig gall bladder	8912.5	61.66	Competition	Falcone *et al.*, 1993
	Guinea pig ileum	2691.5			Pruneau *et al.*, 1995
	Guinea pig ileum		210	Competition	Sequin *et al.*, 1992
	Guinea pig ileum		29	Competition	Farmer *et al.*, 1989
	Guinea pig ileum		8.3	Competition	Lyon *et al.*, 1990
	Guinea pig lung	2818.4			Pruneau *et al.*, 1995
	Guinea pig lung		31 >100μM***	Competition	Farmer *et al.*, 1989
	Guinea pig trachea		>100μM***	Competition	Farmer *et al.*, 1989
	Guinea pig trachea	2630.3			Pruneau *et al.*, 1995
	Guinea pig cultured tracheal smooth muscle cells		12	Competition	Farmer *et al.*, 1991
	Guinea pig nasal turbinate		15	Competition	Fujiwara *et al.*, 1989
	Sheep trachea		29 >100μM***	Competition	Farmer *et al.*, 1989
	Sheep lung		30 >100μM***	Competition	Farmer *et al.*, 1989
	Sheep nasal turbinate		9.5	Competition	Lyon *et al.*, 1990
	Rat lung		9.0	Competition	Tsukagoshi *et al.*, 1995

Table 1.1. The equilibrium dissociation constants for bradykinin B_2 receptor antagonists in a variety of tissues. The values given are from functional or radioligand binding studies. For the values from functional data, unless otherwise stated, the studies investigated the potency of the antagonist to inhibit bradykinin-induced contractions in isolated tissue. The equilibrium dissociation constants were calculated by the Schild method (Schild, 1947). For the binding data, all studies used membrane preparations unless otherwise stated. All binding studies used [^{3}H]-bradykinin as the radioligand unless otherwise indicated. * indicates the presence of multiple binding sites. ** indicates the radioligand used was [^{125}I]para-iodophenyl icatibant. *** indicates binding to the bradykinin B_3 receptor. **** indicates the radioligand was [^{3}H]-NPC 17731.

		Functional studies	Radioligand binding studies		
Ligand	Tissue	Equilibrium dissociation constant (nM)	Equilibrium dissociation constant (nM)	Type of binding experiment	Reference
Icatibant	Human lung		0.73	Competition	Trifilieff *et al.*, 1994
	Human IMR -90 fetal lung fibroblasts		0.4	Competition	Sawutz *et al.*, 1994b
	Human umbilical vien	3.24			Gobeil *et al.*, 1996
	Guinea pig gall bladder		0.011	Competition	Falcone *et al.*, 1993
	Guinea pig ileum	3.8	0.79	Competition	Hock *et al*, 1991
	Guinea pig ileum		0.57	Competition	Sequin *et al.*, 1992
	Guinea pig ileum		0.015**	Saturation	Brenner *et al.*, 1993
	Guinea pig lung		61.2* 0.06*	Competition	Sequin *et al.*, 1992
	Guinea pig lung	3.02			Pruneau *et al.*, 1995
.	Guinea pig brain		120* 0.31*	Competition	Sequin *et al.*, 1992
	Guinea pig trachea	7.41			Pruneau *et al.*, 1995
	Rat lung		0.097	Competition	Tsukagoshi *et al.*, 1995
NPC 17731	Guinea pig ileum		0.12****	Saturation	Burch *et al.*, 1994
	Guinea pig gall bladder		0.30	Competition	Falcone *et al.*, 1993
	Guinea pig lung	63.1			Lach *et al.*, 1994
	Guinea pig epithelium denuded trachea		0.48	Competition	Trifilieff *et al.*, 1993
	Guinea pig epithelium intact trachea		0.46	Competition	Trifilieff *et al.*, 1993
NPC 17761	Guinea pig lung	25.12			Lach *et al.*, 1994
	Guinea pig trachea	50.12			Da Silva *et al.*, 1995
	Guinea pig epithelium denuded trachea		0.036	Competition	Trifilieff *et al.*, 1993
	Guinea pig epithelium intact trachea		0.048	Competition	Trifilieff *et al.*, 1993

Table 1.1 (cont.).

		Functional studies	Radioligand binding studies		
Ligand	Tissue	Equilibrium dissociation constant (nM)	Equilibrium dissociation constant (nM)	Type of binding experiment	Reference
WIN 64338	Human IMR -90 fetal lung fibroblasts		64	Competition	Sawutz *et al.*, 1994a
	Bradykinin - stimulated $^{45}Ca^{2+}$ efflux from human IMR -90 fetal lung fibroblasts	79.4			Sawutz *et al.*, 1994a
	Human umbilical vein	1023.3			Marceau *et al.*, 1994
	Human umbilical vein	Inactive			Gobeil *et al.*, 1996
	Rabbit jugular vein	724.4			Marceau *et al.*, 1994
	Guinea pig ileum	6.5			Sawutz *et al.*, 1994a
	Guinea pig ileum	10.72			Farmer & DeSiato, 1994
	Guinea pig ileum	26.9			Pruneau *et al.*, 1995
	Guinea pig trachea	>1μM***			Farmer & DeSiato, 1994
	Ferret trachea	>1μM***			Farmer & DeSiato, 1994
	Guinea pig trachea	43.7			Pruneau *et al.*, 1995
	Guinea pig trachea	64.3	50.9	Competition	Scherrer *et al.*, 1995
	Guinea pig ileum		34.1	Competition	Scherrer *et al.*, 1995

Table 1.1 (cont.).

Recently, antagonists have been developed which are based on modifying amino acids in positions 7 and 8 of bradykinin. One of the most potent of these antagonists is the compound icatibant (formerly called Hoe 140); DArg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Lembeck *et al.*,1991; Wirth *et al.*,1991; Hock *et al.*, 1991). In functional studies, icatibant is two to three orders of magnitude more potent at antagonising B_2 bradykinin receptor mediated effects ($pA_2 \approx$ 8-9) compared with D-Arg-[Hyp^2, Thi^{5,8}, D-Phe^7]-bradykinin (Wirth *et al.*,1991; Hock *et al.*, 1991) and it has no agonist activity, even at high concentrations (Regoli *et al.*, 1993). In binding studies on a variety of tissues, icatibant has a high affinity for the B_2 receptor: icatibant's binding affinity is at least two orders of magnitude greater than the that of NPC 567 (see table 1.1). It is proposed that the high affinity of icatibant is caused by the amino acids D-Tic^7,Oic^8 which form a large hydrophobic region leading to prolonged occupation of, and high affinity for the bradykinin B_2 receptor (Rhaleb *et al.*, 1992). Icatibant is also highly resistant to enzymatic degradation as it is not a substrate for kininase I or II (Hock *et al.*, 1991).

Two more potent bradykinin B_2 receptor antagonists are NPC 17731 (D-Arg^0[Hyp^3, D-HypE(trans-propyl)^7,Oic^8]-bradykinin) and NPC 17761 (D-Arg^0[Hyp^3, D-HypE(trans-thio-phenyl)^7, Oic^8]-bradykinin). Table 1.1 presents data indicating that, in the guinea-pig, these antagonists have a high affinity for the bradykinin B_2 receptor. Both NPC 17731 and NPC 17761 are highly potent antagonists of the actions of bradykinin in the guinea-pig trachea: a tissue purported to contain bradykinin B_3 receptors (Trifilieff *et al.*, 1993).

Another potent B_2 receptor antagonist is [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin (Lammek *et al.*, 1990). The antagonist is produced by acylation of the N-terminus of the bradykinin B_2 receptor antagonist D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin; this acylation yielding a B_2 receptor antagonist ten times more potent than its parent compound (Lammek *et al.*, 1990).

The first nonpeptide bradykinin B_2 receptor antagonist has recently been developed (Sawutz *et al.*, 1994a). WIN 64338 {[[4-[[2 -[[bis (cyclohexylamino) methylene] amino]-3- (2-naphthyl) -1-oxopropyl] amino] phenyl] methyl] tributylphosphonium chloride monohydrochloride} inhibits the binding of [^{3}H]-bradykinin to the bradykinin B_2 receptor on human IMR-90 cells. It also competitively inhibits bradykinin-induced calcium efflux from IMR-90 cells and inhibits bradykinin-induced contractions of the guinea pig ileum. However, the effect of WIN 64338 on bradykinin-induced effects in functional studies on isolated human tissue is equivocal. In the human isolated umbilical vein, Marceau *et al.* (1994) reported that WIN 64338 behaved as a competitive and selective antagonist at the bradykinin B_2 receptor. However, another study reports that this antagonist is inactive in the same tissue (Gobeil *et al.*, 1996). The reason for the discrepancy is unclear.

1.6.1.2.3 Bradykinin B_2 receptor heterogeneity

The use of bradykinin B_2 receptor antagonists has produced reports of B_2 receptor heterogeneity (table 1.1). A bradykinin B_3 receptor type has been proposed to exist in the guinea-pig lower airways (Farmer *et al.*, 1989). The B_3 receptor is described as having no affinity for [des-Arg^9]-bradykinin, and the effects mediated by this B_3 receptor type are only antagonised by selected bradykinin B_2 receptor antagonists. While no B_3-selective bradykinin receptor agonists or antagonists have yet been developed, certain B_2 receptor antagonists, for example NPC 567, have been shown to be inactive at the B_3 receptor type (Farmer *et al.*, 1989, see table 1.1). The bradykinin B_2 receptor antagonists, NPC 567 and NPC 349 ([D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin), are reported to be inactive at the B_3 receptor as they only weakly inhibit bradykinin-induced bronchoconstriction in guinea-pigs *in vivo* and are virtually inactive in studies with isolated tracheal smooth muscle (Farmer *et al.*, 1989). However, bradykinin-induced contraction of the isolated trachea of the guinea-pig is inhibited by the potent B_2 receptor antagonists icatibant (Field *et al.*, 1992) and NPC 16731 ([D-Arg^0, Hyp^3, Thi^5, D-Tic^7, Tic^8]-bradykinin) (Farmer *et al.*, 1991a). Therefore, it is proposed that icatibant and NPC 16731 are potent bradykinin

receptor antagonists at both the B_2 and B_3 receptors. In binding studies, NPC 567 failed to displace the specific binding of [^{3}H]-bradykinin from the guinea-pig trachea and displaced only approximately 60% of the specific binding from the guinea-pig lung (Farmer *et al.*, 1989). A similar binding profile is reported for the sheep lower airways (Farmer *et al.*, 1989). NPC 16731 fully displaces the specific binding of [^{3}H]-bradykinin to the guinea-pig airways (Farmer *et al.*, 1991a). The NPC 567-resistant binding site for [^{3}H]-bradykinin is purported to be binding to the bradykinin B_3 receptor type. In guinea-pig tracheal smooth muscle cells, cultured in a medium containing radiolabelled calcium, bradykinin stimulated an efflux of $^{45}Ca^{2+}$ (Farmer *et al.*, 1991a,b). The bradykinin-induced calcium efflux was antagonised NPC 16731, but not by NPC 567, again indicating that this tissue may contain a B_3 receptor. The non-peptide B_2 receptor antagonist, WIN 64338, also failed to antagonise bradykinin-induced contractions of isolated guinea-pig trachea, and this is cited as further evidence for the existence of a B_3 receptor (Farmer & DeSiato, 1994). In studies on the contractile responses of the guinea-pig taenia caeci, the response to bradykinin was only weakly inhibited by NPC 567 and NPC 349 (Field *et al.*, 1992). However, this bradykinin-induced contraction was potently antagonised by icatibant. The similarity between the effects of bradykinin receptor antagonists on this tissue and on the guinea-pig lower airways provides evidence for the existence of bradykinin B_3 receptors in the guinea-pig taenia caeci. The existence of the bradykinin B_3 receptor type is not yet fully established, and various studies have failed to confirm the presence of a bradykinin B_3 receptor type in the guinea-pig airways (Pruneau *et al.*, 1994; Trifilieff *et al.*, 1994; Scherrer *et al.*, 1995). This is discussed further in chapter 4. Bradykinin B_3, B_4 and B_5 receptor types have also been proposed to be present in the oesophagus of the opossum (Saha *et al.*, 1990; 1991). Bradykinin has a low potency at the B_4 and B_5 subtypes ($_pD_2$ 5.1-5.5). At these concentrations the effects of bradykinin may be receptor-independent. For example, histamine release induced by bradykinin appears to be receptor-independent (Devillier *et al.*, 1988). Also only two bradykinin receptor antagonists were used in functional studies on the isolated oesophagus of the opossum. The effects of a larger number of bradykinin receptor antagonists must be

investigated before any definitive characterisation of the opossum bradykinin receptor(s) can be made.

Subtypes of the bradykinin B_2 receptor have also been proposed. Regoli *et al.* (1993) propose that species differences in the pharmacological profile of B_2 receptors reflect B_2 receptor subtypes: B_{2A} (rabbit, dog and possibly man) and B_{2B} (guinea-pig, hamster, rat). This classification of the B_2 receptor as B_{2A} and B_{2B} is largely based on [D-Phe^7]-substituted bradykinin B_2 receptor antagonists. For example, NPC 567, is more potent at the B_{2A} subtype than the B_{2B}. [D-Phe^7]-substituted bradykinin B_2 receptor antagonists were also used to propose the existence of B_2 receptor subtypes in the rat vas deferens (Llona *et al.*, 1987; Rifo *et al.*, 1987). The bradykinin B_2 receptor antagonist [Thi^{5,8}, D-Phe^7]-bradykinin, acts as a partial agonist on neuronal presynaptic B_2 receptors, but is an antagonist at postsynaptic muscular B_2 receptors. This lead to the B_2 receptors in the rat vas deferens being classed as muscular or neuronal. However, Regoli *et al.* (1993) demonstrated that only B_{2B} receptors are present in the rat vas deferens on the basis of using B_2 receptor antagonists, devoid of any agonist activity. The different effects of [Thi^{5,8}, D-Phe^7]-bradykinin on the neuronal and muscular tissues of the rat vas deferens probably reflects tissue differences in receptor numbers, or G-protein and second messenger levels. Recently, developments in the molecular biology of the bradykinin receptor has brought into question whether the evidence for bradykinin B_2 receptor subtypes is sound. Hess *et al.* (1994) and Davis *et al.* (1995) showed that the B_2 receptors cloned from different species have different sensitivities to D-Phe^7-containing antagonists. As the evidence for B_2 receptor heterogeneity is largely based on the affinities of D-Phe^7-containing antagonists, much of the evidence proposing subtypes may reflect species divergence in the evolution of the bradykinin B_2 receptor. This will be discussed further in chapter 4. From binding studies, three B_2 receptor subtypes, B_{2A}, B_{2B} and B_{2C}, have been proposed in different tissues of the guinea-pig (Seguin *et al.*, 1992, see table 1.1). By investigating the effect of guanine nucleotides on the binding to these proposed receptors (Seguin & Widdowson, 1993), the B_{2A} binding site is proposed to be coupled to a G-protein, thus providing some evidence that this binding site may be a

functioning receptor. However, the binding of [^{3}H]-bradykinin to the B_{2B} and B_{2C} sites was unaffected by guanine nucleotides and there was no functional data to relate to this binding evidence. Hence, the three binding sites for bradykinin in the guinea-pig may not all represent different functioning receptors.

1.6.1.2.4 Molecular biology and signal transduction mechanisms of the bradykinin B_2 receptor

The human bradykinin B_2 receptor has been cloned from lung fibroblasts (Hess *et al.*, 1992). The cDNA clone codes for a receptor containing seven membrane spanning domains that are the hallmark of G-protein-coupled receptors. The human receptor protein contains 364 amino acids and has a molecular mass of approximately 41 kDa in the non-glycosylated state. The amino acid sequence of the human B_2 receptor clone has 81% sequence homology with the cloned smooth muscle rat B_2 receptor (McEachern *et al.*, 1991), and 98% sequence homology with the cloned murine B_2 receptor (Hess *et al.*, 1994). The human cloned receptor has three potential glycosylation sites on the extracellular domain and four potential phosphorylation sites on the intracellular domain (Burch *et al.*, 1994). Hess *et al.* (1992) transfected the human B_2 receptor cDNA into COS-7 cells and performed binding assays on the membranes. Saturation binding revealed a single binding site for [^{3}H]-bradykinin over the concentration range used (K_d=0.13nM). In competition binding studies, the B_1 receptor ligands, [des-Arg^9]-bradykinin and [des-Arg^9, Leu^8]-bradykinin, were very weak inhibitors of the specific binding of [^{3}H]-bradykinin to the transfected COS-7 cell membranes. Icatibant was the most potent B_2 antagonist used in the competition experiments, almost 300 times more potent than NPC 567 at inhibiting the specific binding of [^{3}H]-bradykinin (Hess *et al.*, 1994). The binding profile of the cloned human B_2 receptor is, therefore, consistent with previous characterisations of the bradykinin B_2 receptor in various tissues (Table 1.1). It is interesting that the cloned mouse B_2 receptor has 60 to 80 fold higher affinity for NPC 567 and [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin when compared with the human cloned receptor (Hess *et al.*, 1994). Davis *et al.*, (1995) reported that cloned rat B_2

receptors are also more sensitive to these antagonists when compared with the human receptor clone. Therefore, there appears to be species differences in the pharmacological profile of the bradykinin B_2 receptor.

Many studies have provided evidence that the bradykinin B_2 receptor is G-protein coupled. The signal transduction, for example the activation of phospholipase A_2 or the production of inositol phosphates, following B_2 receptor activation is sensitive to guanine analogues and pertussis toxin (Burch & Axelrod, 1987; Higashida *et al.*, 1986). The binding of bradykinin to bovine myometrial membranes is also sensitive to guanine nucleotide analogues (Leeb-Lundberg & Mathis, 1990) and the GTPase activity of rat myometrial membranes is increased by bradykinin activation of the B_2 receptor (Liebmann *et al.*, 1990). In the rat myometrium, $G\alpha_{i2}$ has been implicated as the bradykinin-coupled G-protein (Liebmann *et al.*, 1990). Similarly, in rat dorsal root ganglion, $G\alpha_{i2}$, together with $G\alpha_o$, appear to mediate the pertussis-toxin-sensitive ability of bradykinin B_2 receptor activation to open calcium channels (Ewald *et al.*, 1989). The pertussis-toxin-insensitive G_q appears to couple the B_2 receptor to phospholipase C in NG108 cells (Gutowski *et al.*, 1991). The cloning of the human B_2 receptor has demonstrated seven membrane spanning domains, a characteristic of all known G-protein-coupled receptors (Hess *et al.*, 1992).

The signal transduction mechanisms of the bradykinin B_2 receptor have been widely studied in numerous tissues. A brief overview of the main mechanisms will be included here, with particular reference to the mechanisms in the vascular endothelial cell. In nearly all tissues, bradykinin B_2 receptor stimulation causes, via G-protein coupling, activation of phospholipase C which generates diacylglycerides and inositol phosphates (Derian & Moskowitz, 1986; Myers & Larkins, 1989). These second messengers lead to the activation of protein kinase C and cause an increase in cytosolic calcium, respectively. The calcium concentration of the cytosol increases due to depletion of intracellular stores and calcium influx across the plasma membrane. In endothelial cells, the influx of calcium across the plasma membrane is enhanced by hyperpolarisation of the cell (Adams

et al., 1989). The hyperpolarisation is brought about by the activation of potassium channels, which are opened either by G-protein coupling to the B_2 receptor, by an increase in intracellular calcium or by an increase in cyclic AMP (Mombouli & Vanhoutte, 1995). It has been suggested that endothelial-derived hyperpolarizing factor (EDHF) may contribute to kinin-induced opening of potassium channels in endothelial cells (Mombouli & Vanhoutte, 1995), but as the precise identity of EDHF is still unknown, this hypothesis remains uncertain. In many of tissues, including endothelial cells, bradykinin induces, via activation of phospholipase A_2, the release of arachidonic acid and the generation of eicosanoids (Burch *et al.*, 1994). The stimulation of phospholipase A_2 may be a result of raised intracellular calcium (Luckhoff, 1988) or may result from the activation of a G-protein linked to the B_2 receptor (Ricupero *et al.*, 1993). In many systems, eicosanoid generation plays a significant role in the actions of bradykinin, and inhibitors of eicosanoid synthesis often markedly attenuate responses to bradykinin (Regoli & Barabe, 1980). The synthesis of prostaglandins, for example PGI_2, can lead to increases in cellular cyclic AMP. In endothelial cells, the raised intracellular calcium and intracellular alkalization induced by bradykinin can cause the generation of nitric oxide (Luckhoff, 1988; Flemming *et al.*, 1994). The role of nitric oxide in mediating the effects of bradykinin will be discussed further in chapter 5. In fibroblasts, bradykinin causes tyrosine kinase activation and this partly regulates intracellular calcium levels (Lee *et al.*, 1993).

1.6.1.3 The role of neuropeptides in the actions of bradykinin

Bradykinin stimulates the release of neuropeptides from the peripheral terminals of some capsaicin-sensitive nerves (Geppetti, 1993). In certain tissues, the release of neuropeptides, for example calcitonin gene-related peptide (CGRP) and substance P, accounts for some of the effects of bradykinin. The first demonstration that bradykinin mediates its effects by releasing peptides from sensory nerve endings was obtained using the rabbit iris smooth muscle (Butler & Hammond, 1980; Ueda *et al.*, 1984). *In vivo* and *in vitro*, the bradykinin-induced contractile response of the rabbit iris is inhibited by

physical destruction of the neural innervation, by pretreatment with capsaicin (depleting the sensitive neurons of neuropeptides) or by neuropeptide receptor antagonists. The actions of bradykinin in many tissues have now been demonstrated to be mediated, at least in part, by the release of neuropeptides. Examples include bronchoconstriction in the guinea-pig lower airways (Ichinose *et al.*, 1990), plasma extravasation in rat and human skin (Saria *et al.*, 1983; Wallengren & Hakanson, 1992), increases in vascular permeability in the guinea-pig and rat nasal mucosa (Ricciardolo *et al.*, 1994; Bertrand *et al.*, 1993) and CGRP release in the guinea pig isolated heart (Gepetti *et al.*, 1988). The release of neuropeptides by bradykinin is, in some tissues, dependent upon prostaglandin generation (Geppetti *et al.*, 1990), although other studies have reported bradykinin-induced neuropeptide release which is independent of prostaglandin synthesis (Franco-Cereceda, 1989). Bradykinin-induced contractions of the iris smooth muscle in the rabbit are totally abolished by B_2 receptor antagonists (Lembeck *et al.*, 1991), indicating that in this tissue, kinin-induced neuropeptide release may be mediated via B_2 receptor activation.

1.6.1.4 The role of histamine in the actions of bradykinin

Bradykinin and kinin analogues release histamine from rodent mast cells. This effect appears not to be receptor-mediated since kinin antagonists also stimulate histamine release (Devillier *et al.*, 1988). Positive charges at the N-terminal end of the amino acid sequence appear to be crucial for histamine release (Devillier *et al.*, 1989). The histamine release is not a result of cytotoxicity and is dose-dependently inhibited by pertussis toxin or benzalkonium chloride (Bueb *et al.*, 1990b). Bradykinin stimulates the GTPase activity of purified G-proteins and, therefore, it has been proposed that bradykinin releases histamine by direct activation of a pertussis-toxin sensitive G-protein in mast cells (Mousli *et al.*, 1991). Analogues of bradykinin have also been shown to release histamine from human skin mast cells (Cohan *et al.*, 1991).

1.6.1.5 The role of kinins in allergic rhinitis

1.6.1.5.1 Kinins are generated in allergic rhinitis

Bradykinin and kallidin are generated during the pathology of allergic rhinitis (Dolovich *et al.*, 1970; Proud *et al.*, 1983). Allergic subjects, sensitive to ragweed or grass allergen release kinins into the nasal cavity during the early response to nasal allergen challenge. The generation of inflammatory mediators in the nasal cavity can be investigated by analysis of nasal washes taken before and after challenge with allergen. The concentration of kinins present in washes increases after allergen challenge in allergic subjects, but not in matched non-allergic controls. Similarly, there is a significant increase in the level of kinins in the nasal wash during the late reaction (Naclerio *et al.* 1985). The late-phase release of kinins into the nasal cavity is significantly lower than the release in the early response. The kinin generation during both the early- and late-phase reactions correlates with the onset of clinical symptoms.

The evidence for kinin formation in nasal allergy presented so far has been obtained from nasal challenge studies where allergic rhinitis is experimentally induced by spraying allergen into the nasal cavity. However, there is also evidence supporting kinin generation in the nasal cavity of atopic individuals during the pollen season. Svensson *et al* (1990) demonstrated elevated kinin levels in nasal secretions of patients suffering from seasonal allergic rhinitis as a result of natural allergen exposure. Topical application of glucocorticoid reduces the kinin levels in nasal washes from patients with seasonal allergic rhinitis during the pollen season (Svensson *et al.*, 1994). This is interpreted as being the result of the ability of glucocorticoids to reduce plasma exudation into the nasal cavity. Therefore the transudation of kininogen into the nasal cavity would also be decreased thus reducing endogenous kinin formation. Kinin levels are also elevated during experimentally-induced and naturally occurring rhinovirus infections (Naclerio *et al.*, 1988; Proud *et al.*, 1990a).

1.6.1.5.2 Introducing kinins into the nasal cavity mimics some of the symptoms of allergic rhinitis

Exogenous bradykinin introduced into the nasal airways of normal or atopic human subjects induces some of the symptoms of allergic rhinitis: an increase in nasal resistance, raised vascular permeability and nasal pain. Proud *et al.* (1988) showed that introducing bradykinin into the human nasal airway caused an increase in nasal blockage, in nasal secretion and watery eyes as measured by symptom scores. Furthermore, bradykinin challenge increased albumin and TAME-esterase activity in nasal lavage fluid, and caused subjects to have a sore throat (Proud *et al.*, 1988; Doyle *et al* 1990). The nasal blockage, nasal secretion, watery eyes, sore throat, sneezing, albumin release and TAME-esterase release induced by bradykinin challenge appear not to desensitise with repeated nasal bradykinin administration (Churchill *et al.*, 1991).

The plasma exudation (increase in albumin levels of the nasal lavage fluid) induced by bradykinin challenge is significantly higher in patients with seasonal allergic rhinitis, challenged out of season, than in normal subjects (Brunnee *et al.*, 1991). Following bradykinin challenge, normal subjects release significantly more albumin and total protein (marker of protein from all sources) into the nose than subjects with severe perennial allergic rhinitis (Baraniuk *et al.*, 1994b). Bradykinin challenge in normal subjects does not stimulate lysozyme secretion into the nasal cavity (Baraniuk *et al.*, 1994b): lysozyme secretion being a marker for submucosal serous cell secretion. Glycoconjugates are secreted by submucosal mucus cells and epithelial goblet cells. Bradykinin challenge, in normal subjects, stimulates glycoconjugate secretion only at high doses. However, bradykinin challenge in subjects with perennial allergic rhinitis does stimulate significant lysozyme secretion into the nasal cavity and stimulates significantly more glycoconjugate secretion than bradykinin challenge in normal subjects (Baraniuk *et al.*, 1994b). Thus, in perennial allergic rhinitis, there are altered glandular responses to bradykinin.

The ability of bradykinin to cause nasal blockage has been confirmed by demonstrating dose-dependent increases in nasal airway resistance, measured by active posterior rhinomanometry (Doyle *et al.*, 1990; Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a), following bradykinin challenge. By using the technique of acoustic rhinometry, bradykinin challenge can be shown to reduce the patency of the nasal airway (Austin & Foreman, 1994b). Compared to histamine, bradykinin is more potent at causing increases in airway resistance and plasma exudation in the nose (Rajakulasingam *et al.*, 1993). Nasal challenge with kallidin also induces some of the symptoms of rhinitis (Rajakulasingam *et al.*, 1991). B_1 specific agonists [Des-Arg^9]-bradykinin and [Des-Arg^{10}]-kallidin have no effect on nasal airway resistance, albumin release into the nasal cavity or on symptom scores (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a). The actions of kinins appear, therefore, to be mediated via the B_2 receptor.

1.6.1.5.3 The effect of bradykinin receptor antagonists in the human nasal airway

Bradykinin binding sites are present in the nasal mucosa from the inferior turbinate bone. Autoradiography reveals that [^{125}I]-bradykinin binds to small muscular arteries, venous sinusoids and submucosal nerve fibres (Baraniuk *et al.*, 1990b). Antagonists at the B_2 receptor have been investigated with respect to the human nasal airway. Pretreatment with bradykinin B_2 receptor antagonists prior to bradykinin challenge reveals interesting data. Pongracic *et al* (1991) tested the effect of intranasal administration of the bradykinin B_2 receptor antagonist NPC 567 on various responses to nasal provocation with bradykinin. NPC 567, even at the high dose of 1mg per nostril, has no effect on any of the parameters measured; namely symptom scores or albumin and TAME-esterase levels in nasal lavage fluid. It also has no significant effect on bradykinin-induced nasal blockage measured quantitatively by active posterior rhinomanometry (Austin & Foreman, 1994a). However the highly potent bradykinin B_2 receptor antagonist, icatibant, antagonized the actions of bradykinin in the nasal airway. Icatibant, delivered by pump spray into the nasal cavity two minutes prior to bradykinin challenge, significantly antagonized the nasal blockage induced by bradykinin (Austin & Foreman, 1994a).

Bradykinin-induced albumin release into the nasal airway was also antagonized by icatibant. Another bradykinin B_2 receptor antagonist, [1-adamantane acetyl-D-Arg^0,Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin, antagonised bradykinin-induced nasal blockage and albumin release (Austin & Foreman, 1994a). Icatibant also significantly antagonized house dust mite-induced nasal blockage in individuals with allergic rhinitis to this antigen (Austin *et al.*, 1994).

1.6.1.5.4 The role of nerves in the nasal response to bradykinin

The nasal mucosa contains sensory nerves, parasympathetic and sympathetic nerves. The c-fibres contain calcitonin gene-related peptide, substance P, neurokinin A and gastrin-releasing peptide (Lundberg *et al.*, 1987; Sunday *et al.*, 1988). Parasympathetic fibres contain acetylcholine, vasoactive intestinal peptide and peptide histidine methionine (Said & Mutt, 1988). Sympathetic neurones contain noradrenaline and neuropeptide Y (Baraniuk *et al.*, 1992b). In addition, there is evidence for the localisation of neuronal nitric oxide synthase in nerves of the human nasal mucosa (Kulkarni *et al.*, 1994).

The contribution nerves make to the nasal actions of bradykinin is still being investigated. Capsaicin, which stimulates a subcategory of sensory nerves, introduced into the nasal cavity causes nasal discomfort and rhinorhoea: effects also induced by bradykinin challenge (Phillip *et al.*, 1994; Rajakulasingam *et al.*, 1992b). Capsaicin challenge also induces a significant increase in the total protein content of the nasal lavage fluid compared with a vehicle control. These observations suggest that capsaicin-sensitive neurones induce a glandular secretory response. But, whereas bradykinin causes nasal blockage and increased vascular permeability, capsaicin has no such effects. Hence, capsaicin-sensitive neurones may mediate bradykinin-induced nasal discomfort and rhinorhoea, but seem not to be involved in nasal blockage and increased vascular permeability. However, repeated applications of capsaicin, to desensitize capsaicin-sensitive neurones, failed to block the nasal pain produced by administering kallidin to the normal human nasal mucosa (Geppetti *et al.*, 1991). Thus, kinin-induced algesia in

the nose appears to be independent of activity in capsaicin-sensitive neurones. Unfortunately, these studies using capsaicin to desensitize neurons did not quantitatively measure the nasal blockage, rhinorhoea or vascular permeability changes following bradykinin challenge. Repeated application of capsaicin does block the algesic response to nasal challenge to low pH (citric acid), indicating that capsaicin-sensitive neurones in the nose mediate the pain response to hydrogen ions (Geppetti *et al.*, 1993). Pretreating the non-atopic human nasal cavity with the muscarinic receptor antagonist, ipratropium bromide, has no significant effect on bradykinin challenge suggesting that cholinergic pathways do not play a role in the nasal actions of bradykinin in normal non-atopic subjects (Rajakulasingam *et al.*, 1992a). However, parasympathetic nerves do appear to play a role in bradykinin-mediated nasal effects in allergic rhinitis patients . If bradykinin is applied to only one nostril, of subjects with perennial allergic rhinitis, the contralateral unstimulated nasal cavity produces raised levels of total protein and glycoconjugates in lavage fluid (Baraniuk *et al.*, 1994a&b). Normal subjects, treated in this way, do not show any changes in contralateral secretions. The contralateral total protein and glycoconjugate secretion induced by bradykinin is abolished by ipratropium bromide, indicating that parasympathetic nerve reflexes mediate this secretion (Baraniuk *et al.*, 1994a). There is considerable evidence that tachykinin release mediates bradykinin-induced increases in vascular permeability in the guinea-pig and rat nasal mucosa (Ricciardolo *et al.*, 1994; Bertrand *et al.*, 1993). However, there is no evidence yet that supports a role for nerves in the increased nasal vascular permeability or blockage induced by bradykinin in normal or atopic humans. The contribution of nerves to the action of bradykinin in the human nasal airway will be discussed further in chapter 5.

1.6.1.5.5 The role of prostaglandins in the nasal response to bradykinin

In a variety of systems, bradykinin mediates a number of its actions via the generation of prostaglandins. In humans, bradykinin injected intradermally into the skin increases microvascular blood flow and an inhibitor of cyclooxygenase, indomethacin, significantly inhibits this effect. Bradykinin also stimulates prostaglandin generation in human

respiratory epithelial cells and human vascular endothelial cells. In the nasal airways, aspirin pretreatment has no significant effect on bradykinin-induced albumin or TAME-esterase increases in the lavage, or on symptom scores reported by the subjects (Churchill *et al.*, 1991). Baraniuk *et al* (1992a) investigated the effect of ibuprofen on bradykinin-induced total protein, glycoconjugate and albumin release into the nasal cavity of normal human subjects. Ibuprofen is more potent at inhibiting the activity of cyclooxygenase than aspirin. Ibuprofen had no effect on bradykinin-induced total protein or albumin release. However, the glycoconjugate released into the nasal cavity by bradykinin provocation was significantly higher following ibuprofen treatment. They also measured lysozyme release, but as already discussed bradykinin does not release this into the nasal cavity of normal subjects. However, after ibuprofen treatment, bradykinin, 10nmol, (but not 100 or 1000nmol) causes a significant lysozyme release. The lack of a dose-dependent effect by bradykinin on lysozyme release in the presence of ibuprofen is difficult to explain and requires further investigation. It appears, therefore, that inhibition of cyclooxygenase may enhance bradykinin-induced glycoconjugate production, presumably either by blocking the production of inhibitory prostaglandins, promoting the production of lipoxygenase products or by some action unrelated to either of these. Human nasal explants show the opposite response to ibuprofen (Baraniuk *et al.*, 1990b). Ibuprofen treatment of *ex vivo* nasal tissue decreased bradykinin-induced glycoconjugate release. Whilst this shows the difference in results that can occur between *in vitro* and *in vivo* experiments it does not help to resolve the question of the relevance of cyclooxygenase products in the nasal action of bradykinin.

1.6.1.5.6 The role of histamine in the actions of bradykinin in the human nasal airway

As already discussed, bradykinin releases histamine from rodent mast cells and the human nasal mucosa contains a considerable number of these cells. Therefore, the contribution of histamine to the nasal actions of bradykinin has been investigated. Rajakulasingam *et al.* (1992) reported that an orally administered H_1 receptor antagonist, terfenadine, produced no effect on bradykinin-induced increases in nasal airway

resistance, rhinorhoea, nasal pain or the albumin level in the nasal lavage. However, Austin (1994) reported that two chemically different H_1 receptor antagonists, cetirizine and terfenadine, inhibit bradykinin-induced increases in nasal airway resistance, vascular permeability and symptom scores. The role of histamine in the nasal actions of bradykinin will be further discussed in chapter 6.

1.6.2 Histamine

For a long time, histamine has been implicated as a mediator of the acute allergic reaction. In an allergic reaction, histamine is released from mast cells and basophils generating the cardinal signs of inflammation, namely redness (vasodilatation), swelling (increased vascular permeability and vasodilatation leading to tissue oedema), heat (increased blood flow) and pain (histamine can stimulate C-fibres). The role of histamine in allergic rhinitis has been extensively researched and histamine receptor antagonists are one of the most common clinical treatments for allergic rhinitis.

The histamine levels in the nasal lavage fluid increase in both the early and late response to allergen challenge (Naclerio *et al.*, 1983; Naclerio *et al.*, 1985). As already discussed, the source of histamine in the early response appears to be the mast cell, whereas the histamine released during the late response is a result of basophil activation.

Intranasal administration of histamine generates symptoms characteristic of allergic rhinitis: nasal congestion, sneezing and rhinorrhoea. Histamine induces an increase in the nasal airway resistance (McLean *et al.*, 1977) and a reduction in the patency of the nasal airway (Austin & Foreman, 1994b). Changes in these parameters indicates that histamine causes nasal airway congestion. Histamine challenge of the nasal airway also induces a dose-dependent increase the albumin content of the nasal lavage fluid (indicating increased vascular permeability), increased glandular secretions and sneezing (Doyle *et al.*, 1990). Histamine-induced increases in glandular secretion, in vascular permeability and in sneezing are abolished by pretreatment with histamine H_1 receptor antagonists

(Raphael *et al.*, 1989). However, histamine-induced increases in nasal airway resistance are most effectively antagonised by a combination of H_1 and H_2 receptor antagonists (Havas *et al.*, 1986): the combination being more effective than either H_1 or H_2 receptor antagonists alone. A role for the histamine H_2 receptor in nasal airway blockage is supported by the report that the H_2 receptor agonist, impromidine, induces an increase in nasal airway resistance (Shelton & Eiser, 1994). Histamine H_2 receptors have been demonstrated to be present on the vasculature in the human nasal mucosa (Pipkorn, 1990): a site consistent with this receptor playing a role in regulating mucosal blood flow and, therefore, the patency of the nasal airway.

Histamine receptor antagonists are commonly used clinically as a treatment for allergic rhinitis. In experimentally-induced allergic rhinitis, following intranasal administration of grass pollen antigen, histamine H_1 receptor antagonists effectively inhibited rhinorrhoea and sneezing (reviewed by Meltzer, 1995). Pretreatment with H_2 receptor antagonists has also been reported to reduce antigen-induced rhinorrhoea (Holmberg *et al.*, 1989). However, histamine receptor antagonists do not inhibit the nasal blockage induced by antigen challenge (Rokenes *et al.*, 1988; Pipkorn, 1990; Corrado *et al.*, 1987; Hamilton *et al.*, 1994; Thomas *et al.*, 1992). Hence, other mediators may be involved in antigen-induced nasal blockage. The previous section reviewed in detail the role of bradykinin as a mediator of allergic rhinitis. The following sections will briefly review the evidence for other inflammatory mediators in allergic rhinitis.

1.6.3 Metabolites of arachidonic acid

1.6.3.1 Leukotrienes

The enzyme 5-lipoxygenase can act on arachidonate to produce 5-hydroperoxyeicosatetraenoic acid which is converted by a dehydrase to leukotriene A_4. Leukotriene A_4, a short-lived intermediate, can be further converted to either leukotriene B_4 (LTB_4) or a series of cysteinyl-leukotrienes, leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4). LTB_4 causes activation of monocytes, macrophages

and lymphocytes. The cysteinyl-leukotrienes cause contraction of bronchial smooth muscle and vasodilatation in most vessels except for the coronary vessels, where they produce vasoconstriction.

Strong evidence is emerging that leukotrienes play an important role in the pathophysiology of allergic rhinitis (Knapp & Murray, 1994). Leukotrienes are generated both in the early and late response to grass pollen nasal challenge in allergic subjects (Naclerio *et al.*, 1985). The type of leukotriene generated following experimentally-induced allergic rhinitis varies greatly between studies (Knapp & Murray, 1994). Nasal challenge with LTD_4 produces an increase in nasal blood flow (Bisgaard *et al.*, 1986) and nasal congestion (Okuda *et al.*, 1988) without itching or sneezing.

The strongest evidence for the involvement of leukotrienes in allergic rhinitis comes from the use of 5-lipoxygenase inhibitors in the human nasal airway. Knapp (1990) demonstrated that A-64077, a 5-lipoxygenase inhibitor, reduced nasal congestion and reduced the leukotriene B_4 levels in the nasal lavage fluid following nasal challenge with grass pollen in allergic subjects. Therefore, A-64077 appears to inhibit grass pollen-induced nasal congestion by inhibiting 5-lipoxygenase activity in the nasal cavity. In this study by Knapp (1990), the nasal congestion was measured by symptom scores. Howarth *et al.* (1995) showed that the 5-lipoxygenase inhibitor, A-78773, reduced the increase in nasal airway resistance induced by grass pollen challenge. This study used rhinomanometry to measure nasal airway resistance; a technique more quantitative than symptom scores. Inhibition of leukotriene synthesis appears to be the first demonstration that blocking the generation of a specific inflammatory mediator inhibits the nasal congestion caused by challenge with grass antigen. Therefore, the leukotrienes have the potential to provide a target for new treatments of allergic rhinitis.

1.6.3.2 Prostaglandins

The enzyme cyclooxygenase acts on arachidonic acid to liberate cyclic endoperoxides which can be further modified to produce the biologically active prostaglandins.

Prostaglandins are generated during the early response to antigen challenge in subjects with allergic rhinitis. Following nasal challenge with grass pollen antigen in subjects with seasonal allergic rhinitis, the concentrations of prostaglandin (PG) D_2, PGE_2, PGF_2 and 6-keto-$PGF_1\alpha$ in the nasal lavage fluid is increased (Brown *et al.*, 1987). Subjects with allergy to *Dermatophagoides pteronyssinus* (house-dust mite) also have raised levels of prostaglandins in their nasal lavage fluid following antigen challenge (Ramis *et al.*, 1991). Pretreatment with the cyclooxygenase inhibitor, aspirin, significantly reduces the production of the prostaglandins (Brown *et al.*, 1987). Exogenous application of PGD_2 causes vasodilatation of the nasal blood vessels and nasal airway congestion (Doyle *et al.*, 1990). This prostaglandin is more potent than histamine and bradykinin at decreasing the patency of the nasal airway. However, if the release of prostaglandins into the nasal lavage fluid is studied during the late response to grass pollen antigen challenge then no increase in PGD_2 is detected (Naclerio *et al.*, 1985). Nasal challenge with $PGF_{2\alpha}$ increases the patency of the nasal airway (Doyle *et al.*, 1990).

As prostaglandins are generated during experimentally-induced allergic rhinitis, the effect of cyclooxygenase inhibitors on the nasal response to antigen challenge has been investigated. Aspirin, as already discussed, inhibits the antigen-induced increase in the prostaglandin content of the nasal lavage fluid (Naclerio *et al.*, 1985; Walden *et al.*, 1988). However, aspirin has no effect symptoms scores recorded after grass pollen antigen challenge (Naclerio *et al.*, 1985; Walden *et al.*, 1988). Another cyclooxygenase inhibitor, flurbiprofen, failed to inhibit the increase in nasal airway resistance induced by grass pollen antigen challenge (Brooks *et al.*, 1984). Therefore, the generation of prostaglandins does not appear to contribute significantly to the pathophysiology of allergic rhinitis.

1.6.3.3 Platelet activating factor

Barbaro & Zvaifler (1966) reported that histamine is released from rabbit platelets during an acute allergic response. The histamine release was shown to be a result of IgE-mediated activation of the rabbit basophils, causing the release of a soluble factor which caused platelet activation (Benveniste *et al.*, 1972): this mediator was termed platelet activating factor (PAF).

In 1979 PAF was identified as an acetyl-glycerol-ether-phosphorylcholine (Benveniste *et al.*, 1979; Demopolous *et al.*, 1979; Blank *et al.*, 1979).

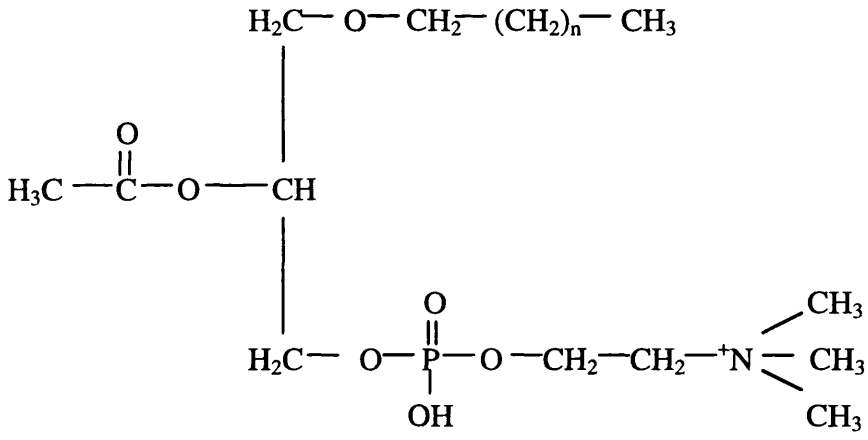

Figure 1.2. The chemical structure of PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). n=14-16.

A number of molecular species belong to the structure shown in figure 1.2 (Pinckard *et al.*, 1984) but those with alkyl groups of C_{16} and C_{18} have been most extensively investigated for biological activity.

1.6.3.3.1 Synthesis of PAF

A large number of human cell types can synthesis PAF. These include neutrophils, monocytes, alveolar macrophages, eosinophils, platelets, lymphocytes, endothelial cells and fibroblasts (Braquet & Rola-Pleszczynski, 1987). The majority of the PAF synthesised by these cells remains within the cell where its function is unclear (Lynch & Henson, 1986). There are two pathways for the synthesis of PAF (reviewed by Page, 1994). In inflammatory and allergic reactions, PAF is generated by the enzymatic actions of phospholipase A_2 (PLA_2) and acetyl CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphorylcholine acetyltransferase. The membrane lipid, alkylacyl-glycerophosphocholine, is converted via hydrolysis by PLA_2 to produce lyso-PAF and arachidonate. The arachidonate liberated by this activity of PLA_2 can be further modified to produce eicosanoids such as prostaglandins and leukotrienes. The lyso-PAF can be converted to PAF by the action of the acetyl transferase enzyme. This pathway is known as the remodelling pathway (figure 1.3). This pathway is also the route by which PAF is broken down by the action of acetyl hydrolase enzymes. Lyso-PAF is therefore the precursor and metabolite of PAF.

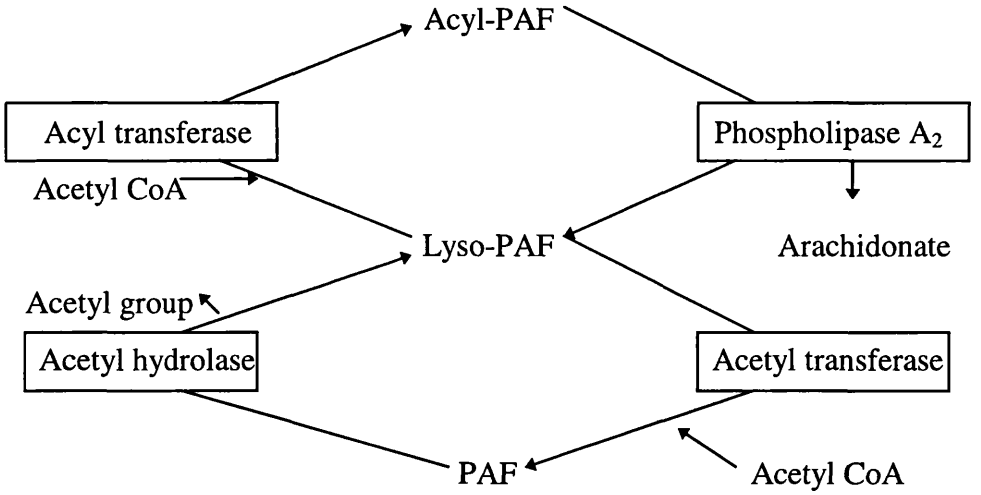

Figure 1.3. The remodelling pathway for the synthesis of PAF

Alternatively, PAF can be generated from alkyllyso-glycerophosphate by acetylation, dephosphorylation and transfer of phosphorylcholine by the action of the enzyme, phosphocholine transferase (figure 1.4). This pathway is known as the *de novo* pathway and although it probably does not play a role in inflammatory and allergic reactions, it is believed to be the source of endogenous PAF required for physiological functions (Snyder, 1990).

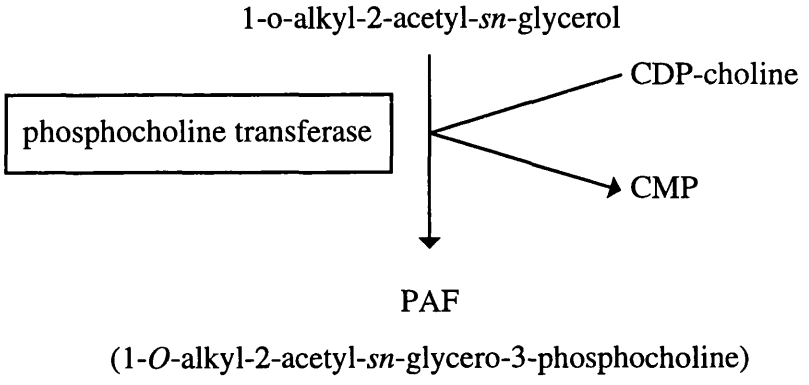

Figure 1.4. The *de novo* pathway for the synthesis of PAF.

1.6.3.3.2 PAF receptors

Binding experiments, using [^{3}H]-PAF, have identified specific PAF receptors in a wide variety of tissues and cells (Chao & Olson, 1993). The first binding experiments identified two binding sites for PAF on human platelets: the higher affinity site being responsible for platelet aggregation (Valone *et al.*, 1982). Specific binding sites for PAF were also identified on many other tissues including smooth muscle cells, neutrophils, macrophages, monocytes, eosinophils and nerves (reviewed by Chao & Olson, 1993). The cDNA for the PAF receptor was first cloned from guinea-pig lung (Honda *et al.*, 1991) and codes for a 342 amino acid protein with seven transmembrane regions characteristic of G-protein-coupled receptors. The PAF receptor was subsequently cloned from human leukocytes (Nakamura *et al.*, 1991), and has 83% amino acid sequence homology with the

guinea-pig receptor. Expressing the human PAF receptor in stable cell lines demonstrated that it is G-protein-coupled to phosphoinositide metabolism. In addition to activation of phopholipase C, the PAF receptor is also coupled to phospholipase A_2 and, therefore, the release of arachidonic acid. PAF receptor stimulation raises intracellular calcium and activates protein kinase C, tyrosine kinases and can increase proto-oncogene expression (Chao & Olson, 1993).

1.6.3.3.3 Actions of PAF

PAF increases vascular permeability in various blood vessels and is a powerful chemoattractant for neutrophils and eosinophils. PAF induces free radical production and leukotriene synthesis in neutrophils and eosinophils (O'Flaherty & Wykle, 1983). In the lower airways PAF can induce bronchoconstriction and a non-specific hyperresponsiveness (Cuss *et al.*, 1986).

1.6.3.3.4 The role of platelet-activating factor in allergic rhinitis

1.6.3.3.4.1 Lyso-PAF and PAF are generated in allergic rhinitis

Following the administration of grass pollen antigen into the nasal cavity of subjects with seasonal allergic rhinitis (sensitive to this antigen), the levels of the PAF precursor and metabolite, lyso-PAF, are raised in the nasal lavage fluid (Miadonna *et al.*, 1989; Shin *et al.*, 1994). Nasal challenge with saline does not increase the lyso-PAF content of the nasal lavage fluid in subjects with seasonal allergic rhinitis and grass pollen antigen nasal challenge does not raise the levels of lyso-PAF in normal, non-atopic subjects (Miadonna *et al.*, 1989; Shin *et al.*, 1994). Although the generation of lyso-PAF is unequivocal, the levels of PAF in the nasal lavage fluid are only marginally raised following grass pollen challenge in sensitive subjects (Miadonna *et al.*, 1989). In fact, Shin *et al.* (1994) failed to detect any increase in the levels of PAF in the nasal lavage following antigen challenge. The enzyme acetylhydrolase, which converts PAF to lyso-PAF, shows significantly raised activity in the nasal lavage fluid following grass pollen antigen challenge in sensitive

subjects (Touqui *et al.*, 1994). Therefore, the high levels of lyso-PAF may be due to PAF being hydrolysed in the nasal cavity. This could explain why the increase in the levels of PAF is small or nonexistent following antigen challenge. The level of phospholipase A_2 in the nasal lavage fluid is also raised following the administration of grass pollen into sensitive nasal cavities (Shin *et al.*, 1994; Touqui *et al.*, 1994). Therefore, the raised levels of lyso-PAF may also be explained by the actions of phospholipase A_2 on phospholipids. Miadonna *et al.* (1989) report the interesting observation that administration of *Dermatophagoides pteronyssinus* (house-dust mite) into the nasal cavity of two subjects sensitive to this antigen causes no increase in the levels PAF or lyso-PAF: an observation which is, as yet, unsubstantiated.

1.6.3.3.4.2 The effect of exogenous PAF on the human nasal airway

As PAF is a mediator of inflammation, and there is some evidence for its generation in allergic rhinitis, the effects of PAF on the human nasal airway have been investigated. Pipkorn *et al.* (1984) reported that PAF at a dose up to 26µg per nostril had no effect on nasal airway resistance, measured by active posterior rhinomanometry. PAF at a dose of 60µg per nostril also has no effect on the resistance to airflow through the nasal cavity (Austin & Foreman, 1993). Both these studies used normal, non-atopic human subjects. However, if the dose of PAF is increased to 157µg or 314µg per nostril (300nmol or 600nmol, respectively) then PAF induces a dose-related increase in nasal airway resistance and in symptom scores for rhinorrhoea, itching, sneezing and burning (Leggieri *et al.*, 1991; Tedeschi *et al.*, 1994b). PAF has been shown to release histamine from the rat kidney (Alessandri *et al.*, 1988), but Leggieri *et al.* (1991) conclude that the effects of PAF at the higher doses are not mediated by histamine release since there is no increase in the histamine content of the nasal lavage following nasal challenge with PAF. However, there are other possible explanations for the failure to detect a rise in histamine, such as histamine being cleared into the venous circulation before it enters the nasal cavity. To further investigate the contribution of histamine, the effect of histamine H_1 receptor antagonists on PAF-induced nasal blockage needs to be investigated.

Administration of PAF into the human nasal airway induces a non-specific nasal hyperresponsiveness; one of the features of allergic rhinitis. The response to histamine, bradykinin and grass pollen antigen is significantly enhanced if the nasal cavity is pretreated with PAF (Andersson & Pipkorn, 1988; Austin & Foreman, 1993). However, PAF pretreatment does not generate hyperresponsiveness if methacholine-induced nasal secretion is measured, indicating that PAF induced hyperresponsiveness may not be totally non-specific (Klementsson & Andersson, 1992). Nasal challenge with PAF causes an increase in the numbers of eosinophils in the nasal cavity (Klementsson & Andersson, 1992; Tedeschi *et al.*, 1994b) and this nasal eosinophillia is accompanied by release of eosinophil cationic protein (ECP) (Tedeschi *et al.*, 1994a; Austin & Foreman, 1993). The time-course of PAF-induced hyperresponsiveness is associated with an increase in ECP in the nasal lavage fluid; implicating eosinophil activation in the development of PAF-induced hyperresponsiveness (Austin & Foreman, 1993). It has been suggested that PAF-induced hyperresponsiveness in the ferret trachea is mediated by the release of oxygen free radicals (Webber *et al.*, 1992). Pretreatment with vitamin E, an oxygen free radical scavenger, caused an attenuation of PAF-induced nasal hyperresponsiveness and a decrease in the ECP levels of the nasal lavage fluid (Austin & Foreman, 1993). These findings indicate that free radical production may contribute to PAF-induced hyperresponsiveness and the activation of eosinophils.

1.6.3.3.4.3 The action of PAF receptor antagonists in the human nasal airway

There is very little data relating to the effect of PAF receptor antagonists in the human nasal airway. Shin *et al.* (1994) briefly report that orally administered WEB 2086, a PAF antagonist, had a small effect on the increase in vascular permeability following histamine challenge. The use of PAF receptor antagonists in the human nasal airway is obviously an area which requires more investigation.

1.6.4 Neuropeptides

In the human nasal mucosa sympathetic, noradrenergic nerves innervate the arteries, venules, veins and glands. Parasympathetic, cholinergic nerves innervate the arterial vessels and glands (Raphael *et al.*, 1988). Noradrenergic stimulation causes vasoconstriction whereas cholinergic stimulation causes arterial vasodilatation and glandular secretion (Raphael *et al.*, 1991). The nerve fibres present in the human nasal mucosa contain neuropeptides. The sensory nerve fibres, which are branches of the trigeminal nerve, contain calcitonin-gene related peptide (CGRP), substance P, neurokinin A and gastrin-releasing peptide (GRP) (Kaliner, 1994). In addition to acetylcholine, the neuropeptides vasoactive intestinal peptide (VIP) and peptide histidine methionine (PHM) are present in the parasympathetic nerves of the human nasal mucosa. The sympathetic nerves contain noradrenaline and neuropeptide Y; a peptide which has very similar effects in the nasal mucosa to noradrenaline.

The contribution of neuropeptides to allergic rhinitis is still being investigated. Immunohistochemical studies demonstrate weak staining for CGRP-, substance P- and neurokinin A-containing nerve fibres in the nasal epithelium. The submucosal glands and blood vessels are innervated by fibres staining for GRP, CGRP, substance P, neurokinin A and VIP. The distribution of neuropeptide receptors has also been studied. The epithelium of the nasal cavity stains for substance P binding sites (Baraniuk *et al.*, 1991) and the submucosal glands stain for GRP, VIP and substance P. The arterioles possess binding sites for all the neuropeptides present in the nasal mucosa except GRP, whereas the venous sinusoids contain only a few receptors for CGRP, substance P and VIP. Most of the receptors present on the nasal arterioles are for CGRP, which causes vasodilatation (Baraniuk *et al.*, 1990a) and neuropeptide Y, which induces vasoconstriction (Baraniuk *et al.*, 1992b). Therefore, the receptors and nerve fibres containing neuropeptides are located in sites which potentially could contribute to the nasal congestion and rhinorhoea, which are signs of allergic rhinitis.

Following nasal challenge with grass antigen, in sensitive subjects, the concentration of substance P, CGRP and VIP in the nasal lavage fluid is raised (Mosimann *et al.*, 1993). Nasal challenge with histamine raises the levels of VIP without affecting the lavage concentrations of substance P and CGRP (Mosimann *et al.*, 1993). As already discussed, VIP is contained in cholinergic neurones and therefore histamine-induced stimulation of parasympathetic neurones may account for the increase in VIP following antigen challenge. The effect of nasal challenge with substance P, CGRP and VIP has also been investigated. All three of these neuropeptides cause an increase in nasal airway resistance following their application to non-atopic and atopic human nasal airways (Chatelain *et al.*, 1995; Lurie *et al.*, 1994). Substance P and neurokinin A cause an increase in the albumin content of the nasal lavage fluid indicating that they increase the vascular permeability in the nasal cavity (Braunstein *et al.*, 1991). However, neurokinin A only causes a slight increase in the nasal airway resistance. CGRP does not increase the albumin content of the nasal lavage, so, although it causes nasal blockage, it does not increase the vascular permeability of the blood vessels of the nasal mucosa (Guarnaccia *et al.*, 1994). Glandular secretion has been investigated using *ex vivo* human nasal tissue. Both GRP and VIP are more potent at stimulating secretion from serous cells than mucous cells whereas substance P and neurokinin A weakly stimulate secretion from mucous cells (Raphael *et al.*, 1991). Substance P has releases histamine from cultured human nasal mast cells *in vitro* (Schierhorn *et al.*, 1995). However, the increase in nasal airway resistance induced by nasal challenge with substance P appears not to be mediated by histamine release since the histamine content of the nasal lavage fluid does not increase following nasal challenge with substance P and histamine H_1 receptor antagonists have no effect on the nasal blockage induced by challenge with substance P (Braunstein *et al.*, 1994).

The role of neuropeptides in allergic rhinitis is unclear and requires the use of selective, potent tachykinin receptor antagonists in the human nasal airway to clarify their significance.

1.6.5 Nitric oxide

The relaxation of many isolated blood vessels is dependent upon the presence of an intact vascular endothelium. This was first demonstrated by Furchgott & Zawadzki (1980), who reported that acetylcholine caused a relaxation of the isolated rabbit aorta which was abolished following removal of the intimal surface. The relaxation in response to acetylcholine can be restored by mounting the endothelium-denuded aortic strip next to a strip with an intact endothelium (Furchgott & Zawadzki, 1980). This indicated that muscarinic receptor activation stimulates endothelial cells to release a substance (or substances) which in turn acts on the smooth muscle cells to cause relaxation: this substance was termed endothelium-dependent relaxation factor (EDRF). Further studies showed that EDRF has a short half-life and its actions were inhibited by haemoglobin and methylene blue. The effects of EDRF can be prolonged by addition of superoxide dismutase and EDRF generation causes an accumulation of cyclic GMP levels blood vessels (Moncada *et al.*, 1991). Nitric oxide has properties identical to EDRF and is released from cultured endothelial cells following stimulation with bradykinin (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Therefore, the identity of EDRF was proposed to be nitric oxide. Since these initial observations, there has been a vast amount of research into the biological actions of nitric oxide (NO) and it has been identified as a signalling mechanism in the cardiovascular and nervous systems and plays an important role in host defence to infection (Moncada *et al.*, 1991).

1.6.5.1 Synthesis of nitric oxide

Nitric oxide is synthesised from the precursor amino acid L-arginine. If endothelial cells are cultured in the absence of L-arginine then the EDRF release induced by bradykinin is significantly reduced. This release is restored by addition of L-, but not D-arginine (Palmer *et al.*, 1988). Furthermore, if the terminal guanidino nitrogen atom of L-arginine is radiolabelled then this radioactivity is incorporated into the nitric oxide released by stimulated endothelial cells (Palmer *et al.*, 1988). The enzymes responsible for synthesising nitric oxide from L-arginine in mammalian tissues are known as nitric oxide

synthases (NOS). These enzymes utililise L-arginine, molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate as substrates to form nitric oxide and L-citrulline: they also require tetrahydrobioterin, flavin adenine dinucleotide, flavin mononucleotide and heme as cofactors (Knowles & Moncada, 1994).

Nitric oxide mediates its effects in target cells such as vascular smooth muscle cells by interacting with the haem moiety of soluble guanylate cyclase and activating the enzyme. This leads to formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). Raised intracellular cGMP appears to cause most of the biological effects of nitric oxide (Moncada *et al.*, 1991).

The study of the biological roles of the L-arginine-nitric oxide pathway has been greatly facilitated by the development of nitric oxide synthase inhibitors (Moncada *et al.*, 1991; Knowles & Moncada, 1994). Two of the most extensively used inhibitors are N^{ω}-monomethyl-L-arginine (L-NMMA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME). Both these compounds are competitive inhibitors of all the isoforms of nitric oxide synthase: being in competition with L-arginine. Therefore, an effect due to L-NMMA and L-NAME administration can be reversed by adding an excess of L-arginine. The D stereoisomers of these compounds are inactive with regard to NOS inhibition. There are two important differences between L-NAME and L-NMMA. L-NAME has been reported to be a low affinity muscarinic receptor antagonist (Buxton *et al.*, 1993), but L-NMMA has no affinity for this receptor type. L-NMMA competes with L-arginine at the amino acid transporter in the cell membrane (Bogle *et al.*, 1992). Therefore, L-NMMA reduces the generation of nitric oxide by competing with L-arginine for entry into the cell and for the active site on nitric oxide synthase. In contrast, L-NAME only competes with L-arginine at the level of nitric oxide synthase. The ability of L-NMMA and L-NAME to inhibit a biological response, together with reversal of the inhibition with L-arginine and the inactivity of D-NMMA and D-NAME would indicate that the specific activation of nitric oxide synthase mediates the response. Experimental observations using NOS inhibitors provide the basis for the majority of the investigations into the role of nitric oxide *in vivo* and *in vitro*. There are, of course, other compounds which can be used to

investigate the role of the L-arginine-nitric oxide pathway in a biological response. Haemoglobin will combine with nitric oxide and inactivate it. Flavoprotein inhibitors and depletors of tetrahydrobiopterin will inhibit the production of nitric oxide. Calmodulin antagonists will inhibit the activity of constitutive nitric oxide synthases (see below) and methylene blue block the production of cyclic GMP. Recently, the genes for the various isoforms of nitric oxide synthase have been cloned and mice with these genes selectively inactivated have been used to clarify the role of nitric oxide *in vivo*.

Following the cloning and characterisation of NOS cDNA, three enzyme isoforms have been proposed: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS).

1.6.5.2 Neuronal nitric oxide synthase

This cytosolic enzyme is calcium and calmodulin-dependent (Knowles & Moncada, 1994). The gene for the enzyme is located on human chromosome 12 and encodes for a protein of 1433 amino acids (Knowles & Moncada, 1994). In humans, this isoform is expressed at high levels in the brain, but it is also found in skeletal muscle (Nakane *et al.*, 1993), where Brenman *et al.* (1995) found it complexed with dystrophin. It is absent in Duchenne's muscular dystrophy and this absence may account for some of the symptoms of this disease. In the brain, evidence suggests that nitric oxide plays an important role in the control of neuronal development and in synaptic plasticity; for example long-term potentiation. Nitric oxide is generated following glutamate NMDA receptor activation (Garthwaite *et al.*, 1988) and has been implicated as mediating the neuronal damage following strokes. Histological studies suggest that nNOS is also widely distributed in the peripheral nervous system and spinal cord. In peripheral nerves nitric oxide has been reported to mediate non-adrenergic, non-cholinergic (NANC) transmission in many tissues: for example in the rat anococcygeus muscle (Li & Rand, 1989), rat gastric fundus (Li & Rand, 1990) and guinea-pig trachea (Li & Rand, 1991). The vasodilatation of blood vessels by NANC nerves may also involve the generation of nitric oxide (Holzer *et al.*,

1995). In the spinal cord, nitric oxide is important in the processing of nociceptive stimuli (Meller & Gebhart, 1993).

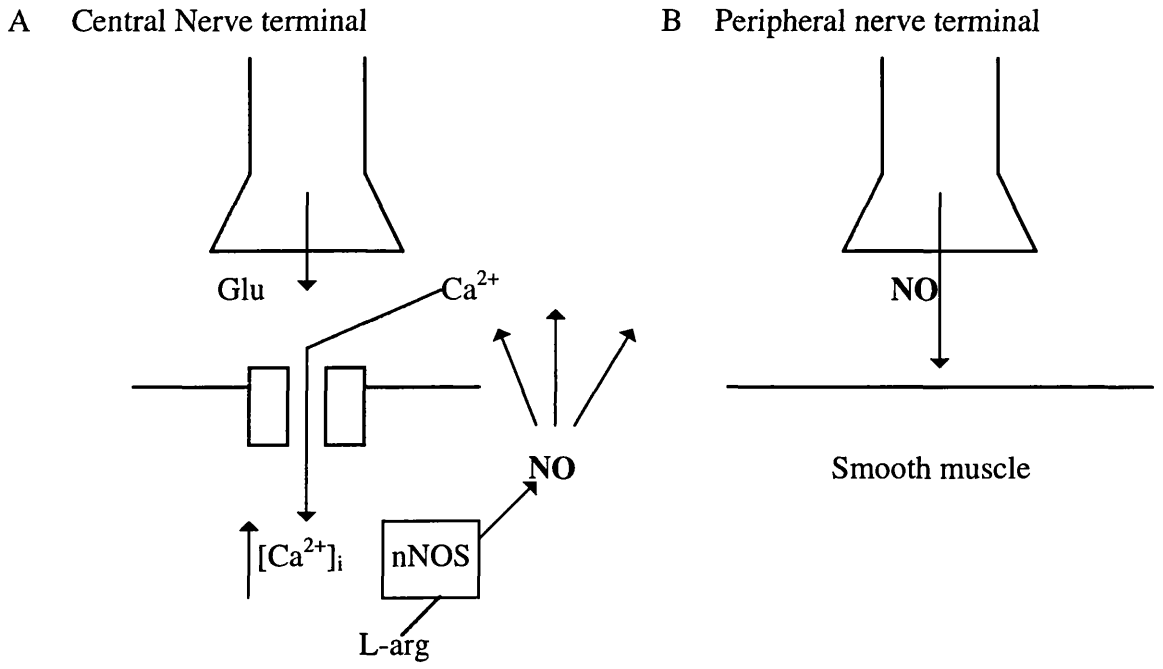

Figure 1.5. The generation of nitric oxide by neuronal nitric oxide synthase (nNOS). Figure A shows the activation of nNOS in a postsynaptic neuron following a rise in intracellular calcium resulting from glutamate NMDA receptor activation. Figure B represents nitric oxide being released from the presynaptic terminal of a peripheral NANC nerve to act on smooth muscle.

Recently, the role of nitric oxide has been considerably clarified by the use of mice with the isoforms of nitric oxide synthase inactivated. Mice in which the gene for nNOS has been inactivated breed normally (Huang *et al.*, 1993). They have dilated stomachs and constricted pyloric sphincters (Huang *et al.*, 1993), are resistant to brain damage following vascular strokes (Huang *et al.*, 1994) and show inappropriate sexual and aggressive behaviour (Nelson *et al.*, 1995). Therefore nNOS appears to be important in regulating behaviour, as well as smooth muscle function. Interestingly, no change was reported in long-term potentiation following nNOS gene knockout; casting doubt on

previous reports suggesting that nitric oxide played a critical role in this mechanism (Snyder, 1995).

1.6.5.3 Endothelial nitric oxide synthase

Endothelial nitric oxide synthase is widespread in the endothelium of blood vessels from various species including humans. Like neuronal nitric oxide synthase it is calcium and calmodulin dependent. However eNOS and nNOS are distinct gene products: eNOS mapping to chromosome 7. Neuronal and endothelial NOS only share 57% amino acid identity. The eNOS N-terminus is shorter than that of nNOS and has a myristoylation site. Following myristoylation, eNOS is primarily associated with the cell membrane (Busconi & Michel, 1993), but after endothelial cell stimulation with bradykinin the enzyme is phosphorylated and becomes cytosolic (Michel *et al.*, 1993).

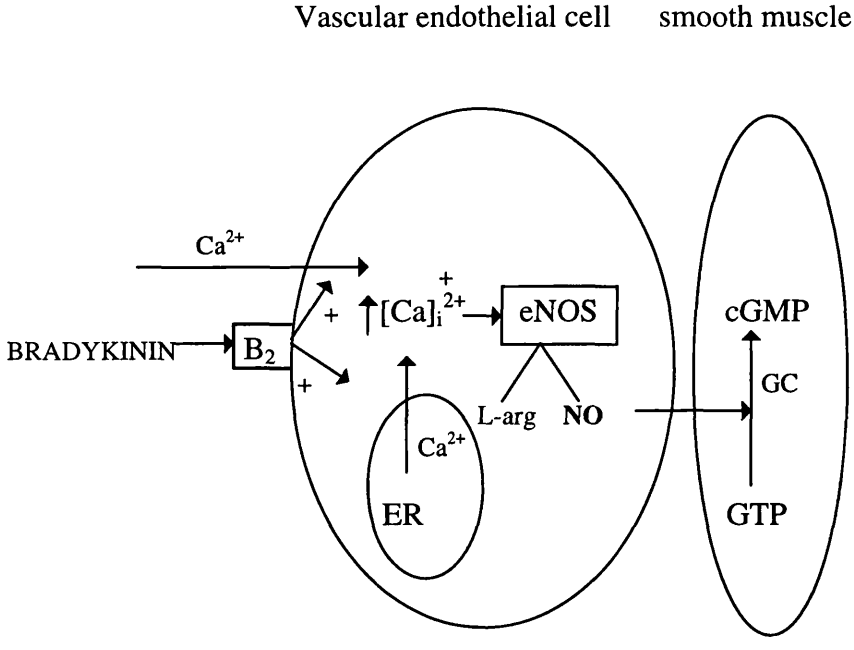

Figure 1.6. The generation of nitric oxide by endothelial nitric oxide synthase (eNOS). Following stimulation of the vascular endothelial cell with bradykinin intracellular calcium rise causing activation of eNOS. The nitric oxide generation diffuses into the surrounding smooth muscle cell and activates soluble guanylate cyclase (GC). This leads to formation of cyclic guanosine monophosphate and relaxation of the smooth muscle.

Endothelial nitric oxide synthase is involved in regulation of blood pressure. Evidence for this includes work with isolated blood vessels showing that sheer stress, neurotransmitters and inflammatory mediators produce vasodilatation via nitric oxide formation. *In vivo*, inhibitors of nitric oxide synthase cause a rise in blood pressure. Mice lacking the gene for eNOS, and therefore not expressing the enzyme, have an elevated blood pressure. Isolated aortic rings from these mice do not dilate in response to acetylcholine (Huang *et al.*, 1995). Therefore, eNOS plays a central role in the vascular response to vasoactive substances such as bradykinin and acetylcholine.

Recently, it has been reported that nitric oxide may be transported in the circulation by haemoglobin (Jia *et al.*, 1996). As already discussed, haemoglobin binds nitric oxide. However, the affinity of nitric oxide for haemoglobin is greater when haemoglobin is also binding oxygen. Therefore, haemoglobin binds nitric oxide in the arterial circulation (when haemoglobin is oxygenated) and releases it in the venous circulation (when haemoglobin becomes deoxygenated). In this way, haemoglobin acts as a carrier for nitric oxide, shunting it from the arterial to the venous circulation.

1.6.5.4 Inducible nitric oxide synthase

The two isoforms of nitric oxide synthase, nNOS and eNOS, previously described are normal constituents of cells. However, following exposure of certain cell types to cytokines or bacterial products, the formation of a nitric oxide synthase is induced which is clearly different from nNOS or eNOS (Moncada *et al.*, 1991).

This inducible nitric oxide synthase (iNOS) has calcium- and calmodulin-independent activity and is not expressed in any healthy, unstimulated cell type (Knowles & Moncada, 1994). Inducible nitric oxide synthase can be expressed in many cell types including macrophages, vascular smooth muscle, heart muscle, gut, immune cells and hepatocytes. Stimuli for its induction include products of infection such as bacterial endotoxin and

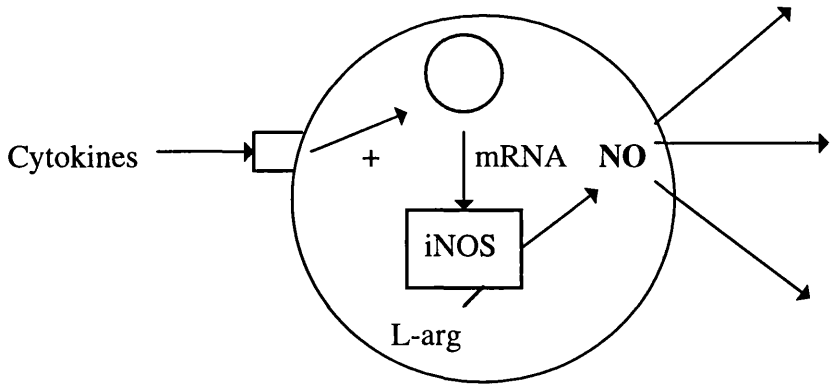

Figure 1.7. The induction of inducible nitric oxide synthase (iNOS) and subsequent generation of nitric oxide. Following cytokine stimulation of a cell, for example a macrophage, the mRNA for iNOS is transcribed and the enzyme translated. Nitric oxide is then generated which contributes to the host's defence mechanisms. This process is calcium independent.

exotoxin, or cytokines such as tumour necrosis factor or interleukin-1 (Vallance & Collier, 1994). Rodent macrophages provide the source of iNOS which is best characterised. The gene coding for iNOS is located on chromosome 17 and the enzyme has a 130 kDa mass (Stuehr *et al.*, 1991). The induction of iNOS is important in host resistance to infection and this is demonstrated using mice with inactivation of the gene coding for the inducible form of nitric oxide synthase. These mice have markedly reduced defences against lymphoma proliferation, microorganisms such as Listeria (MacMicking *et al.*, 1995) and Leishmania (Wei *et al.*, 1995). However, inactivation of iNOS increases the resistance of the mice to endotoxin-induced hypotension and inflammation induced by carrageenan (MacMicking *et al.*, 1995; Wei *et al.*, 1995).

Nitric oxide contributes to the inflammation promoted by inflammatory mediators: for example bradykinin produces vasodilatation via generation of nitric oxide (Palmer *et al.*, 1987). *In vivo*, this is most probably caused by stimulation of endothelial nitric oxide synthase, but since various inflammatory mediators also activate nerves and nNOS occurs in neurons located adjacent to blood vessels, the production of neuronal nitric oxide may also contribute to blood vessel vasodilatation (Snyder, 1995). Induction of iNOS can occur in inflammation and this may further contribute to vasodilatation, plasma

extravasation and tissue damage (Vallance & Moncada, 1993). An example of the role nitric oxide can play in inflammation is provided by experimentally-induced inflammation with carrageenin (Salvemini *et al.*, 1996). Following carrageenin injection into the rat hindpaw, the initial inflammatory response is partly mediated by nitric oxide generated by eNOS. However, 3-10 hours after injection, iNOS is synthesised and the nitric oxide produced by this enzyme is involved in maintaining the inflammatory response. The role of nitric oxide in actions of bradykinin in the human nasal airway and in the nasal inflammation of allergic rhinitis is discussed in chapter 5.

1.6.6 Cytokines

In addition to the release of inflammatory mediators which directly produce the symptoms of allergic rhinitis, cytokines generated in nasal inflammation have an important role in modulating the inflammatory response. Secretion of cytokines upregulates the expression of adhesion molecules on the vascular endothelium and their corresponding ligands on the inflammatory cells; cytokines prime cells to respond to stimuli and enhance the survival of cells within the nasal mucosa (Howarth, 1995). During the initial activation of nasal mast cells by antigen tumour necrosis factor-α (TNFα) is released (Bradding *et al.*, 1995a). TNFα can enhance the expression of adhesion molecules on the endothelial cells so enhance the migration of inflammatory cells into the nasal mucosa. As already discussed, mast cells also release Interleukin-4 (IL-4), a cytokine which upregulates the expression of VCAM-1. This adhesion molecule has enhanced expression in seasonal and perennial allergic rhinitis (Bradding *et al.*, 1993). The release of IL-5 by mast cells will promote eosinophil migration into the nasal mucosa (Howarth, 1995).

Activation of lymphocytes by antigen-presenting cells such as the Langerhan's cells in the nasal mucosa will cause a further release of cytokines. This release will further enhance the inflammatory response. Cytokines can act to recruit eosinophils into the nasal mucosa and these cells can release further cytokines such as IL-8 (Howarth, 1995). IL-8 has been

shown to be chemotactic for mast cells *in vitro* (Hartman *et al.*, 1994), and its release in nasal allergy may contribute to the increase in the numbers of mast cells present in the nasal epithelium of subjects with allergic rhinitis (Bentley *et al.*, 1992).

Therefore, cytokines have the potential to play a central role in the pathogenesis of allergic rhinitis. The development of effective inhibitors of cytokine function will provide a potentially useful treatment for allergic rhinitis.

1.7 Aims of the project

The aim of this project was to characterise of the bradykinin receptor in the human nasal airway and investigate its role in allergic rhinitis. More specifically, the subsequent chapters will present data from investigations into the following:

- Using normal non-atopic human subjects, the effect of bradykinin receptor antagonists on the nasal response to bradykinin was investigated. The data from these *in vivo* studies can be compared with data from radioligand binding studies using the binding of a radiolabelled bradykinin B_2 receptor antagonist to human nasal membranes. From these *in vivo* and *in vitro* approaches, the type of bradykinin receptor in the human nasal airway can be described.

- The role of the bradykinin receptor in allergic rhinitis was studied by investigating the effect of the bradykinin B_2 receptor antagonist, icatibant, on the nasal response to antigen challenge using subjects with allergic rhinitis.

- The role of nitric oxide in the nasal response to bradykinin, histamine and antigen was studied by inhibiting the generation of nitric oxide in the human nasal airway *in vivo*.

- To investigate whether the release of histamine mediates the nasal response to bradykinin, to effect of the histamine H_1 receptor antagonist, cetirizine, on the nasal response to bradykinin was studied *in vivo*. In addition, the release of histamine from isolated human nasal mucosal cells was measured following stimulation with bradykinin.

- Finally, the role of kinins in the hyperresponsiveness induced by nasal challenge with platelet activating factor was investigated by measuring the effect of the bradykinin B_2 receptor antagonist, icatibant, on the nasal response to challenge with platelet activating factor.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Sources of materials

The materials used in this study and their sources are listed below.

MATERIAL	SOURCE
Bradykinin	Calbiochem, Nottingham, UK.
[DesArg^9]-bradykinin	Bachem, Saffron Walden, UK.
Icatibant (Hoe 140)	Hoeschst AG, Frankfurt, Germany.
[DesArg^9]-icatibant	Bachem, Saffron Walden, UK.
[1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] -bradykinin	Bachem, Saffron Walden, UK.
NPC 567	Bachem, Saffron Walden, UK.
NPC 17731	Scios Nova, Sunnyvale, USA.
NPC 17761	Scios Nova, Sunnyvale, USA.
WIN 64338	Sanofi Recherche, Toulouse, France.
Dermatophagoides pternyssinus antigen	Allerayde, Nottingham, UK.
Grass pollen antigens	Allerayde, Nottingham, UK.
Polyethyleneimine	Sigma, Poole, UK.
1,10 phenantroline	Sigma, Poole, UK.
Dithiothreitol	Sigma, Poole, UK.
Bacitracin	Sigma, Poole, UK.
N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES)	Sigma, Poole, UK.
Captopril	Squibb, Princeton, USA.
Bovine serum albumin	Calbiochem, Nottingham, UK.
Ethylene diamine tetraacetic acid (EDTA)	BDH, Poole, UK.
Bradykinin radioimmunoassay kit	Peninsula, St. Helens, UK.

MATERIALS	SOURCE
Albumin radial immunodiffusion plates	Behring, Marburg, Germany.
Albumin standard	Behring, Marburg, Germany.
Protein assay kit	Pierce BCA, Rockford, USA.
Whatman GF/B filters	Whatman, Maidstone, UK.
N^G-nitro-L-arginine methyl ester (L-NAME)	Sigma, Poole, UK.
N^G-nitro-D-arginine methyl ester (D-NAME)	Sigma, Poole, UK.
L-arginine	Sigma, Poole, UK.
N^G-monomethyl-L-arginine (L-NMMA)	Calbiochem, Nottingham, UK.
Histamine	Sigma, Poole, UK.
Ephedrine hydrochloride	Boots, Nottingham, UK.
Lignocaine	Phoenix Pharmaceuticals, Gloucester, UK.
Benzocaine	Astra, Kings Langley, UK.
Calcium ionophore, A23187	Sigma, Poole, UK.
Rabbit anti-human IgE	Dako, Glostrup, Denmark.
Collagenase type 4	Worthington, Twyford, UK.
Cetirizine	UCB, Brussels, Belgium.
Platelet activating factor (C_{16})	Calbiochem, Nottingham, UK.
Substance P	Calbiochem, Nottingham, UK.
N-2-hydroxyehtylpiperzine-N'-2-ethane sulfonic acid (HEPES)	Sigma, Poole, UK.

2.1.2 Dilution of nasal challenge agents.

All agents used in the nasal challenge studies on non-atopic and atopic human subjects were dissolved or suspended in sterile saline (NaCl, 154mM) for both stock solutions and subsequent dilutions. Stock solutions were stored at -20^{0}C and were prepared in appropriate aliquots to avoid thawing and refreezing the stock. When used, bradykinin stock solution was diluted to achieve concentrations between 1mg/ml and 10mg/ml, icatibant was diluted to 0.1-5mg/ml, [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin was diluted to 0.33-2mg/ml and NPC 567 was

diluted to 100mg/ml. Substance P and platelet activating factor were diluted from stock solution to 0.33mg/ml and 0.6mg/ml, respectively. Histamine, as a diphosphate salt, was dissolved to 3mg/ml. L-NAME and D-NAME were dissolved, from solid stock, to 1-100mM concentrations. L-NMMA was dissolved, again from solid stock, to 10 and 100mM; L-arginine to 300mM. All the agents used were diluted or dissolved to their final concentrations immediately prior to use.

The antigens used were *Dermatophagoides pteronyssinus* (house-dust mite) and a mixture of six grass pollen antigens. The grass pollen antigen mixture was composed of *Avena elatior, Dactylis glomerata, Festuca pratensis, Lolium perenne, Phleum pratense* and *Poa pratensis* in equal concentrations. Both house dust mite and grass antigens were dissolved in sterile saline to make a stock concentration of 100,000units/ml, where 1 unit is an arbitrary measure related to the biological potency determined by skin prick tests and compared with histamine, 1mg/ml. The antigen stock solutions were stored at 4^0C and diluted to 5000 units/ml and 10,000units/ml, for house-dust mite and grass pollen respectively, immediately prior to use.

Lignocaine and benzocaine were obtained in sterile saline solution preparation at a concentration of 20mg/ml. Both local anaesthetics were stored at 4^0C.

2.1.3 Subjects

For all studies, except using antigen challenge in atopics, normal, healthy, non-atopic volunteer subjects were used with an age range of 19-52 years. Subjects with any symptoms of nasal infection or allergy were excluded. Subjects were taking no medication at the time of or two weeks prior to the studies. All subjects gave informed consent and the study was approved by the local Ethics Committee at University College London. Experiments were performed in a laboratory at controlled temperature and humidity.

For studies using antigen nasal challenge in subjects with allergic rhinitis, the criteria the subjects had to fulfill was have a clinical history of either seasonal or perennial

allergic rhinitis and a positive skin prick response to the appropriate antigen. Subjects taking part in these studies had not been taking any oral or intranasal therapies for at least 4 weeks prior to the study and had no evidence of nasal polyposis or upper respiratory tract infection. The age range of these atopic subjects was 18-47 years. Patients with asthma were only used if the asthma was very mild. Such patients participated with appropriate medical supervision and regular expiratory peak flow measurements were taken. All subjects gave informed consent and the study was approved by the local Ethics Committee of the Royal National Throat, Nose and Ear Hospital, London.

2.2 Methods

2.2.1 Acoustic rhinometry

Measurements of nasal airway patency were made with an acoustic rhinometry using equipment supplied by gm Instruments (Kilwinning, UK). The apparatus consists of a spark generator that produces an audible sound impulse, a wave tube through which the acoustic pulse is transmitted to the anterior nares of the subject, a microphone with amplifier for measurement of the sound and a computer for acquisition and analysis of data. A plastic tube which fits into one of the anterior nares of a subject's nasal airway fits on the end of the wave tube. The subject is positioned so that the anterior nares of one nostril makes a good seal with this plastic tube. Three sizes of plastic tube were available to ensure a good seal with the anterior nares. For all measurements of nasal airway patency, the position of each subject's head was kept constant by careful positioning. Then the spark generator, located at the other end of the wave tube to the subject, produces the sound impulse which propagates along the tube pass the microphone and into the subject's nostril. The sound is reflected by the structures inside the nasal cavity and this reflected sound is again received by the microphone. The data is displayed on the computer screen as a graph of nasal cross-sectional area (cm^2) against distance into the nasal cavity (cm). The measurement takes less than 10ms.

Acoustic rhinometry measures a number of parameters of nasal airway patency. The minimum cross-sectional area (Amin.) of the nasal cavity is an anatomical measure of nasal airway congestion and often corresponds to the inferior turbinate bone located in the lateral wall of the nasal cavity. Austin & Foreman (1994b) showed that changes in Amin. correlate well with the resistance to airflow through the nasal airway as measured by rhinomanometry. The advantage of acoustic rhinometry is that it is quick to perform, requires minimal subject training (rhinomanometry requires considerable practice by the subject before use) and provides consistent measurements. For acoustic rhinometry, the coefficient of variation (CV) of mean Amin. measurements was found to be approximately half of the CV for nasal airway resistance (NAR) measurements by rhinomanometry (5.7% compared to 9.6% for Amin. and NAR, respectively. n=10). Therefore, acoustic rhinometry provides a simple to use, reliable method of assessing the patency of the nasal airway and this measurement (Amin.) is strongly correlated with functional measures of nasal congestion (Austin & Foreman, 1994b).

2.2.2 Nasal lavage

Nasal lavage involves the instillation of a liquid into the nasal cavity and the subsequent recovery of that liquid. The recovered liquid can then be assayed for various inflammatory mediators, cells and markers of inflammation.

The method used in this study was described by Naclerio *et al.* (1983) as it is easy to use, gives a relatively uniform recovery of approximately 80% of the fluid and has a good tolerance among the subjects. The subjects were in a sitting position with the head extended approximately 30^0 from the horizontal. Warmed (37^0C), sterile saline, 5ml, was instilled into a nostril while the subject held their tongue against the soft palate and abstained from breathing or swallowing. After 10s the subject leaned forward and expelled the lavage fluid into a collection vessel, which was stored on ice until assay. In each experiment, three initial washes were made to remove pre-existing mediators and the third wash was retained and served as the baseline. The lavage fluid was centrifuged at 4^0C for 10min. at 1000g then the supernatant was assayed or stored at -70^0C.

2.2.3 Nasal challenge

The agents used in studies on the human nasal airway *in vivo* were delivered into the nasal cavity using a nasal pump spray (Perfect-Valois, UK Ltd.). The spray delivers 100µl per activation with a 98% degree of accuracy. This spray was placed into each nostril of a subject and activated once; the dose of administered into the nasal cavity was controlled by the concentration of the solution. All solutions were at room temperature and were prepared from stock immediately prior to being used.

2.2.4 Basic nasal challenge protocol

The specific protocol for each experiment is described in the relevant chapters, but a number of details are common to all the nasal challenge studies. For all measurements of the patency of the nasal airway, Amin. was measured three times in both nostrils. As already described, three nasal lavages were initially made to establish the baseline, but following nasal challenge subsequent lavages were only made once in each nostril. The basic design for all the *in vivo* human nasal challenge experiments was a randomised, cross-over study. In these, each subject received both placebo and active treatments on separate occasions. This design was chosen as the nasal response to challenge (particularly with antigen) varies considerably between subjects so for valid data it is very important that each subject acts as their own control. For all the studies, the placebo treatment was the vehicle that the active treatment was dissolved in, i.e. sterile saline. For nasal challenge studies using bradykinin and histamine, measurements of the patency of the human nasal airway and nasal airway lavages were performed 10 minutes after challenge as previous studies have demonstrated that this is the time after challenge when the measured effect is maximal (Austin, 1994). The doses of bradykinin, histamine and antigen used have been demonstrated in previous studies to produce a significant increase in symptom scores reported by the subjects (Austin & Foreman, 1994a; Austin, 1994). Therefore the doses used produce a response, for example an increase in the blockage of the nasal airway, which the subject can feel for themselves.

2.2.5 Data analysis

The Amin. following nasal challenge is expressed as a percentage of the baseline value in order to normalise the data. Such normalisation was done to take into account individual variations in the anatomy of the nasal cavity which give rise to variable baseline measurements. For all experiments the mean baseline value for Amin. is quoted along with the standard error of the mean. Measurements of Amin. are expressed as the mean value for both nostrils to avoid misinterpretation of data caused by nasal cycling.

2.2.6 Statistical analysis of data

For all the nasal challenge studies, the data were analysed by non-parametric statistical tests to determine the probability of the null hypothesis being true. In all studies, the null hypothesis was that there was no difference between the nasal responses in the presence of placebo and active drug. There is the possibility that the *in vivo* responses of the human nasal airway may not have a normal distribution. The sample sizes used are too small to establish whether the data are normally distributed, so non-parametric statistical tests were used to analyse the data. Also, for parametric statistical analysis to be valid, the sets of data to be compared - for example nasal response in the presence of placebo and response in the presence of active drug - must have similar standard deviations. For some of the data presented in subsequent chapters, the two groups of data to be compared have significantly different standard deviations making parametric statistical analysis invalid. The use of non-parametric tests overcomes this problem. When two groups were compared, for example placebo with active treatment, the Wilcoxon sign-rank test was employed. For the analysis of data from more than two treatments, for example a range of drug concentrations, then the Friedman's test was used. Friedman's test is the non-parametric equivalent of the parametric two-way analysis of variance test. A probability value, p, of less than 0.05 was considered significant.

2.3 Biochemical assays

2.3.1 Albumin measurement

The albumin content of the nasal lavage fluid was determined by a radial immunodiffusion assay (Mancini *et al.*, 1965). Nasal lavage samples and human serum albumin standards were loaded into wells on the immunodiffusion plates in volumes of 20µl. The plates were left open for 30 minutes at room temperature to allow the sample to diffuse into the agarose gel of the plate. The plates were then sealed and incubated at room temperature for 48 hours. The diameters of the precipitation rings were measured and a standard curve constructed from the albumin standards (figure 2.1). A standard curve was constructed on every immunodiffusion plate used. The albumin concentration of the samples was interpolated from the standard curve. The assay was sensitive to albumin concentrations in the range 2.5 to 44mg dl^{-1}.

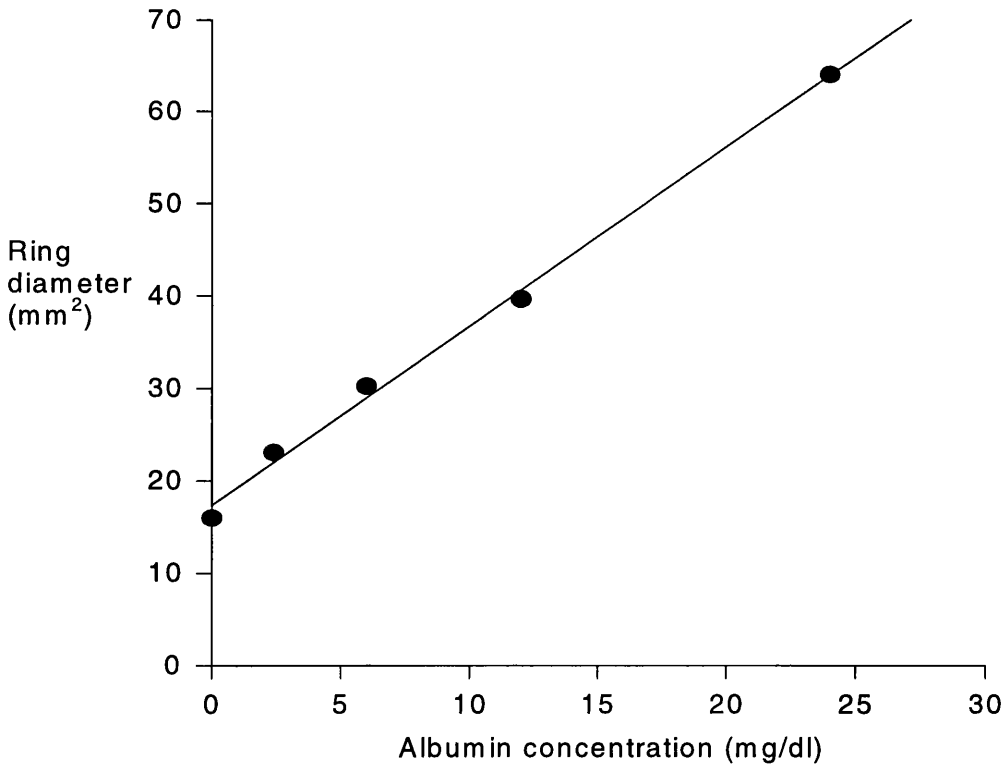

Figure 2.1. A typical standard curve for albumin measured by radial immunodiffusion.

2.3.2 Bradykinin assay

The bradykinin concentration of the nasal lavage fluid was determined by a commercially available bradykinin radioimmunoassay kit (Peninsula Laboratories, INC. USA). This assay is based on the competition of radiolabeled [^{125}I]-bradykinin with unlabelled bradykinin or kallidin (either a standard or unknown) for a limited quantity of antibodies specific for the kinins. As the concentration of unlabelled kinin increases the binding of [^{125}I]-bradykinin to the antibody decreases. Therefore, using known concentrations of unlabelled bradykinin a standard curve can be generated from which the concentration of kinin in unknown samples can be determined (figure 2.2). It is important to note that the assay used was equally sensitive for bradykinin and kallidin.

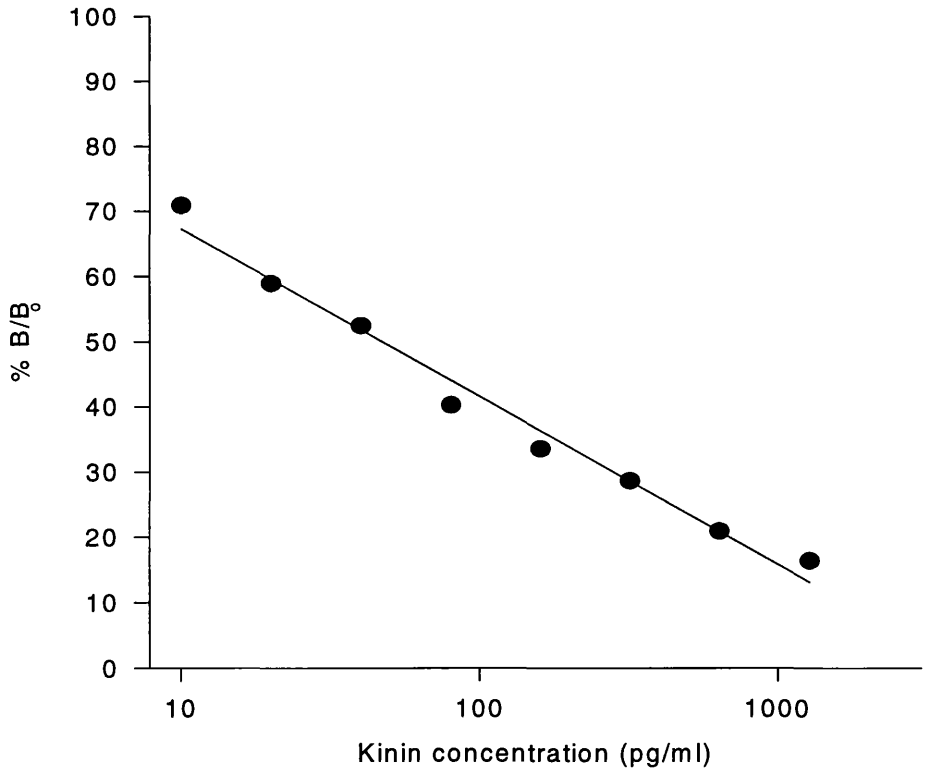

Figure 2.2. A typical standard curve for bradykinin assayed by radioimmunoassay. B and B_0 represent the specific binding of [^{125}I]-bradykinin to kinin-specific antibody in the presence and absence of unlabelled bradykinin.

A brief overview of the assay is as follows:

1) Bradykinin standard or nasal lavage fluid is added to rabbit anti-serum specific for kinin. A new bradykinin standard curve was constructed for every assay. All the nasal lavage samples were assayed at 1:10 and 1:100 dilutions. The tubes were then vortexed and stored overnight.

2) [^{125}I]-bradykinin was added and the tubes again were vortexed and stored overnight.

3) Goat anti-rabbit IgG serum and normal rabbit serum were added to the tubes, the tubes were vortexed and left at room temperature for 90min. The tubes were then centrifuged at 1700g for 20min. at 4^{0}C and the pellets were assayed by the gamma counter.

This assay was sensitive over the range of 1-128pg kinin per tube.

2.3.3 Protein assay

The protein content of the human nasal membrane preparation was assayed a commercially available protein assay kit (Pierce BCA). The method for this protein assay is as follows:

1) Standard protein solution were made by diluting bovine serum albumin in the binding buffer B.

2) 100 parts of protein assay reagent A was mixed with 2 parts reagent B. This formed the protein assay reagent.

3) The membrane preparation (suspended in binding buffer B, 5ml) was diluted 1:2, 1:4 and 1:8 with binding buffer B. Standard protein solution, membrane preparation, and binding buffer B, all at a volume of 100µl, were each mixed with 2ml of the protein assay reagent and incubated for 30min. at 37^{0}C.

4) The mixtures were cooled to room temperature and then their absorbance at 562nm was measured using a flurometer. The flurometer was calibrated so that a water sample was zero. The absorbance of the binding buffer B was subtracted from all

other readings and a standard curve constructed (figure 2.3). From this the protein concentration of the human nasal membrane preparation was determined. Each time a protein assay was performed, a new standard curve was constructed.

Protein assay reagent A: sodium carbonate, sodium bicarbonate and sodium tartrate dissolved in 0.1M sodium hydroxide.

Protein assay reagent B: copper sulphate (4%).

Binding buffer B - N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES), 25 mM; 1,10 phenatroline, 1mM. The pH was 6.8.

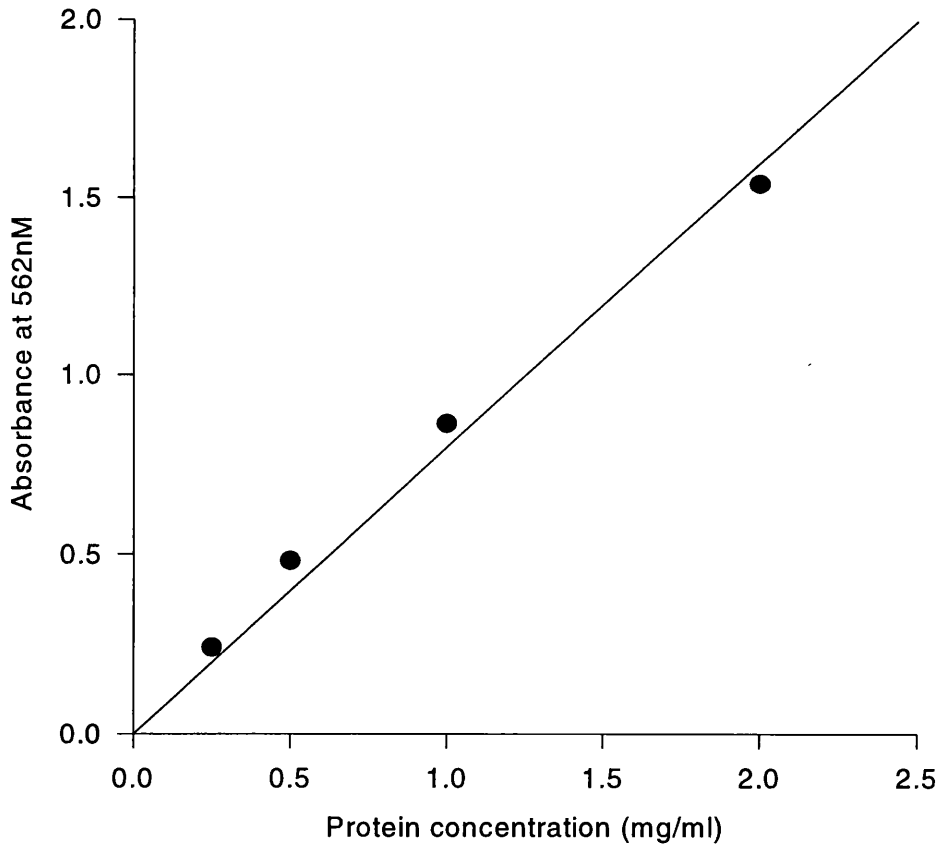

Figure 2.3. A typical standard curve for the protein assay.

2.3.4 Histamine release assay

The histamine released following incubation of human nasal cells with various secretagogues was assayed using a commercial autoanalyser (Technicon Autoanalyser II). The basic principle of histamine assay was first described by Shore *et al.* (1959). Histamine reacts with o-phthaldialdehyde (OPT) under alkaline conditions to form a fluorescent product. This product can be stabilised by acidification and the fluorescence measured by a spectrophotometer. The automated assay used in this study selectively extracts histamine from the samples before the reaction with OPT.

The samples for analysis were first treated with perchloric acid (0.4 M final concentration) to free residual histamine and to precipitate proteins and drugs in the sample. The samples were then centrifuged (200g for 10min. at 4^0C) and the supernatants retained for automatic assay. The automated assay works as follows. The samples were made alkaline and the histamine extracted with butan-1-ol. The organic phase was then separated, washed once in a less alkaline medium, made less polar by the addition of *n*-heptane and the histamine extracted into dilute HCL. The amine was reacted with OPT under alkaline conditions and the product stabilised by acidification. The fluorescence was detected on a chart recorder. The limit of sensitivity of this automated histamine assay was approximately 1ng histamine / ml.

CHAPTER 3

THE EFFECT OF BRADYKININ RECEPTOR ANTAGONISTS ON THE NASAL ACTIONS OF BRADYKININ AND ANTIGEN

3.1 Introduction

There is evidence that bradykinin is a mediator of allergic rhinitis. Bradykinin and kallidin are released into the nasal airway following grass pollen provocation of subjects with seasonal allergic rhinitis (Proud *et al.*, 1983). In non-allergic subjects, challenge of the nasal airway with bradykinin produces some of the symptoms of allergic rhinitis: namely, increased nasal airway resistance and increased vascular permeability of the nasal vasculature (Proud *et al.*,1988; Rajakulasingham *et al.*, 1991; Austin & Foreman, 1994a). The evidence that bradykinin is involved in the pathogenesis of allergic rhinitis is not limited to experimental observations. Kinins appear in the nasal secretions of patients with seasonal allergic rhinitis following grass pollen exposure during the pollen season (Svennsson *et al.*, 1990).

Bradykinin and the closely related peptide kallidin (lys-bradykinin) mediate the majority of their actions via specific receptors. Two types of bradykinin receptor, B_1 and B_2, have been clearly defined. There is also evidence for the existence of a B_3 receptor for kinins in the guinea pig lower airways (Farmer *et al.*, 1989). In the human nasal airway, B_1 receptor agonists are inactive, both in normal and atopic subjects (Rajakulasingam *et al.*, 1991; Austin, 1994). It is proposed that the receptor mediating the actions of bradykinin in the human nasal airway is of the B_2 receptor type (Rajakulasingam *et al.*, 1991).

The bradykinin B_2 receptor antagonist NPC 567 ([D-Arg^0, Hyp^3, D-Phe^7]-bradykinin) fails to antagonise the nasal actions of bradykinin challenge in normal subjects, even at the high dose of 1mg (Pongracic *et al.*, 1991; Austin & Foreman, 1994a). However, a single dose of the potent B_2 receptor antagonist, icatibant, significantly antagonises the nasal actions of bradykinin challenge in normal subjects and house-dust mite challenge in atopic subjects (Austin & Foreman, 1994a; Austin *et al.*, 1994).

Using non-atopic human subjects, this chapter examines the effect of bradykinin receptor antagonists on bradykinin-induced reductions in nasal patency and increases in vascular permeability. In subjects with seasonal and perennial allergic rhinitis, this chapter examines the effect of bradykinin receptor antagonists on the nasal effects of allergen challenge.

3.2 Experimental protocol

All of the protocols used in this chapter are based on a single-blind, randomised, cross-over design.

For the following experiments non-atopic human subjects were used.

3.2.1 Effect of bradykinin on nasal patency and albumin extravasation into the nasal cavity of non-atopic human subjects

The minimum cross-sectional area (Amin.) of the nasal cavity was determined by acoustic rhinometry. Bradykinin was then delivered into the nasal cavity by hand-held pump spray. Ten minutes after bradykinin challenge the Amin. was remeasured.

The study of albumin release was conducted on separate occasions from the study of nasal patency. Nasal lavage was performed, three times per nostril, prior to any treatment: the first two lavages were discarded and the final lavage was used for the measurement of baseline albumin release. Then bradykinin was delivered into the nasal cavity. Ten minutes later another nasal lavage was performed. The albumin content of the nasal lavage was determined by radial immunodiffusion assay.

The doses of bradykinin used for both the studies on nasal patency and albumin release were 100, 300 and 1000μg. Each subject received one of the doses of bradykinin on separate occasions, at least 24 hours apart. The order of the doses was determined randomly; each subject received all of the treatments.

3.2.2 The effect of bradykinin receptor antagonists on the reduction in nasal patency induced by bradykinin in non-atopic human subjects

For the following studies, using bradykinin receptor antagonists in non-atopic subjects, the dose of bradykinin used was 100μg. This dose was chosen as it produces a reproducible and measurable effect but does not mediate its action via histamine release; a possible receptor-independent mechanism (see chapter 6).

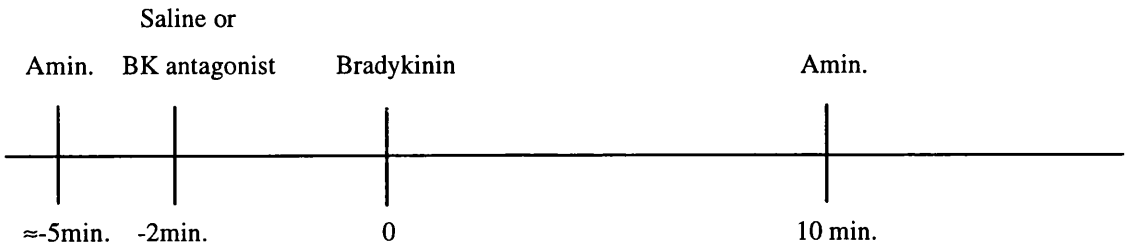

Figure 3.1. Protocol for studying the effect of bradykinin receptor antagonists on the reduction in nasal patency induced by bradykinin in non-atopic human subjects.

The minimum cross-sectional area (Amin.) of the nasal cavity was determined by acoustic rhinometry. Then the nasal cavity was treated with a dose of bradykinin receptor antagonist or vehicle (saline) delivered by hand-held pump spray. On separate occasions, at least 2 days apart, each subject received vehicle or one of the doses of the antagonist being tested. The order of treatments was determined randomly. For each antagonist, each subject received all of the doses. Two minutes after pretreatment, bradykinin, 100μg, was delivered into the nasal cavity, again by hand-held pump spray. Ten minutes after bradykinin challenge the Amin. was remeasured. This protocol is summarised in figure 3.1.

The effect of duration of pretreatment with icatibant, 200μg, on the response to bradykinin, 100μg, was studied. In this case, the interval between the icatibant treatment and the bradykinin challenge was 5-180min. and the baseline Amin. values were recorded 5min. prior to the bradykinin challenge. The Amin. was again measured 10min. after bradykinin challenge.

3.2.3 The effect of bradykinin receptor antagonists on the increase in albumin extravasation into the nasal cavity induced by bradykinin in non-atopic human subjects

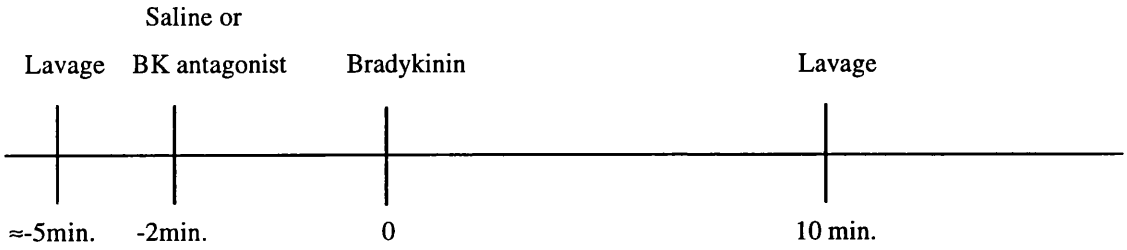

Figure 3.2. Protocol for studying the effect of bradykinin receptor antagonists on the increase in albumin extravasation into the nasal cavity induced by bradykinin in non-atopic human subjects.

The study of albumin release was conducted on separate occasions from the study of nasal patency. Nasal lavage was performed, three times per nostril, prior to any treatment, and the final lavage was used for the measurement of baseline albumin release. Subjects then received either saline or a dose of bradykinin receptor antagonist: the order of which being randomly determined. For each antagonist, each subjects received all the doses. On separate occasions, at least 2 days apart, each subject received vehicle or one of the doses of the antagonist being tested. Two minutes later, bradykinin, 100μg was administered to each nostril and 10 min. later another lavage was performed and the sample retained for albumin assay. This protocol is summarised in figure 3.2.

For both the studies on nasal patency and albumin release the pre-bradykinin challenge treatments administered into the nasal cavity were: saline; icatibant, 10, 30, 100 and 200μg; [1-adamantane acetyl-D-Arg^{0}, Hyp^{3}, Thi^{5,8}, D-Phe^{7}]-bradykinin, 33, 50, 100 and 200μg; NPC 567, 10mg;

3.2.4 Effect of grass pollen antigen on the nasal airway patency of atopic human subjects

For this experiment the human subjects had a history of seasonal allergic rhinitis and a positive skin prick test to grass pollen.

The minimum cross-sectional area (Amin.) of the nasal cavity was determined by acoustic rhinometry. Grass pollen was then delivered into the nasal cavity by hand-held pump spray. Ten minutes after challenge the Amin. was remeasured. The doses of grass pollen antigen used were 100, 500, 1000 and 2000units. Each subject received saline or one of the doses of grass pollen on separate occasions of at least 1 week apart. The order of the doses was determined randomly. Each subject received all of the doses of grass pollen.

3.2.5 The effect of bradykinin receptor antagonists on the nasal responses to antigen challenge in atopic human subjects

For these experiments the human subjects had a history of either perennial allergic rhinitis and a positive skin prick test to house-dust mite; or a history of seasonal allergic rhinitis and a positive skin prick test to grass pollen.

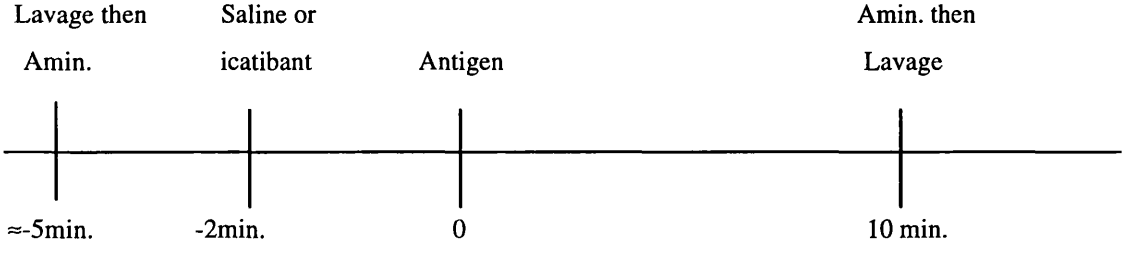

Figure 3.3. Protocol for investigating the effect of bradykinin receptor antagonists on the nasal responses to antigen challenge in atopic human subjects

The protocol was similar to that for the bradykinin study except that mixed grass pollen antigen, 1000units per nostril or house-dust mite antigen, 500units per nostril was used for the challenge. The subjects were treated with icatibant or saline, each subject receiving saline or one of the doses of icatibant on separate occasions at least 1 week apart; the treatment delivered first being determined randomly. For both antigens, each subject received all of the doses of icatibant. As before, there was 2 minutes between treatment and challenge. In addition, nasal lavage and acoustic rhinometry were determined in the same study: nasal lavage preceding the pretreatment Amin. measurement and coming after the post challenge Amin. measurement. This protocol is summarised in figure 3.3. For subjects with allergy to grass pollen antigen, the pre challenge treatments were saline; icatibant 200 and 500μg. For subjects with allergy to house-dust mite antigen the pre-challenge treatments were saline; icatibant 10, 33, 100 and 200μg.

Using the same protocol, subjects with allergy to house-dust mite antigen were pretreated with saline or icatibant, 200μg. Each subject received both treatments on separate occasions at least one week apart. The treatment given first was determined randomly. As before, nasal lavages and Amin. measurements were taken, but the nasal lavages were assayed for their kinin content. EDTA, 40mM was added and the lavages were stored on ice then at -70^{0}C until analysis (Proud *et al.*, 1983). The kinin content of the nasal lavage was determined using a commercially available bradykinin radioimmunoassay kit, the antiserum used for this assay was raised in the rabbit. [^{125}I]-bradykinin was used as the radioisotope and the range of sensitivity of this assay was 1-128 pg of kinin per tube. This assay has equal sensitivity for bradykinin and kallidin.

3.2.6 Data analysis

The dimensions of the nasal airway vary between subjects and also within subjects from day to day and so the data have been normalized by expressing changes in Amin. as a percentage of the baseline control value. The absolute values for the baseline measurements have been given with each set of data. Means are given together with

standard error of the mean (s.e.mean). The appropriate, non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

3.3 Results

3.3.1 The effects of bradykinin and bradykinin analogues on the non-atopic human nasal airway

Administering bradykinin into the nasal cavity of normal, non-atopic subjects causes a dose-dependent reduction in nasal patency and an increase in the albumin released into the nasal cavity (figure 3.4A and 3.4B, for both, $p<0.03$, analysed by Friedman's test). Figure 3.5A shows that bradykinin, 100μg introduced into the nasal airway of normal subjects resulted in a reduction of the minimal cross-sectional area (Amin) of the nasal cavity of 16 ± 5%. Icatibant produced significant dose-related reversal of this action of bradykinin, 100μg ($p<0.01$, analysed by the Friedman test). Figure 3.5A was fitted to the following equation:

$$y = y_{max} \bullet \frac{A^{nH}}{IC_{50}{}^{nH} + A^{nH}} \qquad \text{(Eq 3.1)}$$

where A is the dose of antagonist per nostril, IC_{50} is the dose of antagonist that produces a half-maximal inhibition of the action of bradykinin, y and y_{max} are the response and maximal response (i.e. % of baseline Amin.) and n_H is the Hill coefficient. The IC_{50} for icatibant reversal of bradykinin-induced reduction in Amin. was 20±7μg, n_H=13.4 (Figure 3.5A). Figure 3.5B shows that bradykinin, 100μg induced release of albumin into the nasal cavity and this effect was inhibited in a dose-related manner by icatibant ($p<0.02$, analysed by the Friedman test). Figure 3.5B was fitted to the following equation:

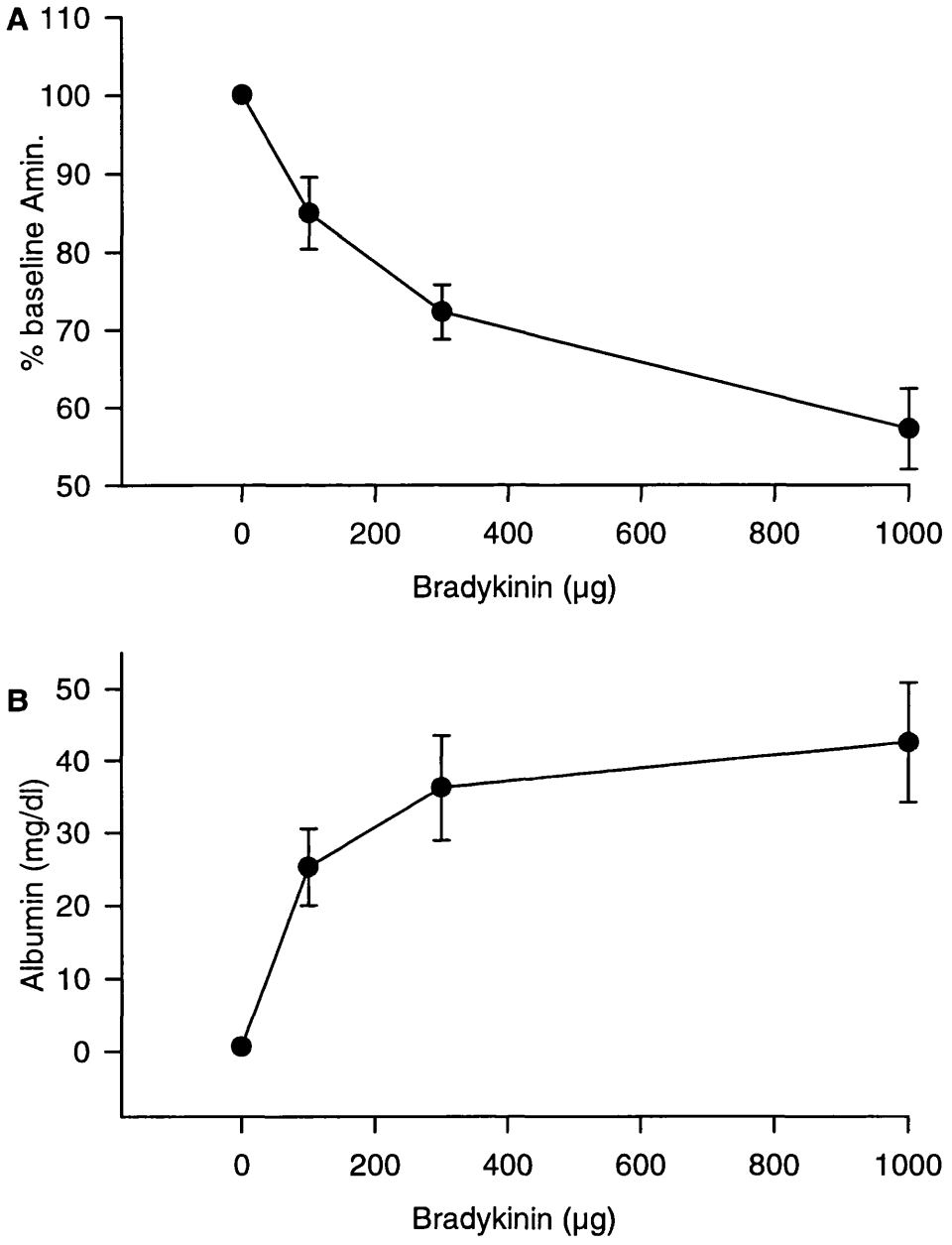

Figure 3.4. Dose-response curves for the effect of bradykinin on: A the minimal nasal cross-sectional area (Amin.) and B albumin extravasation into the nasal cavity. Data are means from 5 subjects. Vertical bars represent s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.51±0.04 cm^2.

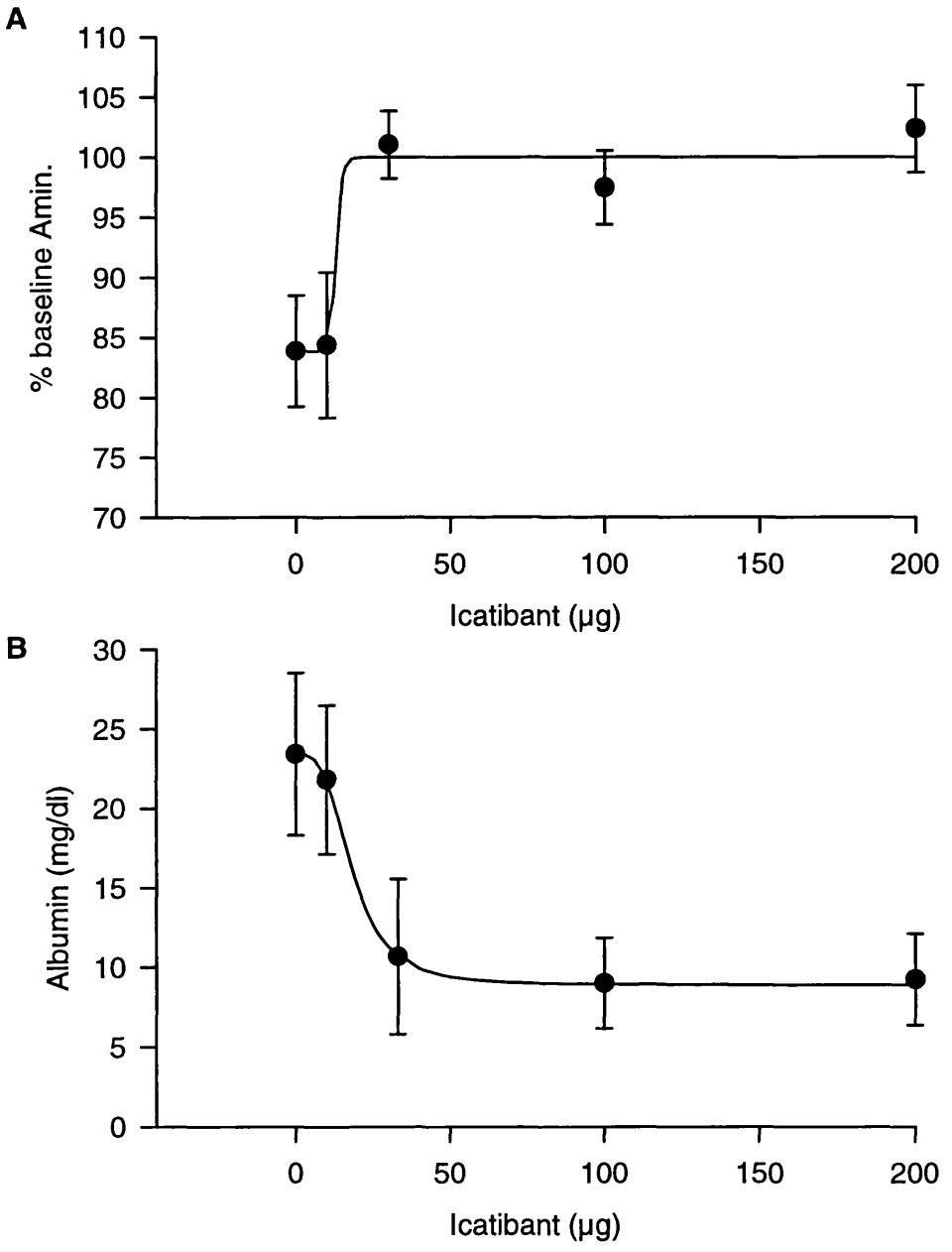

Figure 3.5. Dose-response curves for the effect of icatibant on: A the reduction of the minimal nasal cross-sectional area (Amin.) and B albumin extravasation into the nasal cavity induced by bradykinin, 100µg. Data are means from 5 subjects. The curves have been fitted as described in the results. Vertical bars represent the s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.56± 0.02 cm^2.

$$y = y_0 \bullet \left(1 - \frac{A^{nH}}{IC_{50}{}^{nH} + A^{nH}}\right) \qquad \text{(Eq 3.2)}$$

where y and y_0 represent the response (albumin release) in the presence and absence of antagonist respectively. The other symbols are as in equation 3.1. For icatibant antagonism of bradykinin-induced albumin release the IC_{50} was 25±2μg, n_H=3.3.

Figure 3.6 shows the effect of the duration of pretreatment with icatibant on the reduction in Amin. induced by bradykinin. The effect of icatibant was significant when it was administered 5-120 min. prior to bradykinin ($p<0.05$, assessed by the Wilcoxon paired, sign-rank test, compared to saline pretreatment) and the magnitude of the effect of icatibant decreased with longer pretreatment time.

Figure 3.7 shows the effect of [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin on bradykinin-induced Amin. reduction and albumin release in normal subjects. Figure 3.7A shows that this antagonist significantly antagonised the nasal blockage induced by bradykinin, 100μg, in a dose-related manner ($p<0.01$, analysed by the Friedman test). By fitting to equation 3.1, the IC_{50} was 56±9μg, n_H=2.6. Figure 3.7B shows that the increased albumin extravasation induced by bradykinin, 100μg, is also inhibited by [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin in a dose-dependent manner($p<0.02$, analysed by the Friedman test). By fitting to equation 3.2 the IC_{50} was calculated as 79±7μg, n_H=2.6.

Figure 3.8 shows that NPC 567, 10mg failed to inhibit the bradykinin-induced Amin. reduction and albumin release in normal subjects ($p>0.05$, analysed by the Wilcoxon sign rank test).

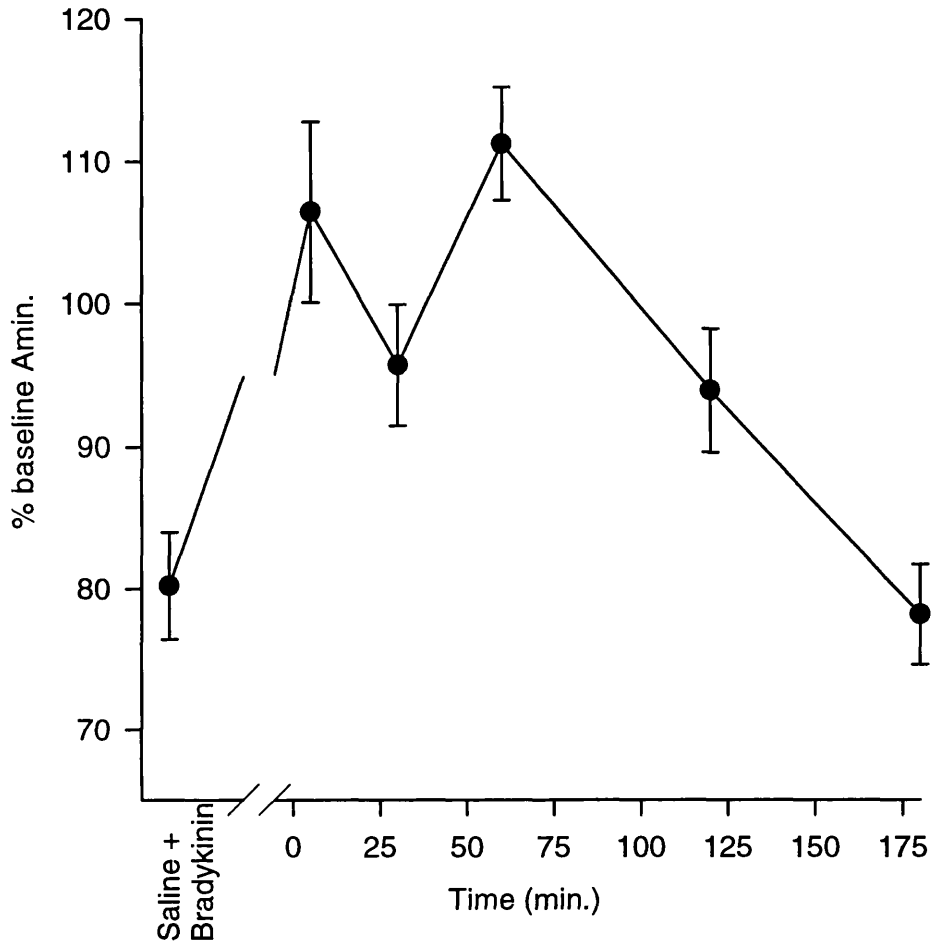

Figure 3.6. Effect of the duration of pretreatment with icatibant on the reduction of Amin., caused by bradykinin, 100 μg. The reduction in Amin. induced by bradykinin, 100μg, with saline pretreatment is also shown. Icatibant, 200μg was administered at the times shown prior to the challenge with bradykinin. The data are means from 5 subjects. Vertical bars represent the s.e.mean. Changes in Amin. have been normalized by expressing them as a percentage of the baseline Amin. for each subject. The mean ± s.e.mean baseline value of Amin., was 0.48 ± 0.03 cm^2.

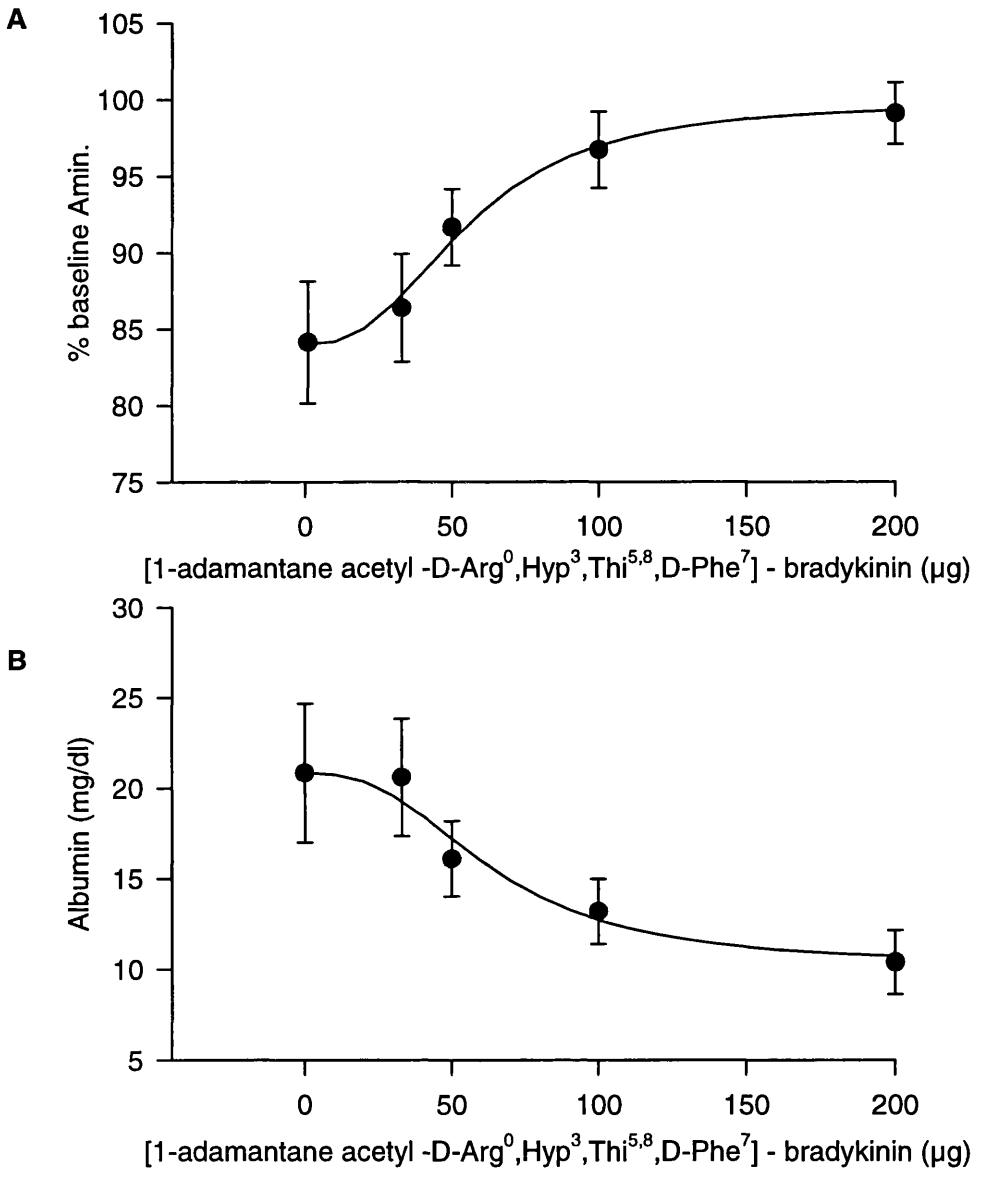

Figure 3.7. Dose-response curves for the effect of [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin on: A the reduction of the minimal nasal cross-sectional area (Amin.) and B albumin extravasation into the nasal cavity induced by bradykinin, 100µg. Data are means from 5 subjects. The curves have been fitted as described in the results. Vertical bars represent the s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.50 ± 0.01cm^2.

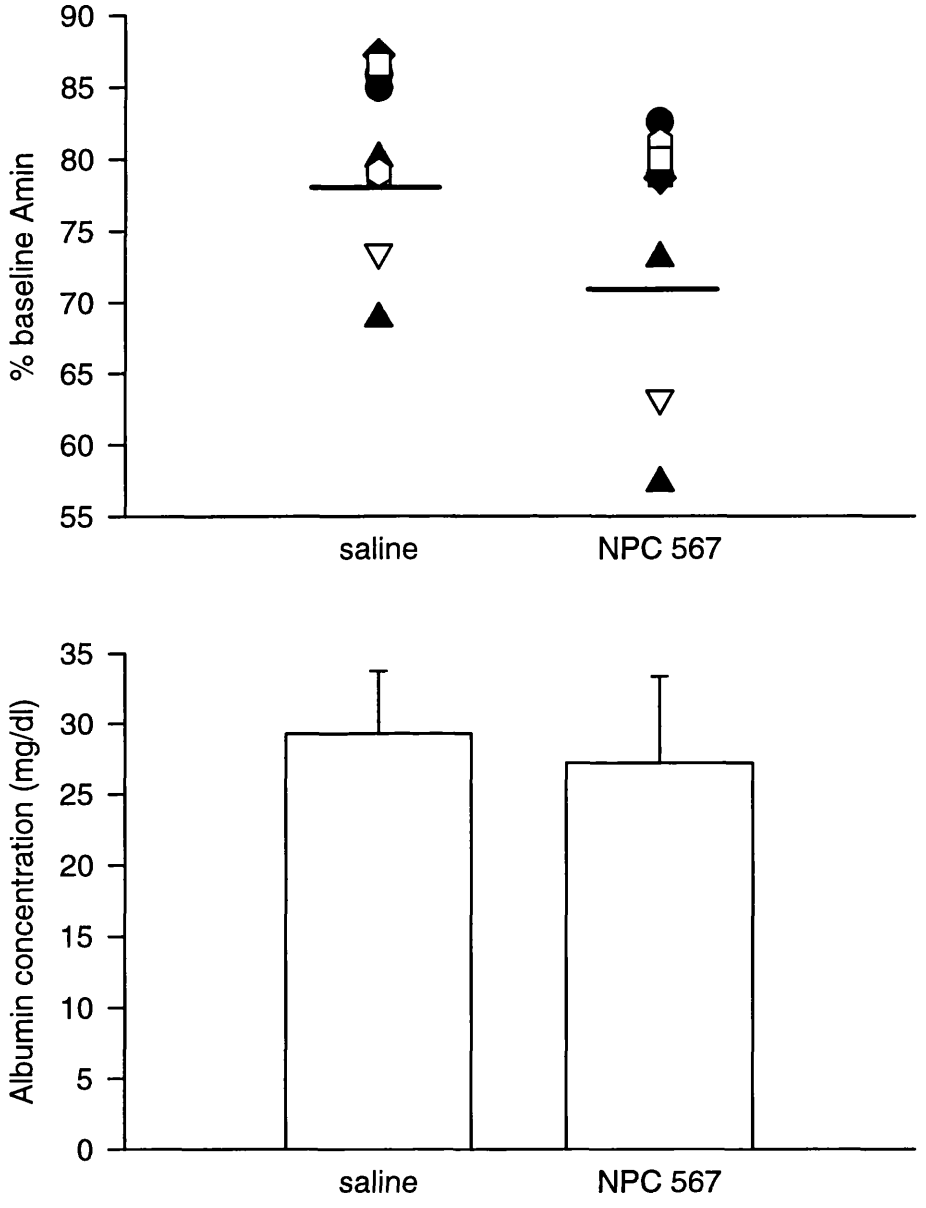

Figure 3.8. Effect of NPC 567 on bradykinin-induced reductions in Amin. and increased albumin extravasation into the nasal cavity. Graph A shows the change in Amin. for each subject following nasal challenge with bradykinin, 100μg. Each symbol represents an individual subject with either saline or NPC 567, 10mg, pretreatment. Changes in Amin. have been normalized by expressing them as a percentage of the baseline Amin. for each subject. Horizontal bars represent the mean. The mean ± s.e.mean baseline value of Amin., was 0.49 ± 0.01cm^2. Graph B show the albumin extravasation into the nasal cavity induced by bradykinin, 100μg, with either saline or NPC 567, 10mg, pretreatment. The mean from nine subjects is shown. Vertical bars represent the s.e.mean.

3.3.2 The effect of bradykinin receptor antagonists on the nasal actions of antigen in atopic human subjects

Figure 3.9 shows that grass pollen causes a dose-dependent reduction in the nasal patency of subjects with allergy to this antigen ($p<0.03$, analysed by the Friedman test). Grass pollen antigen, 1000units, caused a decrease in the minimum cross-sectional area (Amin) of 26±5 %. Previous studies using subjects with perennial allergic rhinitis showed that 500units of house-dust mite antigen produced a decrease in the minimum cross-sectional area (Amin.) of 27±6 % (Austin, 1994). For further challenge studies grass pollen, 1000units and house-dust mite, 500units were used as these doses appear to be approximately equipotent and are near to the mid-point of the dose-response curve for reductions in Amin.

Figure 3.10 shows that icatibant failed to antagonise grass pollen-induced reductions in Amin. and increases in albumin extravasation. Grass pollen challenge (1000units), in subjects with allergy to this antigen, caused a reduction in Amin. of 29.6±2.7 % (figure 3.10A). Icatibant, even at 500μg, failed significantly to antagonise this reduction ($p>0.1$, analysed by Friedman's test; icatibant, 500μg, failed significantly to reverse the decrease in Amin. when compared to saline control, $p>0.2$, analysed by the Wilcoxon sign rank test). Grass pollen challenge in sensitive subjects caused an increase in albumin extravasation into the nasal cavity (figure 3.10B). Icatibant also failed significantly to antagonise this albumin release ($p>0.15$, analysed by Friedman's test; icatibant, 500μg, failed significantly to inhibit the albumin release when compared to saline control, $p>0.2$, analysed by the Wilcoxon sign rank test).

Figure 3.11 shows the dose-response relationship for the inhibition by icatibant of the reduction in Amin. caused by house dust mite antigen challenge in allergic subjects sensitive to this antigen. House-dust mite, 500units, caused a significant reduction of the minimal cross-sectional area (Amin.) of the nasal cavity of 22±5 %. Icatibant significantly reversed this nasal blockage in a dose-related fashion ($p< 0.01$ analysed by Friedman's test). Fitted to equation 3.1 the IC_{50}=24±3μg, n_H=1.1. House-dust mite challenge in sensitive subjects failed to increase albumin extravasation into the nasal

cavity. With saline pretreatment, the baseline albumin concentration in the nasal lavage was 3.1±0.6 mg/dl, after house-dust mite challenge the albumin concentration in the nasal lavage was 3.4±0.8 mg/dl ($p>0.05$, analysed by the Wilcoxon, sign-rank test, n=10)

Figure 3.12 shows the bradykinin concentrations recovered by nasal lavage and the changes in Amin. following house dust mite antigen challenge of subjects with perennial allergic rhinitis, sensitive to this antigen. House dust mite antigen challenge significantly reduced the Amin. (figure 3.12A) and elevated the bradykinin concentration in nasal lavage fluid (figure 3.12B). Pretreatment of subjects with icatibant, 200μg 2min. prior to antigen challenge significantly reduced the bradykinin level in nasal lavage fluid and reversed the effect of antigen challenge on Amin.

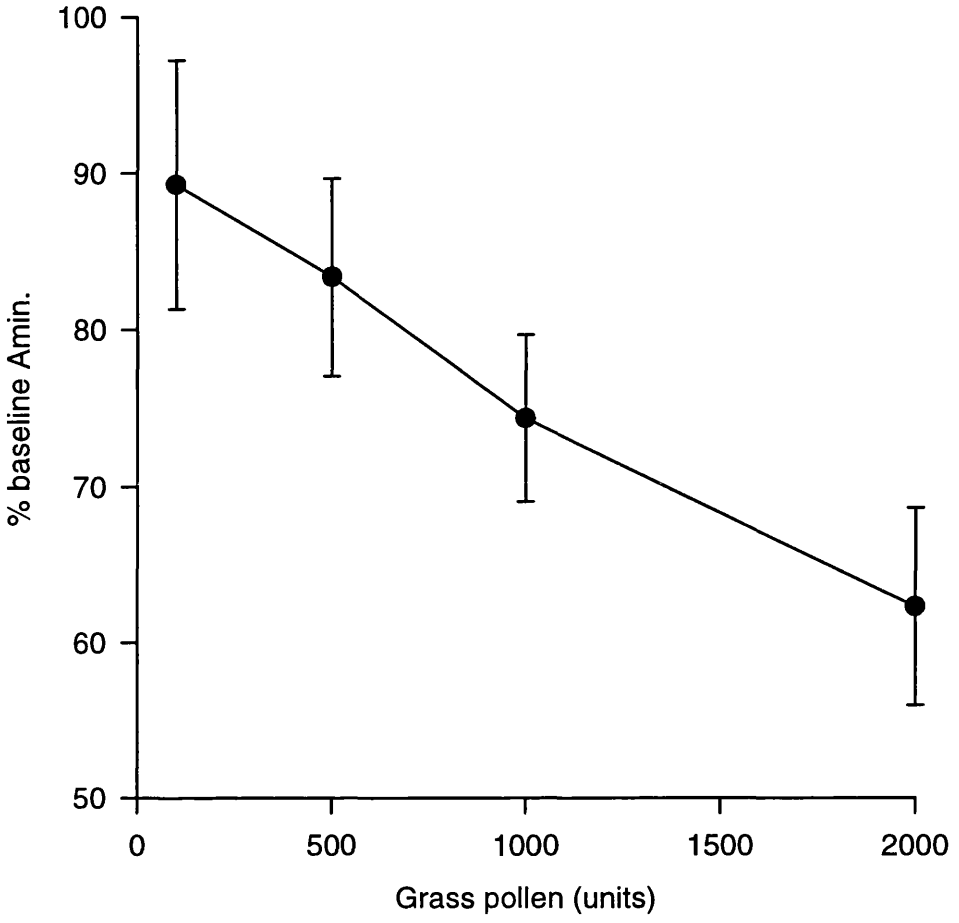

Figure 3.9. Dose-response curve for the effect of grass pollen antigen on the minimal nasal cross-sectional area (Amin.) Data represent the means from 5 subjects. Vertical bars represent the s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.45±0.05 cm^2.

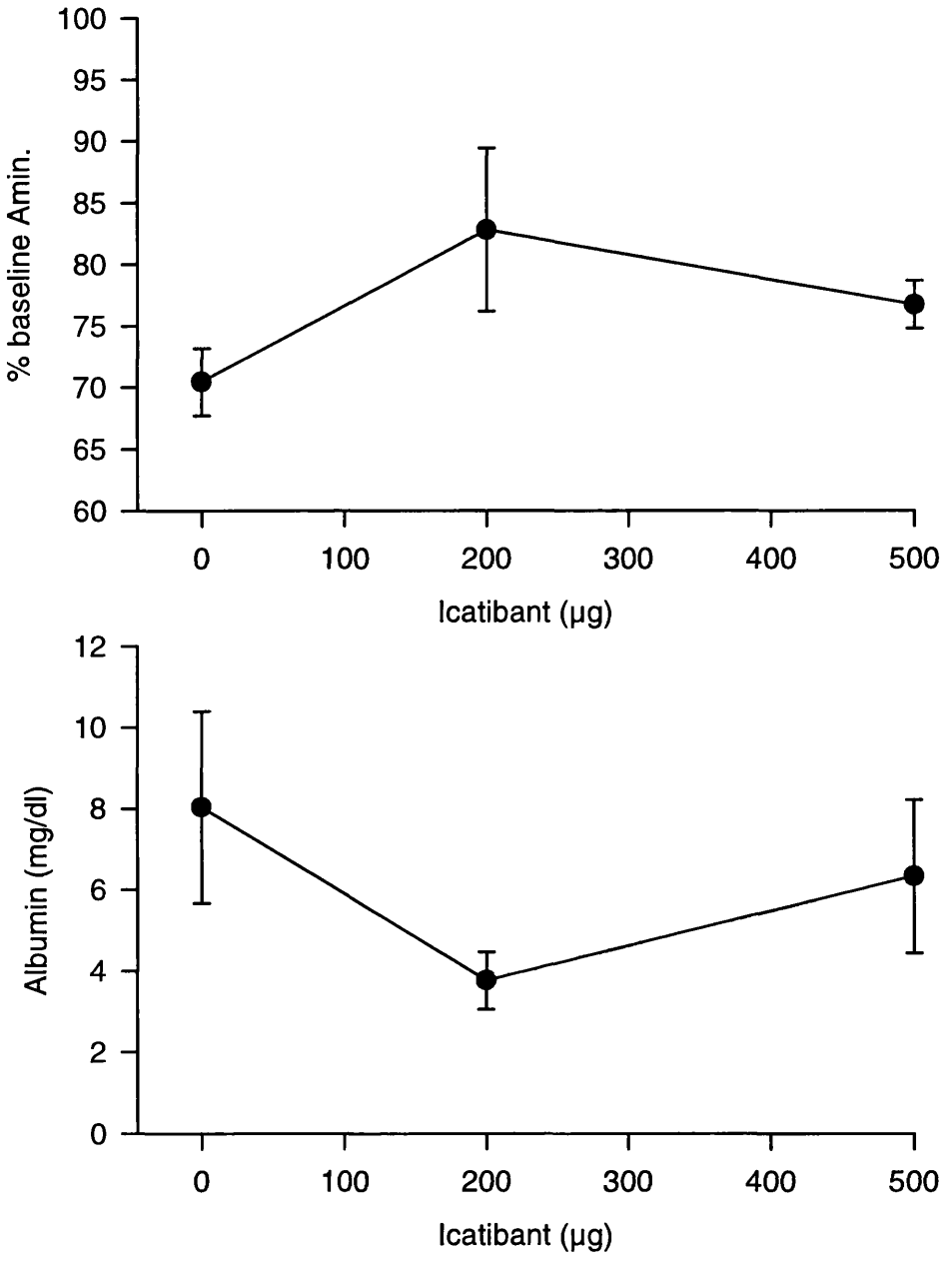

Figure 3.10. Dose-response curves for the effect of icatibant on: A the reduction of the minimal nasal cross-sectional area (Amin.) and B albumin extravasation into the nasal cavity induced by grass pollen antigen, 1000 units. Data represent the means from 10 subjects. Vertical bars represent the s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.5± 0.1 cm^2.

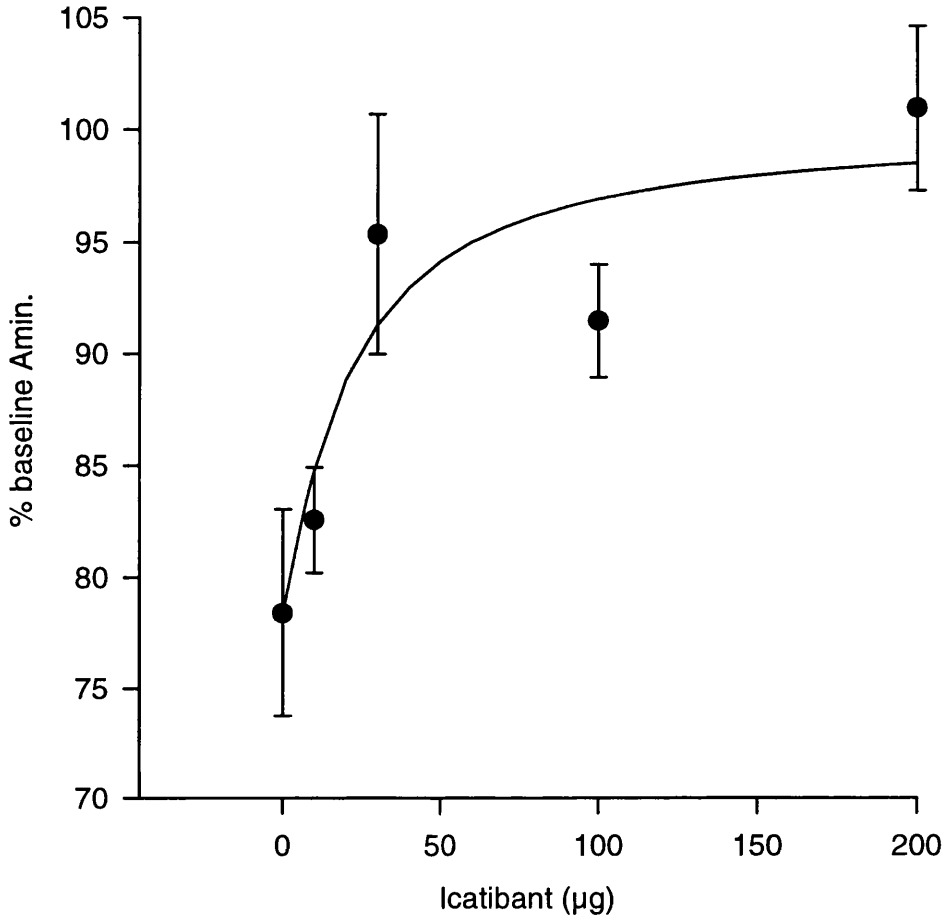

Figure 3.11. Dose-response curve for the effect of icatibant on the reduction of the minimal nasal cross-sectional area (Amin.) induced by house-dust mite, 500 units (HDM). Data represents the means from 10 subjects. Curves have been fitted as in the results. Vertical bars represent the s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean. baseline value for Amin. was 0.48 ± 0.02cm^2.

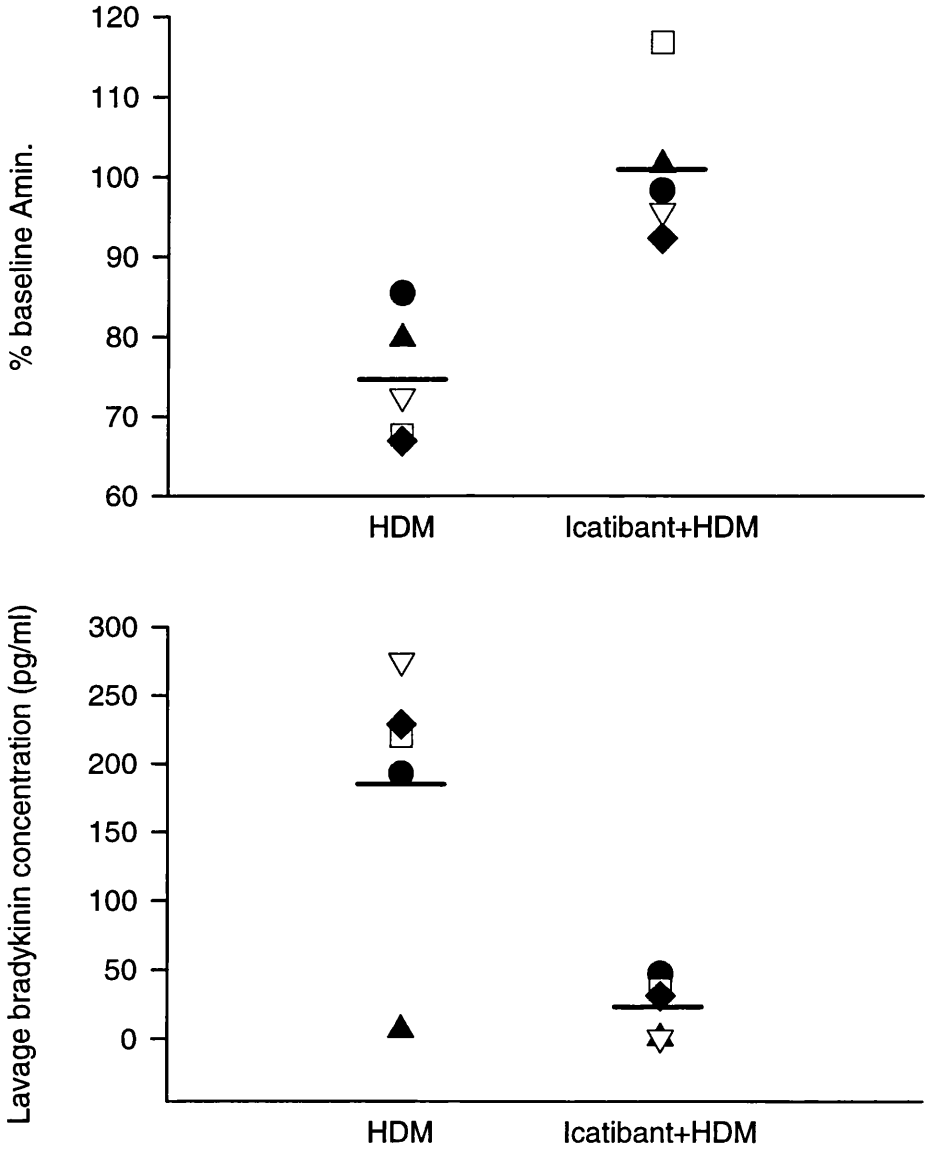

Figure 3.12. The effect of icatibant on the reduction in Amin. and bradykinin release into the nasal cavity induced by house-dust mite, 500 units (HDM). Graph A shows the change in Amin for each subject following nasal challenge with house-dust mite antigen. Each symbol represents an individual subject pretreated with either saline or icatibant, 200μg. Changes in Amin. have been normalized by expressing them as a percentage of the baseline Amin. for each subject, horizontal bars represent the mean. The mean ± s.e.mean baseline value of Amin. was 0.50 ± 0.03cm^2. Graph B shows the kinin concentration in the nasal lavage following HDM challenge. The mean ± s.e.mean baseline value of kinin concentration was 25.7 ± 10.6pg.ml^{-1}.

3.4 Discussion

Bradykinin, administered intranasally, causes a dose-related reduction in the patency of the nasal airway and an increase in the albumin released into the nasal cavity. This is consistent with the findings of other studies (Proud *et al.*, 1988; Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a). The increase in the albumin release is probably due to increased plasma extravasation from the blood vessels of the nasal mucosal. Increased plasma extravasation could result from an increase in vascular permeability, but, in addition, an increase in blood flow into the permeabilized vascular bed will increase the extravasation. The reduction in nasal airway patency may be due to vasodilatation of the nasal vasculature, and/or tissue oedema. This oedema results from plasma leaking out of the mucosal blood vessels into the surrounding tissues. Both of these events would lead to swelling of the tissue lining the nasal cavity and therefore blockage of the airway. The swelling is particularly great in the mucosa covering the inferior turbinate bone, which is located on the lateral wall of the nasal cavity, as this is the site of cavernous sinusoids analogous to the erectile tissue of the penis. Bradykinin may also induce a reduction in the patency of the nasal airway by increasing the mucous secretion into the nasal cavity, therefore again causing blockage. The effect of bradykinin on mucus secretion was not studied.

Previous studies have shown that bradykinin B_1 receptor agonists have no effect on human nasal airway resistance or albumin release into the nasal cavity (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a). These studies have been performed both in normal non-atopic human subjects (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a) and in atopic human subjects with chronic allergic rhinitis (Austin, 1994). Therefore, although the B_1 selective metabolite of bradykinin, [DesArg^9]-bradykinin, can be generated in the human nasal airway (Proud *et al.*, 1987a), it appears that the B_1 receptor is not involved in the nasal actions of bradykinin. Bradykinin is a highly potent agonist at the B_2 receptor and autoradiographic studies of human nasal tissue have shown that [^{125}I]-bradykinin binds to vascular and neural sites (Baraniuk *et al.*, 1990b). This evidence, together with the lack of effect of bradykinin B_1 receptor agonists, strongly suggests that the

nasal actions of bradykinin are mediated by the B_2 receptor, although a role for the putative B_3 receptor type is a possibility. The first part of this discussion will use bradykinin B_2 receptor antagonists to elucidate further the characteristics of the human nasal bradykinin receptor.

Bradykinin-induced reductions in nasal patency and increases in albumin extravasation are antagonised by icatibant. This has been reported previously (Austin & Foreman, 1994a), but only a single dose of 100μg was used. In this study, a range of doses are used showing that icatibant antagonises the nasal effects of bradykinin in a dose-dependent manner. From fitted antagonist dose-response curves, estimates of the doses of antagonist required to reduce the response to a given dose of antigen or bradykinin by 50% have been determined. Clearly, these doses which produce a 50% inhibition will depend on the dose of agonist and are, therefore, only of use in calculating relative activities. The IC_{50} values for the inhibition by icatibant of bradykinin-induced Amin. reduction and albumin leakage are 20 and 25μg (15 and 19nmol) respectively. The duration of action of icatibant, 200μg, was also studied and icatibant had no significant effect on bradykinin-induced Amin. reduction if delivered 3 hours prior to kinin challenge. This time-course is consistent with the time course of the nasal action of icatibant reported in other studies (Proud, 1994). It seems unlikely that the factor limiting the duration of icatibant action is enzymatic degradation as studies have shown this antagonist to be relatively stable against breakdown by enzymes (Hock *et al.*, 1991). Proud (1994) proposed that the factor limiting the duration of action of icatibant is its mucocilliary clearance from the nasal cavity. As icatibant is a polar molecule, it maybe poorly absorbed across the nasal mucosa so becoming a target for mucocilliary clearance (Proud, 1994). However, as icatibant has a very rapid onset of action in the nasal cavity (extremely effective when given only 2 minutes prior to bradykinin challenge), this strongly argues against icatibant being poorly absorbed into nasal tissues. For whatever reason, the short duration of action of icatibant may limit its therapeutic value.

Bradykinin-induced reductions in nasal patency and increases in albumin extravasation are also antagonised by [1-adamantane acetyl-D-Arg^0, Hyp^3, $Thi^{5,8}$, D-

Phe^7]-bradykinin. This antagonist inhibits the effects of bradykinin nasal challenge in a dose-related manner and IC_{50} values for the inhibition of bradykinin-induced Amin. reduction and albumin leakage are 56 and 79μg (38 and 54nmol) respectively. Pongracic *et al* (1991) tested the effect of intranasal administration of the kinin B_2 receptor antagonist NPC 567 ([D-Arg^0,Hyp^3,D-Phe^7]-bradykinin) on various responses to nasal provocation with bradykinin. Even at 1mg (0.8μmol) per nostril, NPC 567, has no effect on any of the parameters measured, namely symptom scores or albumin and TAME-esterase levels in nasal lavage fluid. It also had no significant effect on bradykinin-induced nasal blockage measured quantitatively by active posterior rhinomanometry (Austin & Foreman, 1994a). NPC 567 has a relatively low affinity for the kinin B_2 receptor and a low potency at antagonising B_2 receptor mediated effects. Therefore, the dose of NPC 567 used in previous investigations may not have been sufficiently high to significantly antagonise the nasal actions of bradykinin. In this study, no effect was detected with 10mg (8μmol) of NPC 567.

There are various possible explanations for the failure of NPC 567 to antagonise the nasal actions of bradykinin. Bradykinin-induced reductions in nasal patency and increases in albumin extravasation appear to be, at least partly, receptor-mediated effects as they are antagonised by icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin. The ability of these antagonists, but the failure of NPC 567 to antagonise the nasal actions of bradykinin may be due to icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin having a higher affinity for the bradykinin B_2 receptor (reviewed in table 1.1). Several groups have utilised the cloning of the human bradykinin B_2 receptor to explore the receptor pharmacology. Human bradykinin B_2 receptor cDNA and genomic clones have been expressed in stable cell lines and functionally characterised by measuring second messenger generation. This has revealed some interesting data on the potency of B_2 receptor antagonists but must be interpreted with caution as results from reconstituted receptors in stable cell lines do not necessarily reflect the *in vivo* situation. Eggerickx *et al.* (1992) reported that NPC 567 was unable to antagonise bradykinin-induced increases in intracellular calcium in transfected CHO cell lines. However, this response was antagonised by icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8},

D-Phe[7]-bradykinin (Eggerickx *et al.*, 1992). Another study, using hepatoma H4 cells transfected with human bradykinin B_2 receptor cDNA, reported that NPC 567 antagonised bradykinin-induced increases in inositol trisphosphate, but was 1000 times less potent than icatibant (Davis *et al.*, 1995). By transfecting Chinese hamster ovary cells with the human B_2 receptor, Hess *et al.* (1994) reported that NPC 567 is approximately 300 times less potent than icatibant at displacing the binding of [^{3}H]-bradykinin. In *ex vivo* human lung tissue, NPC 567 has approximately 350 times lower affinity than icatibant for the B_2 receptor (Trifilieff *et al.*, 1994) and thus is consistent with the data from cloned receptors. It appears, therefore, that NPC 567 has a low affinity for human B_2 receptors and this may explain the failure of NPC 567 to antagonise the reduction in nasal patency induced by bradykinin. NPC 567 is a substrate for degradation by carboxypeptidase N (Proud *et al.*, 1987a), whereas icatibant is resistant to enzymatic degradation (Hock *et al.*, 1991). The breakdown of NPC 567 may limit its potency in the human nasal airway. The ability of NPC 567 to gain access to the nasal kinin receptor could be poorer than that of the other antagonists, but there is no evidence to support this. NPC 567 has been shown to release histamine from human skin mast cells (Cohan *et al.*, 1991) and induce an inflammatory response when injected into human skin, which is mediated in part by histamine release (Kindgen-Miles & Klement, 1992). Chapter 6 shows that bradykinin releases histamine from human nasal tissue and this release contributes to the ability of bradykinin to cause reductions in nasal patency. In rodent mast cells bradykinin-induced histamine release is receptor independent and histamine is released by bradykinin receptor antagonists as well as agonists. Therefore, the high dose of NPC 567 used in this chapter may stimulate histamine release in the nasal cavity. The released histamine would reduce the patency of the nasal airway and this would oppose the reduction of the effect of bradykinin by NPC 567. Unfortunately, cost precluded any further experiments with a higher dose of NPC 567, therefore the absence of an effect of NPC 567 *in vivo* in human subjects cannot be explained. Alternatively, the human nasal kinin receptor could be an example of the putative B_3 receptor type. NPC 567 has no affinity for the B_3 receptor proposed to be present in the guinea pig lower airways (Farmer *et al.*, 1989). However, icatibant is a potent antagonist at this receptor (Field *et al.*, 1992), which may explain its ability to

antagonise the nasal actions of bradykinin. The issue of whether or not NPC 567 interacts with the human nasal kinin receptor is addressed using a different approach to further characterise the nasal kinin receptor in the next chapter.

As icatibant antagonized bradykinin-induced nasal blockage and albumin extravasation in normal subjects, this antagonist is a useful tool to examine the role of bradykinin in perennial and seasonal allergic rhinitis. Icatibant, 200μg, significantly antagonised house dust mite-induced nasal blockage in individuals with allergic rhinitis to this antigen (Austin *et al.*, 1994). This study shows that house dust mite challenge caused nasal blockage in sensitive subjects, and this antigen-induced nasal blockage is dose-dependently antagonised by icatibant. In previous studies, symptom score measurements revealed that only nasal blockage was increased after house-dust mite challenge (not sneezing, itching or running) and this increase in blockage was significantly antagonized by icatibant (Austin *et al.*, 1994). Interestingly, house dust mite challenge in sensitive subjects does not cause an increase in albumin levels in the nasal lavage fluid. Since bradykinin increases albumin release in the nasal cavity of normal subjects this lack of albumin release after antigen challenge is difficult to explain if bradykinin is considered to be a major mediator of perennial allergic rhinitis. It is also interesting to note that subjects with severe perennial allergic rhinitis secrete significantly less albumin into the nasal cavity following bradykinin challenge than is secreted by normal subjects (Baraniuk *et al.*, 1994b). Hence, there is evidence that in perennial allergic rhinitis, nasal vascular responses are altered compared with normal subjects. The significant inhibition of nasal blockage by icatibant following antigen challenge together with the lack of effect of house dust mite challenge on nasal vascular permeability, suggests that the main kinin-mediated component in perennial allergic rhinitis is nasal blockage, in which there is little or no contribution from swelling caused by plasma extravasation. Histamine H_1 receptor antagonists, while reducing the pruitus, sneezing and rhinorrhoea which occur in perennial allergic rhinitis, have a negligible effect on nasal congestion (Meltzer, 1995). This indicates that histamine is not a major mediator in the nasal blockage of perennial rhinitis.

A central role for bradykinin in the pathogenesis of perennial allergic rhinitis may be explained, in part, by the observation that, *in vitro* and *in vivo*, house dust mite proteases possess the ability to activate the kallikrein-kinin system and generate bradykinin (Stewart *et al.,* 1989, 1991, 1993, Takahashi *et al.,* 1990, Maruo *et al.,* 1991, Matsushima *et al.,* 1992). The ability of mite proteases to generate bradykinin could not, by itself, sufficiently explain the aetiology of perennial allergic rhinitis as it provides no explanation of why only some of the population are affected. However, together with the model of type 1 hypersensitivity, it could explain the ability of icatibant effectively to antagonise house-dust mite-induced nasal blockage. To determine whether these proteases are responsible for kinin generation in perennial allergic rhinitis, it is necessary to be able to inhibit their kallikrein-like activity without affecting the antigenic properties of the house dust mite. In this context, antigenicity relates to the ability of house-dust mite to initiate the formation of specific IgE and then cross-link cell-bound IgE. Very specific mite protease inhibitors would be required which neither affect the ability of the mite antigens to cross-link IgE nor inhibit endogenous plasma and tissue kallikrein. Alternatively, the central role for kinins in perennial allergic rhinitis may be explained at the level of the receptor. A study investigating bradykinin B_2 receptor regulation on human synovial cells found that the receptors were upregulated by pretreatment with the cytokine, interleukin-1 (Bathon *et al.*, 1992). It is possible that this receptor upregulation may occur in inflammation *in vivo*. Cytokines are generated in allergic rhinitis, so the effects of bradykinin may be enhanced in perennial allergic rhinitis by an increase in B_2 receptor number. However, the ability of interleukin-1 (IL-1) to increase the number of bradykinin B_2 receptors is still equivocal. Tsukagoshi *et al.* (1995) reported that although application of IL-1β induced rat lower airway hyperresponsiveness to inhaled bradykinin, there was no change in bradykinin B_2 receptor number.

Grass pollen administered intranasally caused nasal blockage in grass-sensitive subjects. Icatibant, even at the highest dose, 500μg, failed significantly to inhibit this response. Hence, this initial evidence suggests that the nasal blockage in seasonal allergic rhinitis may not be mediated by kinins to the same extent as perennial allergic rhinitis. Histamine H_1 receptor antagonists do not significantly reduce the nasal

blockage in seasonal allergic rhinitis (Rokenes *et al.*, 1988; Pipkorn, 1990; Corrado *et al.*, 1987; Hamilton *et al.*, 1994; Thomas *et al.*, 1992), indicating that H_1 receptor activation does not play a significant role either. Inhibiting 5-lipoxygenase has been reported to attenuate grass pollen-induced increases in nasal airway resistance, indicating that leukotriene synthesis may play a significant role in the nasal blockage of seasonal rhinitis (Howarth *et al.*, 1995). Grass pollen challenge of subjects with seasonal allergic rhinitis causes an increase in the albumin content of the nasal lavage, which is also unaffected by icatibant pretreatment. The increase in albumin extravasation into the nasal cavity induced by grass pollen is antagonised by histamine H1 receptor antagonists (Shin *et al.*, 1992; Naclerio *et al.*, 1990), implicating histamine as a mediator of grass-induced plasma extravasation. It should be noted that allergen challenge in perennial rhinitis produces no albumin release, as discussed above, and this is a further difference between the two types of rhinitis. The failure of icatibant to antagonise the nasal blockage and the increase in vascular permeability induced by grass pollen is consistent with the observations of Akbary and Bender (1993) who presented evidence that icatibant does not block pollen-induced seasonal allergic rhinitis, as measured with symptom scores reported by patients during the pollen season.

The action of icatibant may not just be at the level of antagonism at the bradykinin receptor. The kinin level in the nasal lavage is significantly raised following house-dust mite challenge in subjects with perennial allergic rhinitis. Although this has been demonstrated with grass pollen challenge in subjects with seasonal rhinitis (Proud *et al.*, 1983), the data in this chapter are the first demonstration that kinin levels are also elevated in experimentally-induced perennial allergic rhinitis. This observation is interesting in light of the fact the house-dust mite challenge causes no albumin extravasation into the nasal cavity. Albumin extravasation is a measure of plasma extravasation, a process which allows the kinin precursor, plasma kininogen, and the kinin-generating enzyme, plasma kallikrein, to gain access to the nasal cavity (Baumgarten *et al.*, 1985). The lack of plasma extravasation suggests that plasma kininogen and kallikrein are not involved in the kinin generation during experimentally-induced perennial allergic rhinitis. Therefore, as plasma kallikrein

appears not to be involved, the kinin forming enzyme in perennial allergic rhinitis may be tissue kallikrein. This is supported by the work of Nishiyama (1994), who reported a significant increase in tissue kallikrein activity in the nasal cavity following house dust mite challenge. Another interesting aspect of this study is the finding that icatibant reduces the levels of bradykinin in the nasal airway following antigen challenge of allergic subjects. There is some evidence that icatibant inhibits kallikrein in an animal model (Dr. K. Wirth - unpublished) but it was less potent in this respect than as a kinin B_2 receptor antagonist. Three other bradykinin B_2 receptor antagonists, B4307, B4308 and B3852 have also been reported to inhibit the kinin-forming activity of human urinary kallikrein (Spragg *et al.*, 1989). These antagonists inhibited the kallikrein activity dose-dependently in the micromolar concentration range, therefore are less potent as inhibitors of kallikrein than as B_2 receptor antagonists since they have K_i values in the nanomolar range for the bradykinin B_2 receptor. The data presented in this chapter suggest that *in vivo*, icatibant may inhibit kallikrein as well as blocking the kinin receptor and that both of these effects may contribute to the reversal by icatibant of the antigen-induced nasal blockage in subjects with allergic rhinitis. As already discussed, antigen challenge of subjects with perennial allergic rhinitis does not result in albumin leakage into the nasal cavity. It is unlikely, therefore, that the action of icatibant on bradykinin release into the nasal airway results from blockage of a B_2 receptor-mediated increase in vascular permeability and the consequent fall in the supply of plasma kininogen or kallikrein.

Summary

- The aim of this study was to investigate the effect of bradykinin receptor antagonists on the nasal actions of bradykinin and antigen.

- Intranasal administration of bradykinin caused a dose-dependent decrease in the patency of the human nasal airway and an increase in the albumin content of the nasal lavage.

- The bradykinin B_2 receptor antagonists icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin dose-dependently inhibited the nasal actions of bradykinin, 100μg.

- Another bradykinin B_2 receptor antagonist, NPC 567, 10mg, failed to inhibit the nasal actions of bradykinin, 100μg.

- The ability of icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin to antagonise the actions of bradykinin, but the failure of NPC 567 to do so may be explained by the low affinity of NPC 567 for the bradykinin B_2 receptor. Alternatively, the human nasal bradykinin receptor may represent a B_2 receptor subtype.

- Nasal challenge with house dust mite antigen and grass pollen antigen in subjects with perennial and seasonal allergic rhinitis, respectively, produced a reduction in the patency of the human nasal airway. In addition, grass pollen antigen challenge also increased the albumin content of the nasal lavage fluid. House dust mite antigen nasal challenge of subjects with perennial allergic rhinitis did not increase the albumin content of the lavage fluid.

- The bradykinin B_2 receptor antagonist, icatibant, also dose-dependently inhibited the reduction of nasal patency induced by house-dust mite antigen in subjects with perennial allergic rhinitis. Icatibant failed to inhibit the nasal actions of grass pollen antigen in subjects with seasonal allergic rhinitis.

- Following house-dust mite challenge, pretreatment with icatibant significantly reduced the kinin content of the nasal lavage fluid compared with saline pretreatment.

- Therefore, the reduction in nasal airway patency induced by house-dust mite antigen is mediated, at least in part, by kinin generation. However, the reduction in nasal airway patency and increase in lavage albumin induced by grass pollen antigen is independent of kinin generation. In addition to receptor antagonism, icatibant may also inhibit kallikrein activity in the human nasal airway.

CHAPTER 4

CHARACTERIZATION OF THE BRADYKININ RECEPTOR IN THE HUMAN NASAL AIRWAY USING THE BINDING OF [^{125}I]-ICATIBANT

4.1 Introduction

In human subjects *in vivo*, bradykinin nasal challenge induces an increase in albumin extravasation and a reduction in the patency of the nasal airway (Austin & Foreman, 1994a). These actions are antagonised by the bradykinin B_2 receptor antagonists, icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin (Austin & Foreman, 1994a). The bradykinin B_1 receptor agonist, [DesArg^9]-bradykinin, is inactive in the human nasal airway (Rajakulasingam *et al.*, 1991), indicating that the actions of bradykinin are mediated via activation of the bradykinin B_2 receptor. However, the bradykinin B_2 receptor antagonist NPC 567 fails to antagonise the actions of bradykinin nasal challenge, even at high dose (Pongracic *et al.*, 1991; Austin & Foreman, 1994a). The inactivity of NPC 567 may be explained by its relatively low affinity for the bradykinin B_2 receptor. Alternatively, the human nasal bradykinin receptor may represent a B_2 receptor sub-type or an example of the bradykinin B_3 receptor type, proposed to be present in the guinea-pig lower airways (Farmer *et al.*, 1989). The use of a wider range of bradykinin B_2 receptor antagonists in humans *in vivo* has been precluded by the manufacturers. Also making estimates of antagonist potency *in vivo* is excessively time-consuming, expensive and cannot make the precision of radioligand binding.

The technique of radioligand binding provides an alternative method of characterising the human nasal bradykinin receptor. In other species this technique has been employed to characterise the nasal bradykinin receptor. Using radioligand binding, the bradykinin binding site on the guinea-pig and sheep nasal turbinate tissue has been characterised as belonging to the B_2 receptor type (Fujiwara *et al.*, 1989; Lyon *et al.*, 1990). These binding studies used a radiolabelled agonist, bradykinin, as the

radioligand. It is preferable to use an antagonist as the radioligand in binding studies as the binding of an agonist to a receptor causes a conformational change in the receptor state (Del Castello & Katz, 1957).

This chapter examines the binding of the radiolabelled antagonist, [^{125}I]-icatibant, to membranes isolated from human nasal turbinate bones. From this the bradykinin receptor present in the human nasal airway can be further characterised.

4.2 Experimental protocol

The tissue used was inferior turbinate bones removed at routine surgery for hypertrophy of the nasal turbinates. Clinical histories indicating the precise diagnosis for the patients undergoing surgery were not available. Soft tissue was dissected from bone and the bone discarded. The soft tissue was chopped with scissors and then homogenized in buffer A using a polytron blender at 4^{0}C. Five 10 second bursts of homogenization were performed in homogenisation buffer A. The homogenate was centrifuged at 2500g for 10 min. at 4°C and the supernatant was then recentrifuged at 50,000g for 10 min. at 4°C. The pellet was resuspended and washed twice by alternate centrifugation and resuspension in a volume of 10ml of buffer A. The pellet was finally resuspended in 5ml of buffer B and the protein content was determined by the Pierce BCA protein assay. The protein concentration of the preparation was adjusted to 1mg.ml^{-1}.

To 100µl of membrane preparation, [^{125}I]-icatibant was added in 50µl of buffer A and a further 50µl was added containing either buffer, unlabelled icatibant or displacing drug, depending on the protocol. For the competition and saturation binding studies the reaction mixture was incubated at room temperature (20°C) for 1 hour, and then 2ml of ice-cold buffer A was added and the whole mixture immediately filtered through a Whatman GF/B filter under reduced pressure. The Whatman GF/B filters had been previously treated with polyehtyleneimine, 0.1% for 1 hour. The filters were washed three times with 3ml of ice-cold buffer A and then transferred to a gamma spectrometer for assay of the ^{125}I.

For the association kinetic binding studies the reaction mixture was membrane preparation, 100µl; [^{125}I]-icatibant, 50µl, (final concentration, 0.2nM); and 50µl of buffer or excess unlabelled icatibant. This was incubated at 4^{0}C for 0 - 45 minutes. The reaction was terminated and the binding counted as described for the competition and saturation studies. The reaction mixture for the dissociation binding studies was the same as the association studies and again the experiment was conducted at 4^{0}C. The mixture was incubated for 1 hour to allow equilibrium to be reached, then unlabelled icatibant, 1µM, was added to initiate dissociation. Aliquots were taken at intervals between 0-20 min. after the excess unlabelled ligand had been added, the reaction was terminated and the binding measured.

For all the binding studies, specific binding of [^{125}I]-icatibant was defined as the binding inhibited by unlabelled icatibant, 1µM. In each binding experiment, the specific activity of [^{125}I]-icatibant was measured.

The composition of the buffers used was based on the work of Trifilieff *et al.* (1994) and was as follows:

Buffer A - N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES), 25mM; 1,10 phenatroline, 1mM; bacitracin 140µg.ml^{-1}; dithiothreitol, 1mM; captopril 10µM; bovine serum albumin, 0.1%. The pH was 6.8.

Buffer B - TES, 25 mM; 1,10 phenathroline, 1mM. The pH was 6.8.

4.2.1 Data analysis

The data were analyzed by non-linear, least squares, curve fitting using CVFIT (courtesy of Prof. D. Colquhoun, Department of Pharmacology, University College London) and Sigma Plot (Jandel) and, for comparison, by linearizing transformations of the binding curves. The equations for fitting and linear transformation are given in the results section. For all the data, each separate experiment was fitted and the values calculated were used to calculate the mean and s.e.mean.

4.3 Results

Figure 4.1 shows a protein standard curve, the amount of protein being determined by flurometric absorbance at 562nm. Also shown is a typical concentration of protein found in 5ml of a preparation of human nasal membranes.

Figure 4.2 shows the total, non-specific and specific binding of [^{125}I]-icatibant to a human nasal membrane preparation, (protein content, 100μg), as a function of [^{125}I]-icatibant concentration. The specific binding is the binding of [^{125}I]-icatibant which is inhibited by excess unlabelled icatibant. The specific binding was fitted to the following equation:

$$B = B_{max} \bullet \frac{[L]}{K_L + [L]} \qquad \text{(Eq 4.1)}$$

where B is the amount of [^{125}I]-icatibant specifically bound, B_{max} is the amount of [^{125}I]-icatibant specifically bound at equilibrium, [L] is the concentration of [^{125}I]-icatibant and K_L is the equilibrium dissociation constant for [^{125}I]-icatibant. The specific binding was found to be saturable and could be accounted for by binding to a single high affinity site (figure 4.3.) over the range of concentrations used. By rearranging equation 4.1:

$$\log\left(\frac{B}{B_{max} - B}\right) = n_H \log [L] - \log K_L$$

and plotting $\log(B/B_{max}-B)$ against log[L], the Hill coefficient (n_H) was calculated to be 1.01± 0.07 (figure 4.4).

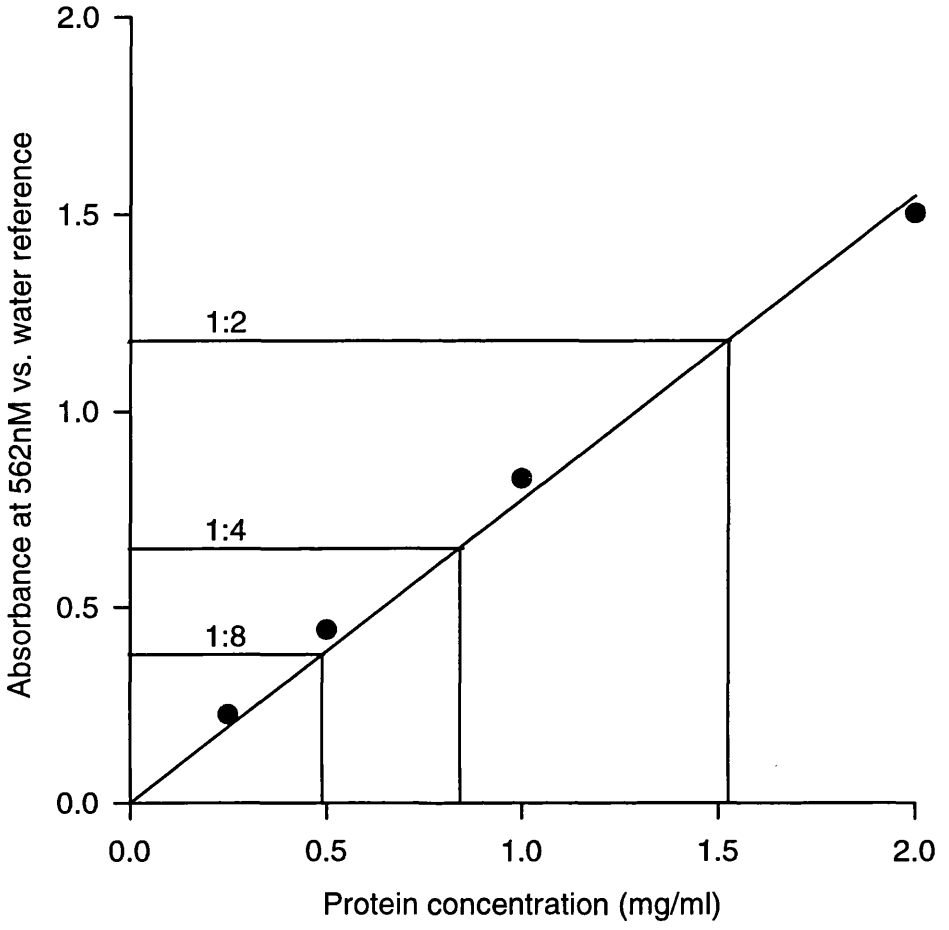

Figure 4.1. The protein content of a human nasal membrane preparation. The figure shows a standard curve for protein concentration; the ordinate axis represents the flurometric absorbance, compared to water at 562nm, of standard protein concentrations. Also shown are the absorbances of a typical human nasal membrane preparation, diluted 1:2, 1:4 and 1:8. The membranes were suspended in 5ml of buffer B.

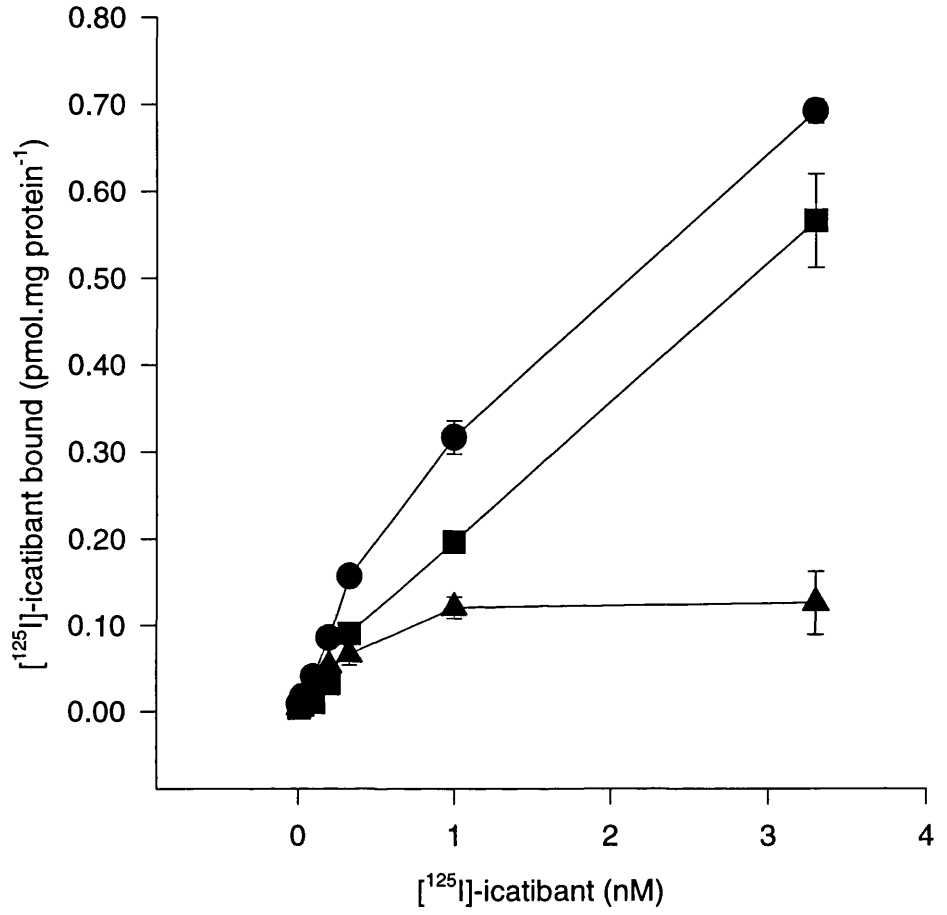

Figure 4.2. The total, non-specific and specific binding of [^{125}I]-icatibant to human nasal membrane preparations. The binding of [^{125}I]-icatibant in the absence and presence of 1μM unlabelled icatibant corresponds to the total (●) and non-specific binding (■), respectively. Specific binding (▲) is also shown. Data are the means of six membrane preparations, vertical bars show the s.e.mean.

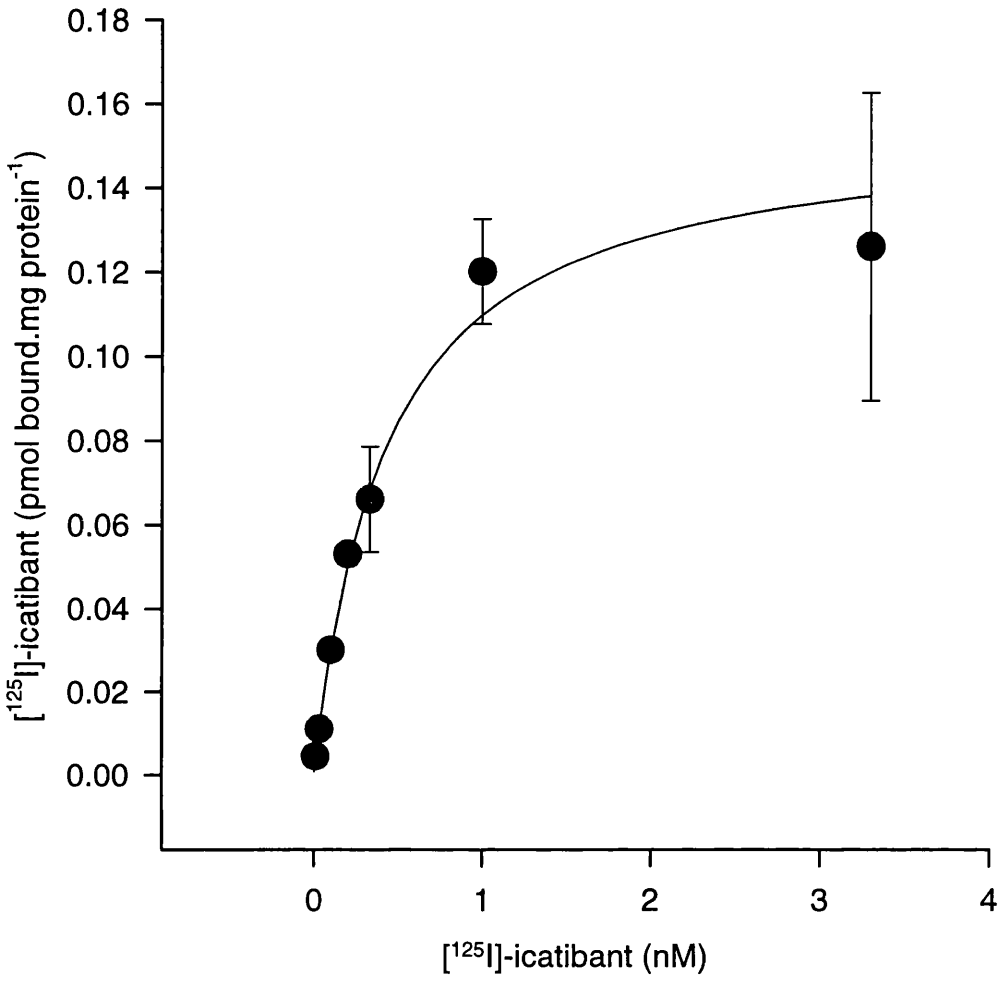

Figure 4.3. The receptor specific binding of [^{125}I]-icatibant to human nasal membranes, defined as that binding displaced by unlabelled icatibant, 1μM. The data are fitted as described in the results, the Hill value is constrained to unity. Data are the means of six membrane preparations, vertical bars show the s.e.mean.

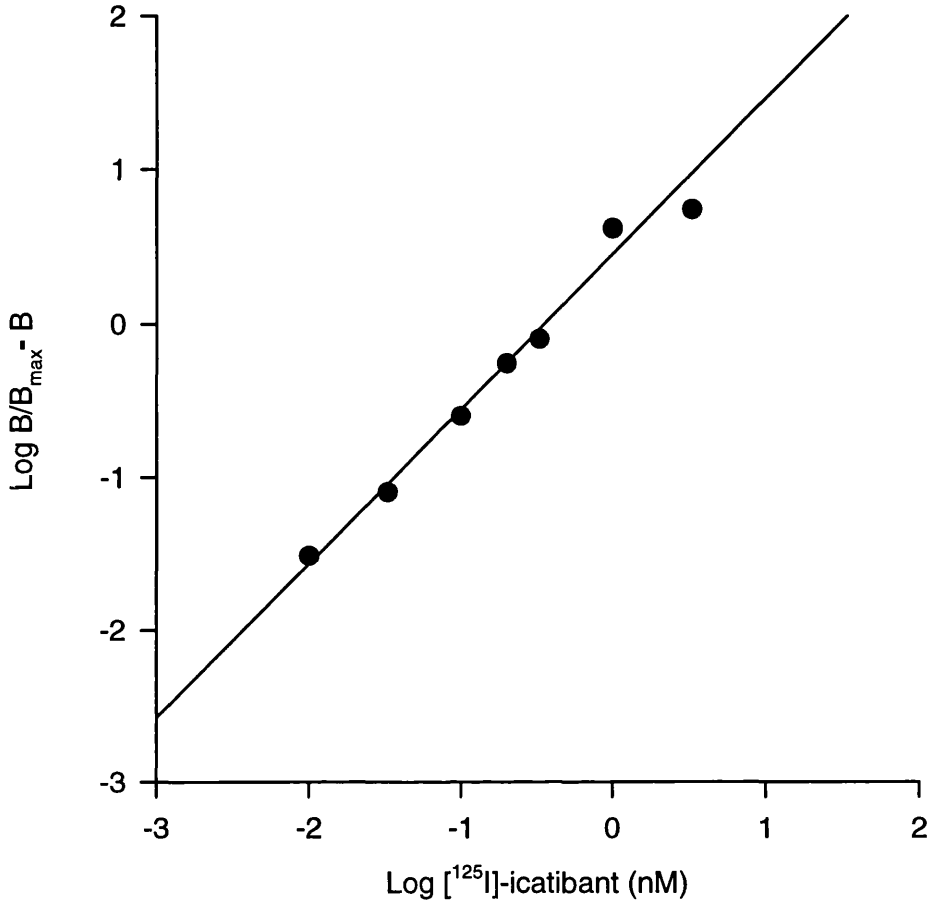

Figure 4.4. The receptor specific binding of [^{125}I]-icatibant to human nasal membranes represented as a Hill plot. The data are fitted as described in the results. On the ordinate, B represents the specific binding at each [^{125}I]-icatibant concentration, B_{max} is the specific binding of [^{125}I]-icatibant when the binding sites are saturated. Data are the means of six membrane preparations.

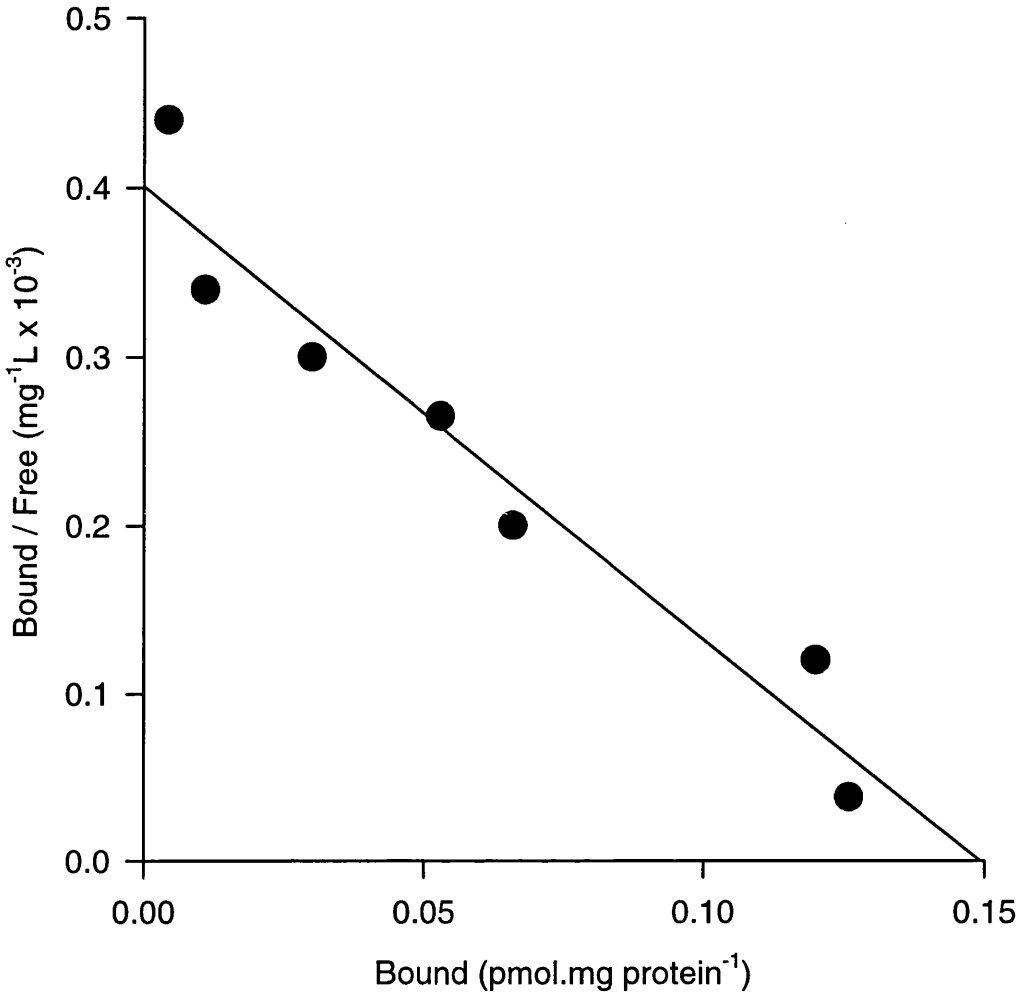

Figure 4.5. Scathard plot of the receptor specific binding of [^{125}I]-icatibant to human nasal membranes. The data are fitted as described in the results. On the axis, bound is the specific binding of [^{125}I]-icatibant and free is the concentration of [^{125}I]-icatibant added. Depletion was calculated to be negligible. Data are the means of six membrane preparations.

Using curve-fitting and constraining the Hill coefficient to unity, the estimates of the equilibrium dissociation constant of icatibant, K_L and of B_{max} were found to be 0.46± 0.08nM and 0.136 ± 0.003pmol.mg protein^{-1} respectively (n=6; mean ± s.e.mean). Equation 4.1 can also be rearranged to give:

$$\frac{B}{[L]} = \frac{B_{max}}{K_L} - \frac{B}{K_L}$$

By plotting B/[L] against B a straight line is generated and K_L can be calculated from the negative reciprocal of the gradient. The intercept on the ordinate axis represents B_{max}. This graph is known as a Scathard plot. Scathard analysis of the specific saturation binding of [^{125}I]-icatibant to human nasal membranes yielded an equilibrium dissociation constant of 0.36± 0.08nM and a B_{max} of 0.15± 0.01pmol.mg protein^{-1} (figure 4.5).

The rate of dissociation of [^{125}I]-icatibant, 0.2nM, with the binding site on human nasal membranes, at 4° C, is shown in Figure 4.6. The data are fitted to the following equation:

$$B_t = B_0 \bullet e^{k_{-1} t}$$

Where B_0 and B_t are the amounts of [^{125}I]-icatibant specifically bound initially (t=0) and at times (t) after initiating dissociation, k_{-1} is the dissociation rate constant. The dissociation of [^{125}I]-icatibant occurred over a time period of 20min. with a half-time for dissociation of approximately 5min. Using curve fitting analysis, the mean value of the dissociation rate constant (k_{-1}) was 0.14 ± 0.01min^{-1}. The data was also fitted to a straight line by plotting $\ln(B_t / B_0)$ against time.

$$\ln\left(\frac{B_t}{B_0}\right) = k_{-1} t$$

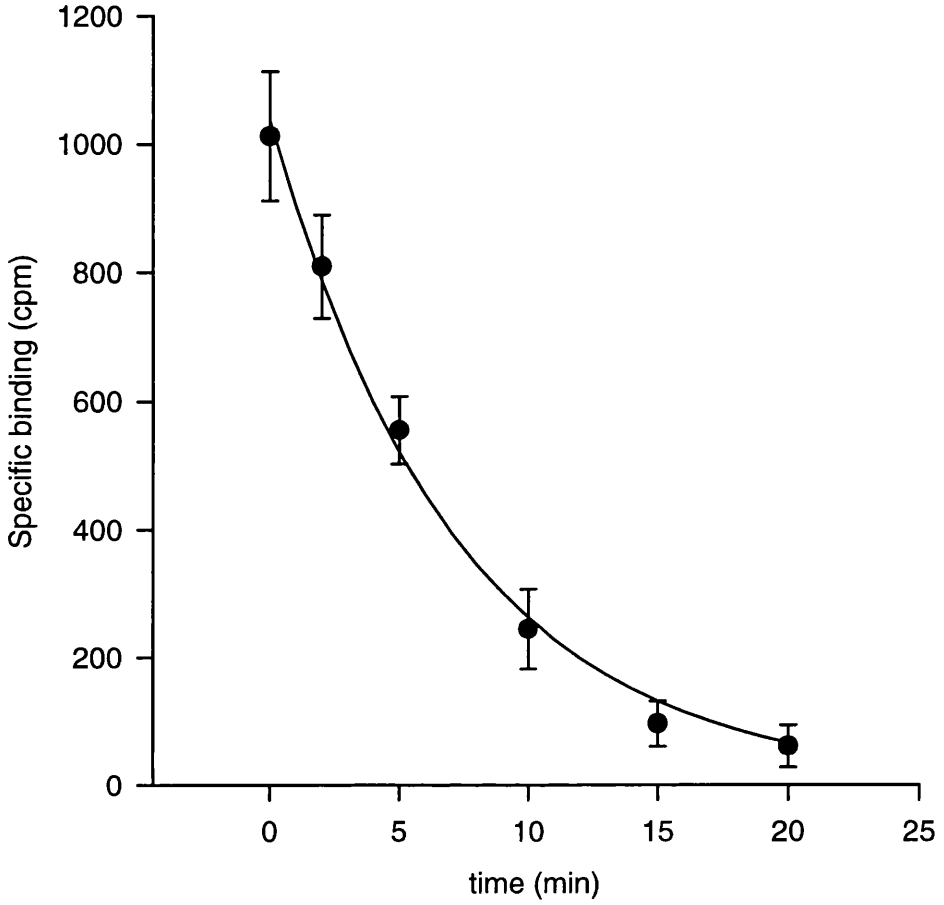

Figure 4.6. The rate of dissociation of [^{125}I]-icatibant, 0.2nM, with human nasal membrane binding sites, following the addition of excess unlabelled icatibant at time 0. The data are fitted as described in the results. Specific binding is defined as that binding displaced by unlabelled icatibant, 1μM. The experiment was performed at 4^{0}C and the binding is shown as counts per minute. Data represent the mean of triplicate experiments. The vertical bars represent the s.e.mean.

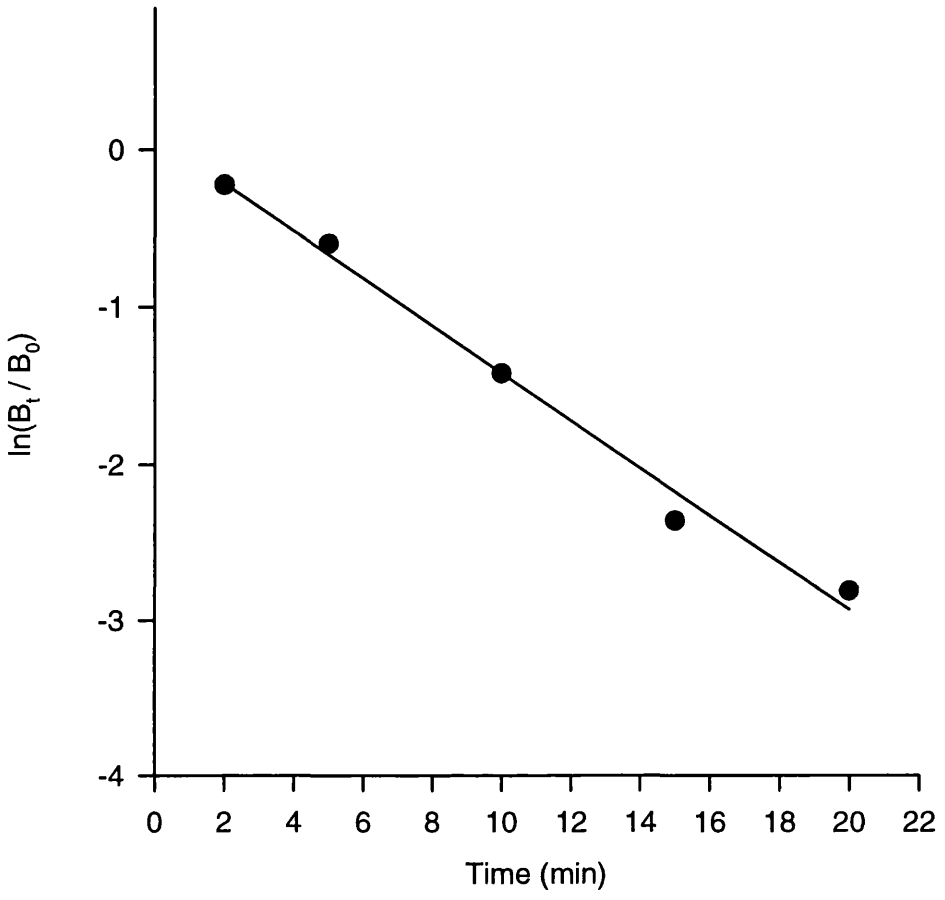

Figure 4.7. Linear transformation of the rate of dissociation of [^{125}I]-icatibant with human nasal membrane binding sites, following the addition of excess unlabelled icatibant at time 0. The data are fitted as described in the results. Specific binding is defined as that binding displaced by unlabelled icatibant, 1μM. On the ordinate axis, B_t represents the binding at each time point and B_0 is the binding at equilibrium (time 0).

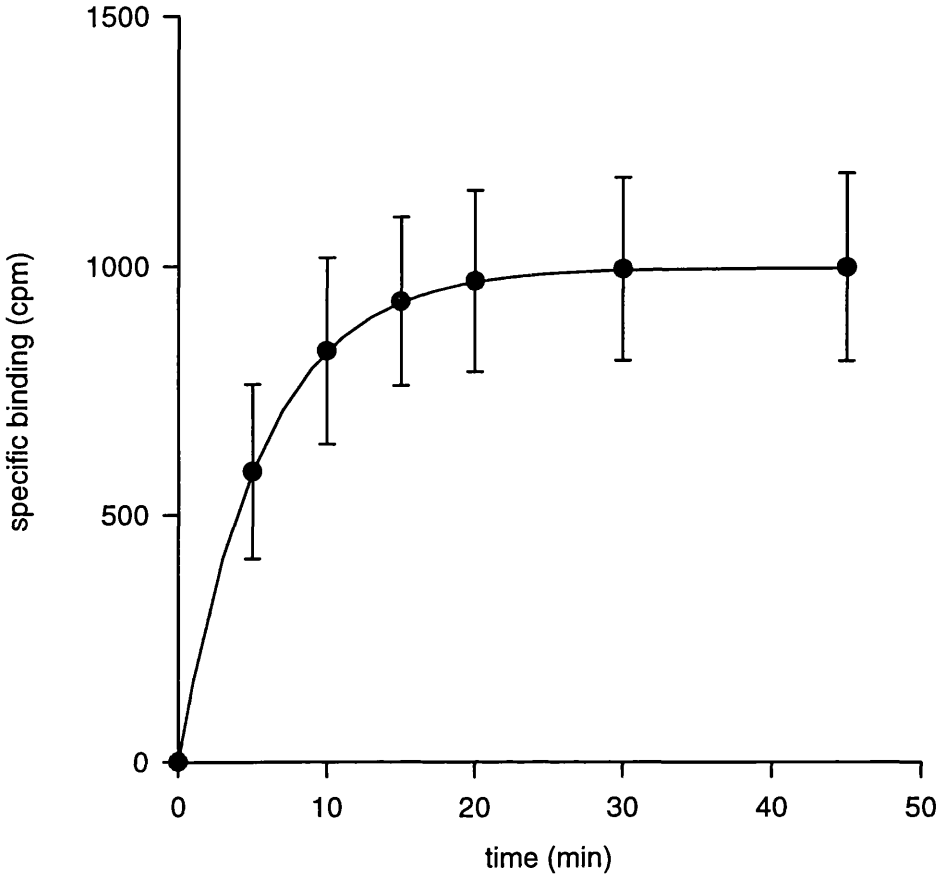

Figure 4.8. The rate of association of [^{125}I]-icatibant, 0.2nM, with human nasal membrane binding sites. The data are fitted as described in the results. Specific binding is defined as that binding displaced by unlabelled icatibant, 1μM. The experiment was performed at 4^{0}C and the binding is shown as counts per minute. Data represent the mean of triplicate experiments. The vertical bars represent the s.e.mean.

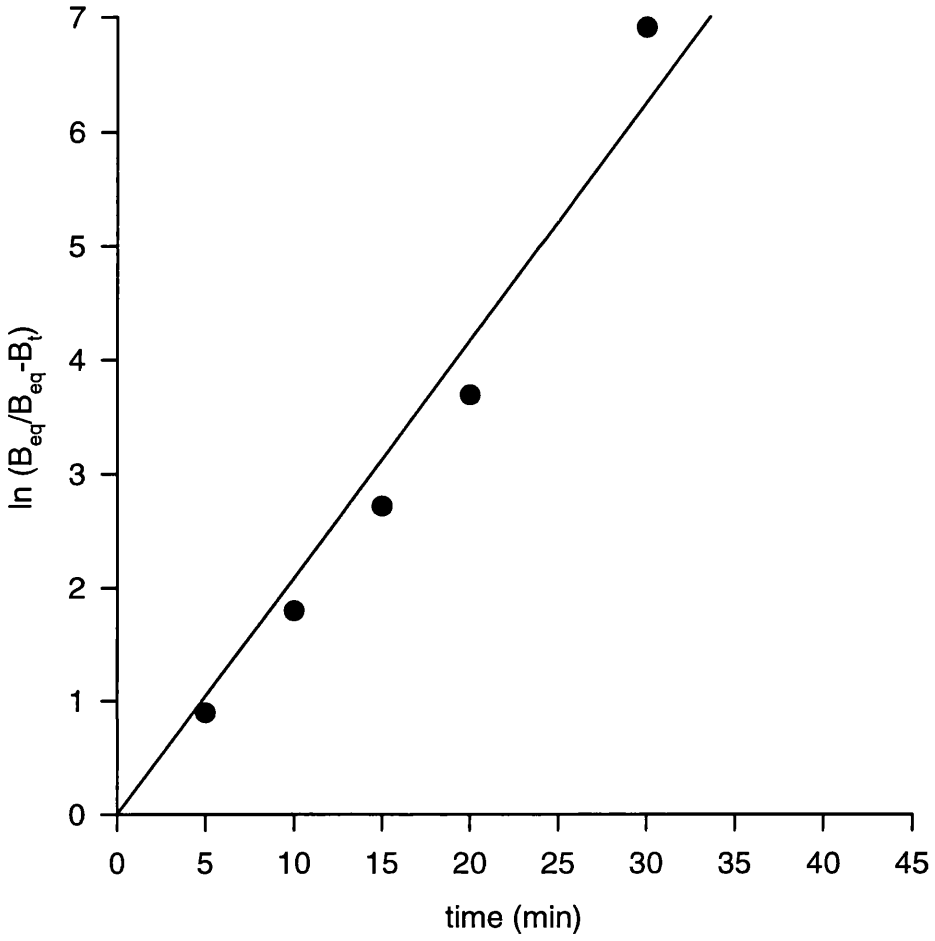

Figure 4.9. Linear transformation of the rate of association of [^{125}I]-icatibant, 0.2nM, with human nasal membrane binding sites. The data are fitted as described in the results. Specific binding is defined as that binding displaced by unlabelled icatibant, 1μM. On the ordinate axis, B_t represents the binding at each time point and B_{eq} is the binding at equilibrium.

Therefore the slope of figure 4.7 gave an another estimate of the dissociation rate constant. The mean value of k_{-1} was $0.15 \pm 0.03 min^{-1}$.

The rate of association of [^{125}I]-icatibant, 0.2nM, with the binding site on human nasal membranes, at 4°C, is shown in Figure 4.8. The data were fitted to the following equation:

$$B_t = B_{eq} \bullet (1 - e^{-k_{on} t})$$

where B_t is the specific binding of [^{125}I]-icatibant at time t, B_{eq} is the binding at equilibrium and k_{on} is the observed onset rate constant. The relationship between k_{on} and the association and dissociation rate constants (k_{+1} and k_{-1}) is:

$$k_{on} = k_{+1} [L] + k_{-1}$$

where [L] is the concentration of [^{125}I]-icatibant. Specific binding increased over a time period of 20min. with a half-time for association of approximately 3.5min. (figure 4.8). Constraining k_{-1} to $0.14 min^{-1}$, curve fitting analysis gave a mean value for the association rate constant (k_{+1}) of $0.2 \pm 0.06 nM^{-1} min^{-1}$. The data were also fitted to a straight line by plotting $\ln(B_{eq} / B_{eq} - B_t)$ against time (figure 4.9.).

$$\ln\left(\frac{B_{eq}}{B_{eq} - B_t}\right) = k_{on} t$$

Therefore, the mean value of observed onset rate constant (k_{on}) obtained from the slope of the fitted straight line was $0.21 \pm 0.06 min^{-1}$.

The equilibrium dissociation constant for [^{125}I]-icatibant calculated from the rate constants (k_{-1} / k_{+1}) generated by curve fitting was 0.7nM.

The equilibrium dissociation constant was also calculated using k_{on} and k_{-1} from linear analysis as follows. Substituting k_{on} and k_{-1} values into the equation:

$$k_{on} = k_{+1}[L] + k_{-1}$$

the values of k_{-1} and k_{+1} were applied to estimate the equilibrium dissociation constant (k_{-1} / k_{+1}) The value of the equilibrium dissociation constant for [^{125}I]-icatibant obtained in this way was 0.5nM.

The displacement of specific [^{125}I]-icatibant binding, from human nasal membranes, by unlabelled icatibant itself and by other agonists and antagonists at bradykinin receptors was also studied. Figures 4.10 and 4.11. show the displacement of the specific binding of [^{125}I]-icatibant, 0.2nM, by various bradykinin receptor ligands. The data are fitted to the equation:

$$B_I = B_0\left(1 - \frac{[I]}{[I] + IC_{50}}\right)$$

where the specific binding of [^{125}I]-icatibant in the presence of a competing ligand is B_I, the binding of [^{125}I]-icatibant in the absence of competing ligand is B_0, [I] is the concentration of the competing ligand and IC_{50} is the concentration of inhibitor which reduces the specific binding of [^{125}I]-icatibant by 50%. The IC_{50} of a competing ligand is related to its equilibrium dissociation constant (K_i) by the following equation:

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[L]}{K_L}\right)}$$

the concentration and equilibrium dissociation constant of [^{125}I]-icatibant are [L] and K_L, respectively. All the Hill coefficients (table 4.1) calculated are not significantly different from unity, analysed by regression analysis ($p>0.05$). Therefore, the curves are fitted to the data by constraining the Hill coefficient to unity. Figures 4.12 and 4.13 show the displacement of [^{125}I]-icatibant, 0.2nM, expressed as a Hill plot:

$$\log\left(\frac{B_I}{B_0 - B_I}\right) = \log IC_{50} - \log [I]$$

again, in figures 4.12 and 4.13, the Hill coefficient is constrained to unity.

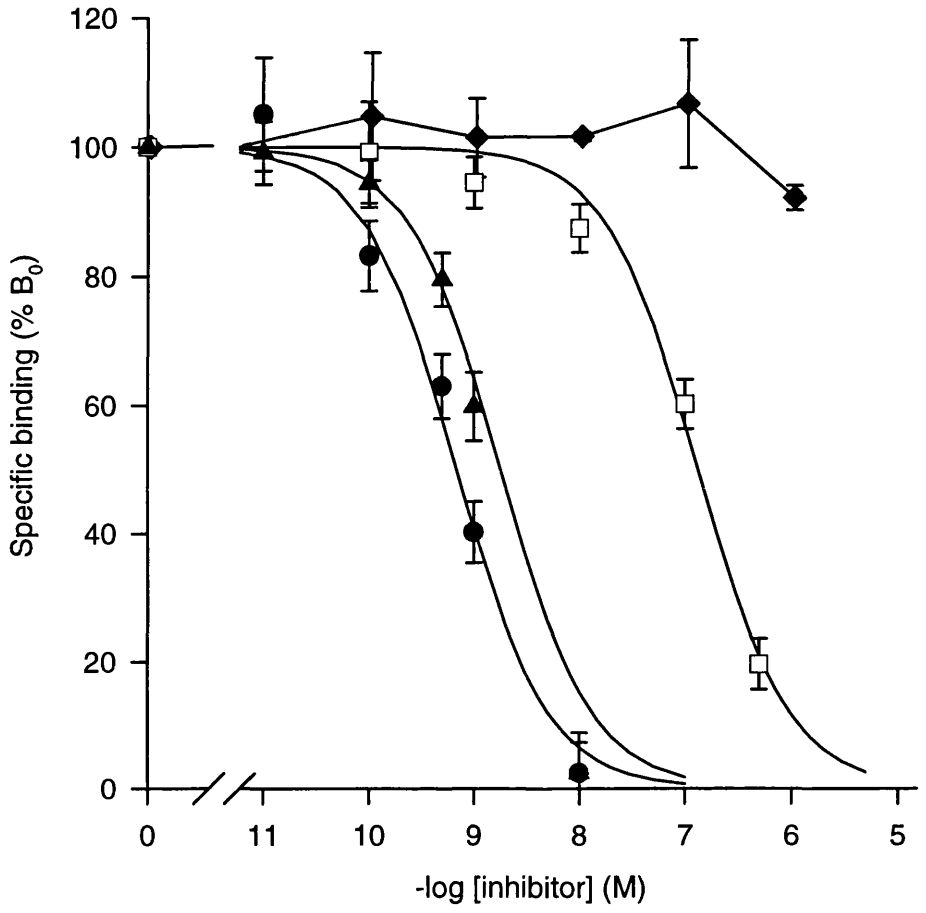

Figure 4.10. Competitive inhibition of the specific binding of [^{125}I]-icatibant to human nasal membranes. Specific binding is again defined as that binding displaced by unlabelled icatibant, 1μM. The data are fitted as described in the results. Data represent the mean of triplicate experiments, vertical bars show the s.e.mean. The Hill coefficient is constrained to 1. [^{125}I]-icatibant, 0.2nM, was displaced by unlabelled icatibant (●), bradykinin (▲), NPC 567 (□) and [DesArg^9]-bradykinin (◆).

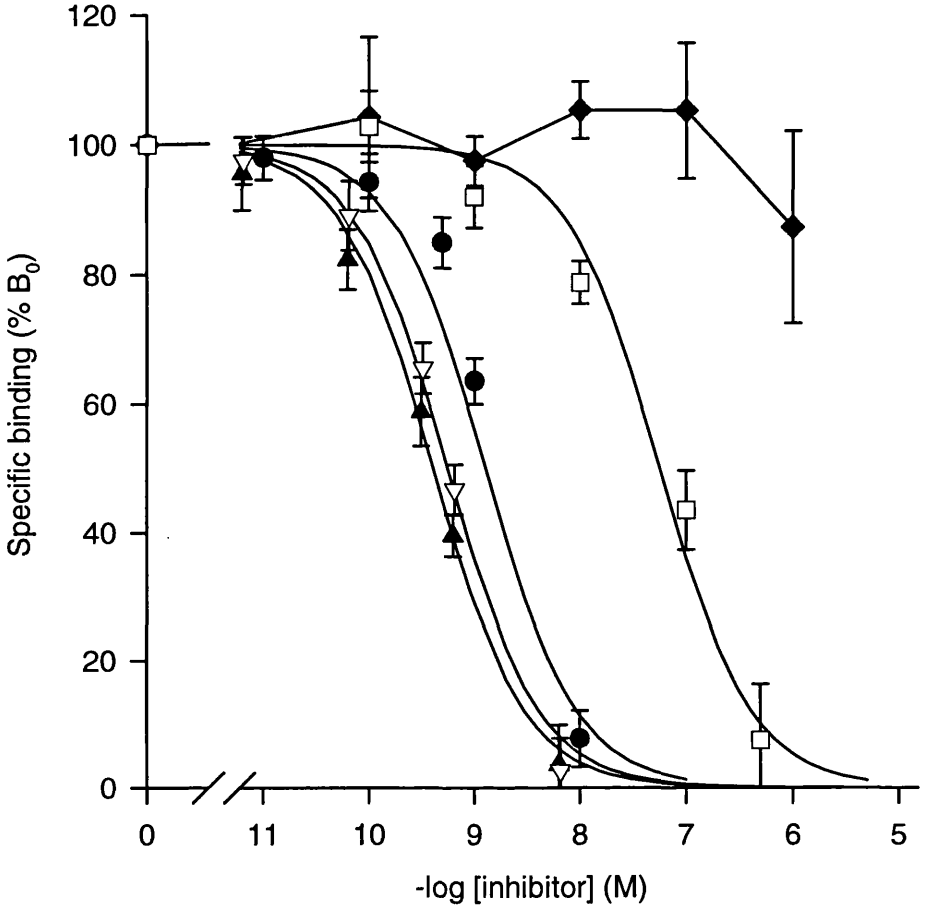

Figure 4.11. Competitive inhibition of the specific binding of [^{125}I]-icatibant to human nasal membranes. Specific binding is again defined as that binding displaced by unlabelled icatibant, 1μM. The data are fitted as described in the results. Data represent the mean of triplicate experiments, vertical bars show the s.e.mean. The Hill coefficient is constrained to 1. [^{125}I]-icatibant, 0.2nM, was displaced by NPC 17761 (▲), NPC 17731 (▽), [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin (●), WIN 64338 (□) and [DesArg^9]-icatibant (◆).

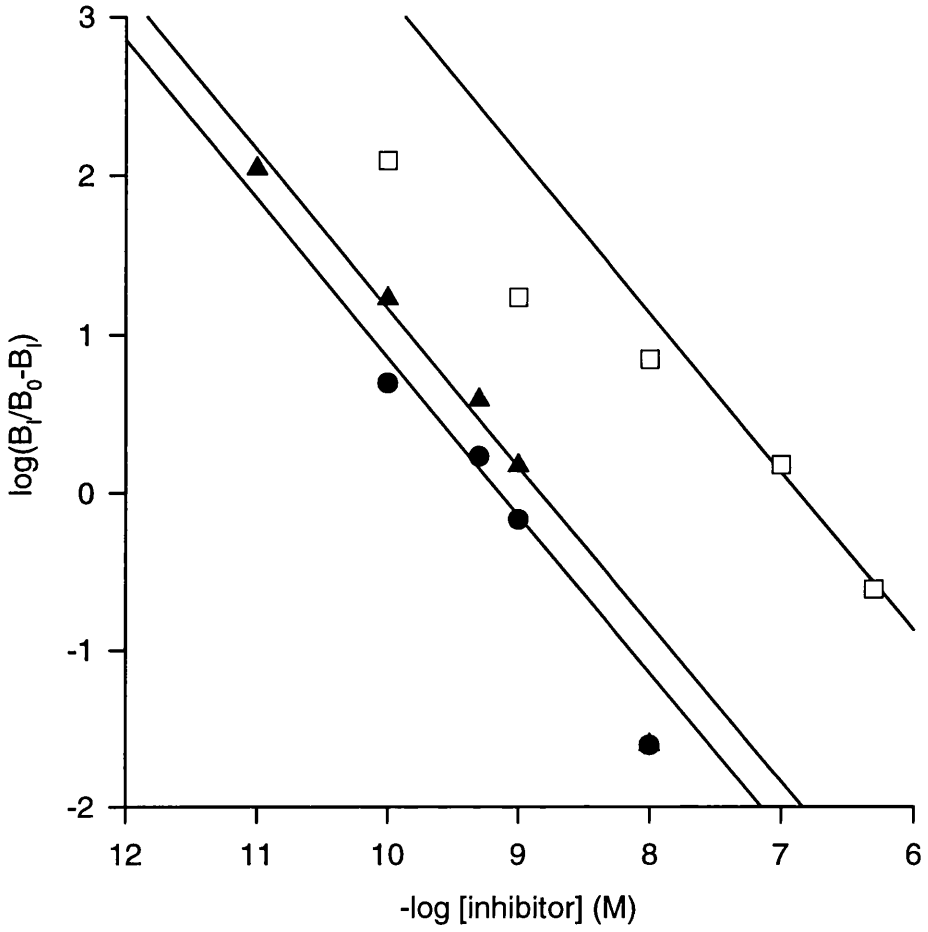

Figure 4.12. Hill plot showing competitive inhibition of the specific binding of [^{125}I]-icatibant to human nasal membranes. The data are fitted as described in the results. Data represents the mean of triplicate experiments. On the ordinate, B_I represents the % [^{125}I]-icatibant specific binding at each inhibitor concentration compared to B_0 , B_0 is % specific binding of [^{125}I]-icatibant in the absence of inhibitor, i.e. 100%. The Hill coefficient is constrained to 1. [^{125}I]-icatibant, 0.2nM, was displaced by unlabelled icatibant (●), bradykinin (▲) and NPC 567 (□).

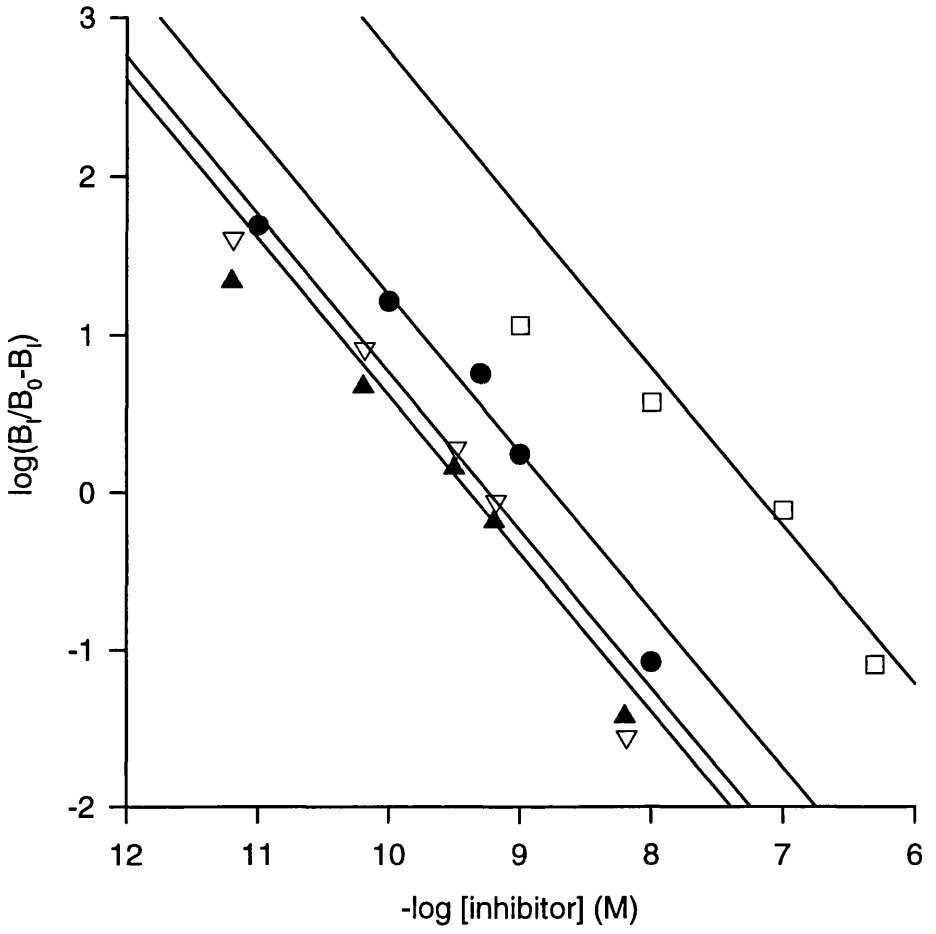

Figure 4.13 Hill plot showing competitive inhibition of the specific binding of [^{125}I]-icatibant to human nasal membranes. The data are fitted as described in the results. Data represents the mean of triplicate experiments. On the ordinate, B_I represents the % [^{125}I]-icatibant specific binding at each inhibitor concentration compared to B_0 , B_0 is % specific binding of [^{125}I]-icatibant in the absence of inhibitor, i.e. 100%. The Hill coefficient is constrained to 1. [^{125}I]-icatibant, 0.2nM, was displaced by NPC 17761 (▲), NPC 17731 (▽), [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin (●) and WIN 64338 (□).

	Curve-fitting		Hill plot	
Compound	K_i (nM)	Hill coefficient	K_i (nM)	Hill coefficient
B_2 antagonists				
NPC 17761	0.27±0.02	0.96±0.06	0.27±0.02	0.94±0.05
icatibant	0.48±0.02	1.06±0.09	0.47±0.05	1.02±0.12
NPC 17731	0.38±0.02	1.13±0.09	0.38±0.02	1.13±0.06
[Ad]-BK	1.20±0.04	1.38±0.07	1.19±0.16	1.36±0.08
WIN 64338	41.24±8.54	0.86±0.03	40.87±7.04	0.83±0.07
NPC 567	90.47±3.93	0.89±0.07	90.01±9.31	0.91±0.08
B_1/B_2 agonist				
bradykinin	0.99±0.13	1.45±0.05	0.97±0.12	1.47±0.08
B_1 selective agonist				
[Des-Arg^9]-bradykinin	>1000		>1000	
B_1 selective antagonist				
[Des-Arg^9]-icatibant	>1000		>1000	

Table 4.1. Inhibition constants (Ki) and Hill (n_H) coefficient from the displacement of [^{125}I]-icatibant binding to a membrane preparation from human inferior turbinate bones by selective agonists and antagonists at bradykinin receptors. The concentrations of [^{125}I]-icatibant used was 0.2nM. [Ad]-BK is [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin. The values are calculated by non-linear, least squares curve fitting and linear transformation (Hill plot) methods. Each value is the mean ± s.e. mean from 3 separate experiments.

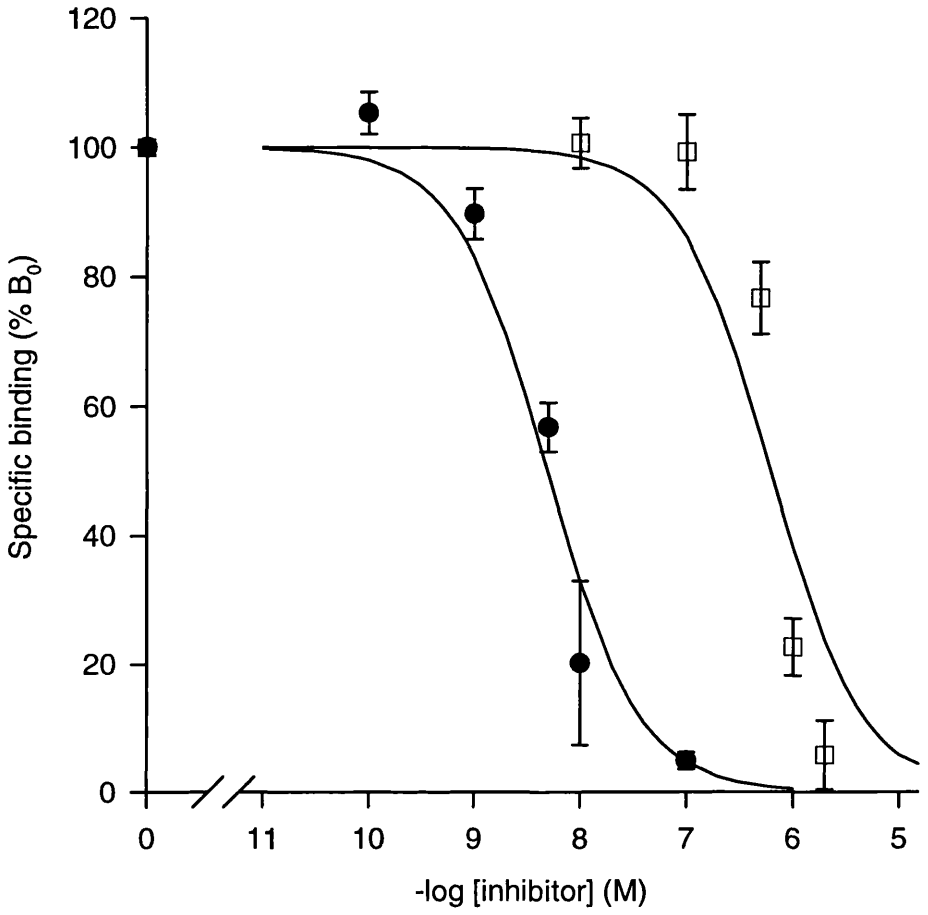

Figure 4.14. Competitive inhibition of the specific binding of [^{125}I]-icatibant to human nasal membranes. Specific binding is again defined as that binding displaced by unlabelled icatibant, 1μM. The data are fitted as described in the results. Data represent the mean of triplicate experiments, vertical bars show the s.e.mean. The Hill coefficient is constrained to 1. [^{125}I]-icatibant, 2.0nM, was displaced by unlabelled icatibant (●) and NPC 567 (□).

The K_i values and the Hill coefficients obtained from these displacement studies by non-linear, least squares curve fitting and linear transformation (Hill plot) are compared in table 4.1.

The specific binding of [^{125}I]-icatibant, 2.0nM, to the human nasal bradykinin receptor was also fully displaced by NPC 567 and unlabelled icatibant (figure 4.14). Analysed by non-linear, least squares curve fitting the equilibrium dissociation constants, K_i, for NPC 567 and icatibant were 63.31 ± 8.88nM and 0.85 ± 0.13nM, respectively. The Hill coefficient for NPC 567 was 2.85± 0.62 and for icatibant was 1.22 ± 0.17.

4.4. Discussion

Icatibant (formerly known as Hoe 140), DArg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin, is a potent bradykinin B_2 receptor antagonist (Lembeck *et al.*,1991; Wirth *et al.*,1991; Hock *et al.*, 1991). The previous chapter demonstrated that low doses of this compound antagonise bradykinin-induced nasal blockage and albumin release into the nasal cavity, suggesting that it has a high potency at the human nasal bradykinin receptor. Another bradykinin B_2 receptor antagonist, NPC 567, failed to antagonise the nasal actions of bradykinin, which may result from the relatively low affinity of NPC 567 for the bradykinin B_2 receptor. Alternatively, the failure of NPC 567 to antagonise the actions of bradykinin in the human nasal airway may reflect the presence of a bradykinin B_2 receptor subtype or the B_3 receptor (Farmer *et al.*, 1989). As icatibant antagonises the effects of bradykinin nasal challenge and has a relatively high affinity for both B_2 and B_3 bradykinin receptors (Field *et al.*, 1992), [^{125}I]-icatibant was used as the radioligand for binding experiments on human nasal membranes. It is preferable to use an antagonist rather than an agonist in binding studies. This is due to the agonist producing a conformational change (isomerisation) in the receptor after binding, which leads to activation of the receptor. In the case of the bradykinin receptor, the isomerisation maybe in order to promote the binding of G-proteins. The equilibrium dissociation constant for the isomerisation step affects the values calculated from binding. Therefore, the values calculated from binding studies using agonists are referred to as effective equilibrium dissociation constants.

Over the range of concentrations used, [^{125}I]-icatibant has demonstrated the presence of a single, saturable binding site in a membrane preparation from the mucosa of the human nasal turbinate bone. The equilibrium dissociation constants for icatibant obtained from the saturation binding curve at 20°C (curve fitted-0.48nM, Scathard analysis-0.36nM) and, independently from the association and dissociation rate constants at 4°C (curve fitted-0.7nM, linear transformation-0.5nM), are consistent with other estimates in human lung of 0.73nM (Trifilieff *et al.*, 1994) and in guinea-pig ileum of 0.79nM (Hock *et al.*, 1991).

The presence of multiple [^{125}I]-icatibant binding sites on the human nasal membranes cannot be excluded by using only a low concentration of radioligand in displacement studies as there maybe high and low affinity binding sites for [^{125}I]-icatibant; as proposed to be present in the guinea-pig lung and brain (Seguin *et al.*, 1992). Under such conditions a low concentration of [^{125}I]-icatibant would bind predominately to the high affinity site giving rise to displacement curve fitted by a single-site model (Swillens *et al.*, 1995). In order to exclude the possibility of multiple [^{125}I]-icatibant binding sites with equilibrium dissociation constants in the nanomolar range, displacement studies were conducted with two concentrations of radioligand. The specific binding of [^{125}I]-icatibant, 0.2 and 2nM, was fully displaced by unlabelled icatibant and NPC 567. The equilibrium dissociation constants and Hill coefficients calculated from this displacement study are consistent with the presence of a single bradykinin receptor of the B_2 type.

The B_1 selective ligands, [DesArg^9]-bradykinin and [DesArg^9]-icatibant do not displace the specific binding of [^{125}I]-icatibant over the range of concentrations used, indicating that the radioligand does not bind to bradykinin B_1 receptors in human nasal membranes. These data are in accord with the observations that B_1-selective agonists have no effect on nasal blockage or albumin extravasation in human subjects *in vivo* (Rajakulasingham *et al.*, 1991; Austin & Foreman, 1994a)

The value for the effective equilibrium dissociation constant for bradykinin which we obtained from binding data was 1nM and this is similar to the value of 1.1nM reported for human lung (Trifilieff *et al.*, 1994) and 1.2nM reported for human skin (Schremmer-Danninger *et al.*, 1995) but rather higher than the value of 0.3 nM reported for guinea-pig ileum (Scherrer *et al.*, 1995). Multiple binding sites for bradykinin have been reported in the guinea pig lung, ileum and brain (Trifilieff *et al.*, 1991; Seguin *et al.*, 1992). However, the displacement of [^{125}I]-icatibant from the human nasal membranes by bradykinin is consistent with the presence of a single binding site.

NPC 567 is the least potent of the B_2 receptor antagonists that we used, giving a K_i value of 90nM. In human lung, a higher value of 270nM was obtained (Trifilieff *et al.*,1994). The value for guinea-pig ileum has been reported as varying from 8 to 210nM (Seguin *et al.*, 1992; Farmer *et al.*, 1989; Farmer *et al.*,1991; Lyon *et al.*, 1990). The nonpeptide bradykinin B_2 receptor antagonist, WIN 64338, exhibited a K_i of 41nM in the present studies on human nasal membranes which is comparable with a value of 64nM in human fibroblasts (Sawutz *et al.*, 1994a) and 34nM in guinea pig ileum (Scherrer *et al.*, 1995).

In guinea-pig trachea, reports claim that both NPC 567 and WIN 64338 fail to antagonise bradykinin-induced contractions and have equilibrium dissociation constants of more than 100μM and 1μM respectively, which is partly the basis for the claim that a B_3 receptor exists in this guinea-pig tissue (Farmer *et al.*, 1989; Farmer & DeSiato, 1994). However, the equilibrium dissociation constants of NPC 567 and WIN 64338 reported by Farmer and his colleagues in guinea-pig trachea have been challenged. Other studies have reported that NPC 567 antagonises bradykinin-induced contraction of the guinea pig trachea and fully displaces, in a two binding site model, the specific binding of [^{3}H]-bradykinin in the guinea pig airways (Pruneau *et al.*, 1995; Trifilieff *et al.*, 1991). Scherrer *et al.*,(1995) have obtained a value of 50nM for the K_i of WIN 64338 and showed that the antagonism of bradykinin-induced contraction of the guinea pig trachea was non-competitive. Farmer *et al.* (1989) reported that another bradykinin B_2 receptor antagonist, NPC 349, only weakly inhibited bradykinin-induced bronchoconstriction in the guinea pig *in vivo* and cited this as further evidence for the presence of a B_3 receptor. However, another study reported that this antagonist abolishes the bronchoconstrictor effect of bradykinin in the guinea pig lower airways (Jin *et al.*, 1989). In this study, both NPC 567 and WIN 64338 fully displaced icatibant binding to human nasal membranes, with a K_i value consistent with the presence of a B_2 receptor.

[1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin has been reported to be a potent bradykinin B_2 receptor antagonist. It antagonises the vasodepressor action of bradykinin in the rat (Lammek *et al.*, 1990) and the nasal actions of bradykinin

challenge in humans (Austin & Foreman, 1994a). The data in this chapter present the first measurement of the equilibrium dissociation constant of [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin at the bradykinin B_2 receptor. This antagonist has a K_i value of 1.2nM and therefore has the lower affinity than icatibant, NPC 17731 or NPC 17761. However, it still has approximately 75 times higher affinity than NPC 567 for the human nasal bradykinin receptor.

D-Arg^0[Hyp^3, D-HypE(trans-propyl)^7, Oic^8]-bradykinin (NPC 17731) and D-Arg^0[Hyp^3, D-HypE(trans-thio-phenyl)^7, Oic^8]-bradykinin (NPC 17761) are potent bradykinin B_2 receptor antagonists (Kyle *et al.*, 1991). NPC 17731 had a equilibrium dissociation constant of 0.4nM in the human nasal membranes which is, interestingly, similar to the value reported for guinea-pig trachea of 0.5nM (Trifilieff *et al.*, 1993) where there is supposed to be a B_3 receptor. The K_L for [^{3}H]-NPC 17731 in the guinea-pig ileum has been reported as 0.1nM (Burch *et al.*, 1994). On the other hand, the value for the K_i of NPC17761 in human nasal membranes was 0.3nM which is nearly an order of magnitude greater than the value of 0.05nM reported for guinea-pig trachea (Trifilieff *et al.*, 1993). It should also be mentioned that, with the exception of the studies reported in this chapter and the study using [^{3}H]-NPC 17731 in the guinea-pig ileum, the K_i values determined in other tissues and cited in this discussion have been obtained from binding studies with the labelled agonist, bradykinin and not with a labelled antagonist. However, as the data presented in this chapter was obtained by using an antagonist as the radioligand, the values calculated in this study, with the exception of the K_i for the B_2 agonist bradykinin, are true equilibrium dissociation constants.

The K_i values from data presented in this chapter are in good agreement with values published for other human tissues but there are some discrepancies with values in guinea-pig ileum and, more particularly with the values from guinea-pig trachea where the existence of a B_3 receptor has been proposed. Differences in the pharmacology of human and non-human B_2 receptors have been reported using cloned B_2 receptors expressed in cell lines. Both the rat and mouse cloned B_2 receptors have a higher affinity for [D-Phe^7]-substituted B_2 antagonists (for example, NPC 567) than

the cloned human B_2 receptor (Hess *et al.*, 1994; Davis *et al.*, 1995). While expressing receptors in cell lines does not represent the *in vivo* situation it is interesting that binding studies on *ex vivo* tissue, to an extent, support this species difference. Hess *et al.*, (1994), using cloned human B2 receptors expressed in Chinese hamster ovary cells, reported that icatibant is approximately 300 times more potent at displacing the binding of [^{3}H]-bradykinin than NPC 567. The data reported in this chapter demonstrate that icatibant has approximately 200 times higher affinity than NPC 567 for the bradykinin receptor in the human nasal airway. Therefore, this study is consistent with the data reported from binding studies using cloned human B_2 receptors. It is also interesting that the affinities of [D-Phe^7]-substituted B_2 antagonists have been used to propose bradykinin B_2 receptor subtypes and the B_3 receptor (Saha *et al.*, 1990; 1991; Regoli *et al.*, 1993; Seguin *et al.*, 1992; Farmer *et al.*, 1989). In light of the species differences in cloned receptors, the proposed B_2 receptor heterogeneity may be explained by species divergence in the evolution of the bradykinin B_2 receptor gene. In summary, the data presented in this chapter are consistent with the bradykinin receptor in the human nose being a B_2 receptor.

Chapter 3 presented data on the effect of bradykinin receptor antagonists on the nasal actions of bradykinin in human subjects *in vivo*. For the antagonism of bradykinin-induced changes in nasal patency (Amin), icatibant was 2.5 times more potent than [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin, and for the antagonism of bradykinin-induced albumin extravasation icatibant was 2.8 times more potent than [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin. These potency ratios were calculated using the dose of antagonist, in moles, which produced a 50% inhibition of the *in vivo* effect of bradykinin, 100µg. From the binding data, icatibant was 2.5 times more potent than [1-adamantane acetyl-D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin. Thus, there is good agreement between the functional relative activities *in vivo* and the relative activities from the binding data for these two compounds. NPC 567 has previously been shown not to block the nasal actions of bradykinin at a dose of 1mg (0.8µmol) (Austin & Foreman, 1994a) and chapter 3 demonstrates no effect at 10mg (8µmol). Taking 15nmol as the dose of icatibant producing 50% inhibition of the effect of bradykinin on Amin., the equivalent dose of NPC 567, based on relative

potencies from the binding data, would be 3μmol (4mg). One might, therefore, have expected to see an effect with NPC 567 at a dose of 10mg, but this assumes that access of both icatibant and NPC 567 to the receptor are the same. They may, of course, differ in their susceptibility to metabolism or in their ability to diffuse to the receptor. Furthermore, *in vivo* studies may not represent equilibrium conditions and NPC 567, at high concentration, could stimulate histamine release from human nasal tissue. Finally, while this chapters binding data shows that NPC 567 displaces [^{125}I]-icatibant from human nasal membranes it does not demonstrate whether NPC 567 is an antagonist. While NPC 567 has been shown to be a B_2 receptor antagonist in many tissues, there is some evidence that it may act as an agonist in human tissues. NPC 567 evokes a pain response when injected into human skin which was unaffected by histamine H_1 receptor antagonists and therefore not mediated by histamine release (Kindgren-Milles & Klement, 1992). At the cellular level, NPC 567 acts as an agonist when intracellular calcium levels are measured in CHO cells transfected with the human B_2 receptor gene (Eggerickx *et al.*, 1992) and in human IMR-90 lung fibroblasts (Sawutz *et al.*, 1992). The ability of high doses of NPC 567 to act as an agonist in the human nasal airway and cause nasal blockage could be simply resolved by challenging with 10mg of NPC 567 alone. Unfortunately, this experiment was precluded by cost so remains unresolved.

Summary

- The aim of this study was to characterise the bradykinin receptor in the human nasal airway using [^{125}I]-icatibant binding to a membrane preparation from human nasal turbinate bones and to compare Ki values from binding displacement with antagonists with the functional effects of these drugs in vivo.

- In a membrane preparation from human nasal turbinate bones removed during surgery, [^{125}I]-icatibant labelled a single, saturable binding site. The equilibrium dissociation constant (at 20°C) for [^{125}I]-icatibant binding to the receptor was 0.46 ± 0.08nM, calculated by non-linear least squares curve-fitting. The Bmax was 0.136 ± 0.003 pmol.mg protein^{-1}.

- The association rate constant for [^{125}I]-icatibant binding to the receptor was 0.2 ± 0.06nM.min^{-1} and the dissociation rate constant was 0.14 ± 0.01min^{-1}. These values were determined at 4°C and calculated from the data by non-linear least squares curve fitting. The equilibrium dissociation constant calculated from these rate constants was 0.7nM. The equilibrium dissociation constant for [^{125}I]-icatibant binding, calculated by both saturation and kinetic studies, is consistent with the presence of the bradykinin B_2 receptor in human nasal tissue.

- Bradykinin and the B2 receptor antagonists NPC 567, NPC 17731, NPC 17761, [1-adamantane acetyl -D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin WIN 64338 and icatibant displaced [^{125}I]-icatibant binding, the Ki values from binding displacement are consistent with values expected from a B2 receptor. The B1 agonist [des-Arg^9]-bradykinin and the B1 antagonist [des-Arg^9]-icatibant failed to displace [^{125}I]-icatibant binding at concentrations up to 1μM. The relative affinities of icatibant and [1-adamantane acetyl -D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin correlate well with the relative potencies calculated *in vivo* in the previous chapter.

- In conclusion, there is a bradykinin B2 receptor in the human nasal airway which mediates nasal blockage and plasma extravasation induced by either bradykinin or antigen challenge.

CHAPTER 5

THE ROLE OF NITRIC OXIDE IN THE ACTIONS OF BRADYKININ, HISTAMINE AND ANTIGEN IN THE NORMAL AND ATOPIC HUMAN NASAL AIRWAYS

5.1 Introduction

As previously discussed, there is evidence that bradykinin is a mediator of allergic rhinitis. Histamine is also considered to be a mediator of allergic rhinitis since its application to the nasal airway causes blockage (Britton *et al.*, 1978). Histamine is also detected in increased amounts in nasal lavage following the antigen-challenge of subjects with allergic rhinitis (Naclerio *et al.*,1983), and pretreatment with an histamine H_1 receptor antagonist, of subjects with allergic rhinitis, reduces some of the effects of antigen-challenge (Rokenes *et al.*,1988).

In certain tissues the vasodilator effects of bradykinin and histamine have been shown to be endothelium-dependent (Cherry *et al.*, 1982; Toda & Okamura, 1989), and both substances are able to initiate the formation of the vasodilator, nitric oxide from endothelial cells (Palmer *et al.*,1987; Furchgott & Vanhoutte, 1989).

The kinin-induced endothelial-dependent relaxation of vascular smooth muscle can be mediated through B_1 or B_2 receptors (Sung *et al.*,1988; Wiemer & Wirth, 1992), but nitric oxide can also be generated as a result of bradykinin-induced phospholipase A_2 stimulation (Kontos *et al.*, 1984). Bradykinin-induced relaxation of vascular smooth muscle is mediated via nitric oxide or a related species in several tissues: for example, the pig coronary artery (Cowan & Cohen, 1991; Vials & Burnstock, 1992), rat kidney (Fulton *et al.*, 1992) and guinea-pig isolated heart (Kelm & Schrader, 1988). In the hamster cheek pouch, the increase in microvascular permeability induced by bradykinin is dependent on nitric oxide generation (Feletou *et al.*, 1996). In the rat mesentery, the effects of bradykinin appear to be mediated by nitric oxide released from non-adrenergic, non-cholinergic neurones (Yu *et al*, 1993). In the human, nitric

oxide has been shown to mediate bradykinin-induced vasodilatation in a number of tissues including the arterial bed of the forearm (O'Kane *et al* 1994, Cockcroft *et al* 1994), opthalmic artery (Haefliger *et al.*, 1992) and the corpus cavernosum (Azadzoi *et al.*, 1992). Bradykinin has been shown to activate constitutive nitric oxide synthase in cultured human vascular endothelial cells (Fleming *et al.*, 1994).

In many systems, histamine mediates its effects indirectly by the generation of nitric oxide. Histamine causes vasodilatation, via nitric oxide, of guinea-pig gastrointestinal blood vessels (Beyak & Vanner, 1995), monkey basilar arteries (Fujiwara *et al.*, 1992), guinea-pig coronary arteries (Kelm *et al.*, 1993) and human pulmonary arteries (Ortiz *et al.*, 1992). The oedema formed in guinea-pig skin following intradermal histamine injection is inhibited by nitric oxide synthase inhibitors (Teixeira *et al.*, 1993). The hypersecretion of mucin by cultured guinea-pig tracheal epithelial cells stimulated with histamine is dependent upon the generation of nitric oxide (Adler *et al.*, 1995). The increased vascular permeability induced by histamine has been shown to result from nitric oxide generation activating soluble guanylate cyclase, both in isolated porcine blood vessels (Yuan *et al.*, 1993) and hamsters *in vivo* (Mayhan, 1994).

The enzyme which generates nitric oxide, nitric oxide synthase, has been shown to be present in the human nasal airways. Inferior turbinate bones obtained from surgery on patients with no history of allergic rhinitis show high levels of immunostaining for endothelial nitric oxide synthase (eNOS) in the vascular endothelium, surface epithelium and submucosal glands (Furukawa *et al.*, 1996). This distribution of eNOS was confirmed by investigating the distribution of eNOS mRNA using *in situ hybridization.* Inducible nitric oxide synthase (iNOS) showed moderate levels of immunostaining localised to the same structures as eNOS, but iNOS mRNA was only present in the inflammatory cells of the nasal mucosa (Furukawa *et al.*, 1996). Neuronal nitric oxide synthase has also been reported to be present in the extrinsic perivascular and periglandular nerve fibres of the human nasal mucosa, suggesting a role for nitric oxide in local blood flow and mucous secretion (Kulkarni *et al.*, 1994). Interestingly, neuronal nitric oxide synthase immunoreactivity has also been located in

mast cells of the normal human nasal mucosa (Bacci *et al.*, 1994). The production of nitric oxide in the human nasal airway has been shown to increase after nasal challenge with house dust mite in subjects with perennial allergic rhinitis (Garrelds *et al.*, 1994) and in seasonal allergic rhinitis (Martin *et al.*, 1996). Nitric oxide production has also been demonstrated in the human paranasal sinuses (Lundberg *et al.*, 1995).

The aim of the studies described in this chapter is to investigate the role of nitric oxide in the nasal actions of bradykinin and histamine in normal subjects and of antigen in subjects allergic to house-dust mite and grass pollen.

5.2 Experimental protocol

5.2.1 Protocol for measuring the effect of local anaesthetics and L-NAME on the resting nasal airway patency

To study whether L-NAME, lignocaine or benzocaine have any effect on the resting nasal airway patency the vasoconstrictor ephedrine was used as a positive control. The effect of ephedrine, 2.5μmol, L-NAME, 1μmol, lignocaine, 10mg or benzocaine, 10mg, on the unstimulated nasal airway was studied by administering one of the treatments into the nasal airway and measuring the minimum nasal cross-sectional area (Amin.) 30 min. after challenge. The Amin. was also measures 5 min. prior to challenge. Each subject received ephedrine or one of the treatments on separate occasions at least 24 hours apart.

5.2.2 Protocol for nasal challenge with bradykinin and histamine in normal human subjects

The basic protocol was a double-blind, cross-over experiment in which each subject received a control treatment on one occasion and an experimental treatment on a

separate occasion. At least one week separated the two treatments in an individual subject. For these experiments normal, non-atopic subjects were used.

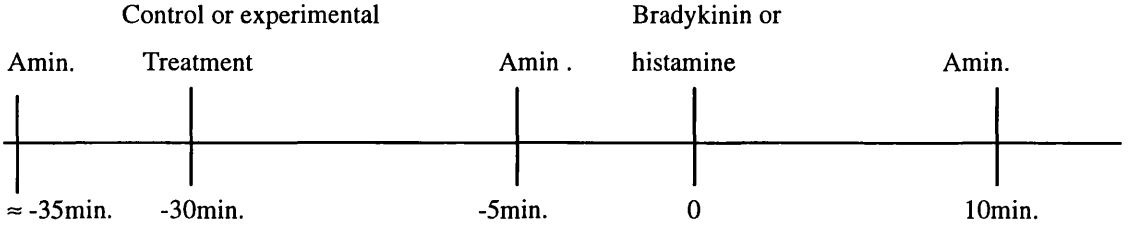

Figure 5.1. Protocol for investigating the effect of local anaesthetics and inhibitors of nitric oxide synthase on the reduction in nasal airway patency induced by bradykinin and histamine.

For the study of nasal patency, an initial value of Amin., was determined as previously described. The value of Amin. determined at this point was used only to compare with the baseline value determined later in the protocol and to demonstrate no change induced by the treatment alone and no change with time. Subjects then received either the control or experimental treatment: which treatment was given first was determined on a random basis. Twenty-five minutes after the treatment, Amin. was redetermined and this value served as the baseline against which changes induced by histamine or bradykinin were judged. Thirty minutes after the treatment, histamine, 300μg or bradykinin, 300μg, were administered into each nostril and Amin. was redetermined 10 min. later. The doses of histamine and bradykinin were chosen, on the basis of pilot studies, to be approximately equipotent (Austin, 1994). The measurement of Amin. 10 min. after bradykinin or histamine was chosen on the basis of prior studies showing that this was the time of maximum change in Amin.(Austin, 1994). This protocol is summarised in figure 5.1.

The protocol was followed to study the effect on histamine- or bradykinin-induced changes in Amin. of: L-NAME, 0.1, 1, 10μmol; D-NAME, 0.1, 1, 10μmol; L-arginine, 30μmol; L-arginine, 30μmol, plus L-NAME, 1μmol; L-NMMA, 1, 10μmol; lignocaine 10mg; benzocaine 10mg. The control for each of the treatments was saline.

Each compound at each dose, or its appropriate control, constituted a separate treatment in the above protocol.

The protocol was varied to study the effect of the duration of pretreatment with L-NAME, 1μmol, on the response to bradykinin. In this case, the interval between the L-NAME treatment and the bradykinin challenge was 0 (L-NAME and bradykinin together), 2, 15, 30 or 60 min. and the baseline Amin. values were recorded 5 min. prior to the bradykinin challenge.

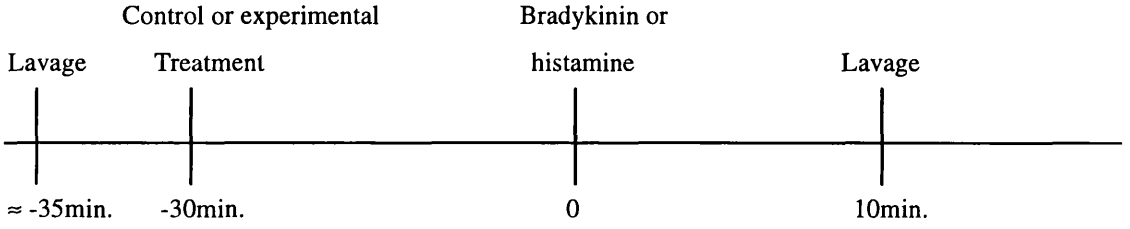

Figure 5.2. Protocol for investigating the effect of local anaesthetics and inhibitors of nitric oxide synthase on the increase in nasal mucosal vascular permeability induced by bradykinin and histamine.

The study of albumin release was conducted on separate occasions from the study of nasal patency. Again, a double-blind, cross-over design was used. Nasal lavage was performed prior to any treatment, and the lavage was used for the measurement of baseline albumin release. Subjects then received either the control or experimental treatment: which was given first being randomly determined. Lavage was not performed after the treatment in order that the treatment was not washed from the nasal airway. Thirty minutes later, bradykinin, 300μg, or histamine, 300μg, was administered to each nostril and 10 min. later another lavage was performed and the sample retained for albumin assay. This protocol is summarised in figure 5.2.

The protocol was undertaken for the following treatments: L-NAME, 0.1, 1, 10μmol; D-NAME, 0.1, 1, 10μmol; L-arginine, 30μmol; L-arginine, 30μmol, plus L-NAME, 1 μmol; lignocaine, 10mg; benzocaine 10mg. In each case the control treatment was saline.

The protocol was varied to study the effect of L-NAME, 1μmol, on the albumin release in the absence of histamine or bradykinin challenge, by omitting the challenge with these agents from the protocol.

5.2.3 Protocol for nasal antigen challenge studies in atopic human subjects

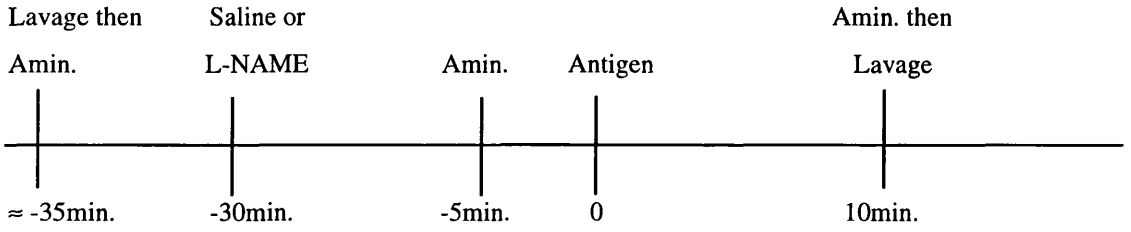

Figure 5.3. Protocol for investigating the effect of L-NAME on the nasal responses induced by antigen.

For the antigen provocation study, a single-blind cross-over design was employed and the first treatment for each subject was allocated randomly. The protocol was similar to that for the histamine and bradykinin study except that mixed grass pollen antigen, 1000u per nostril or house-dust mite antigen, 500u per nostril was used for the challenge. The subjects were treated with L-NAME, 1μmol or saline, the treatment given first being determined randomly. As before there was 30 minutes between treatment and challenge. In addition, nasal lavage and acoustic rhinometry were determined in the same study: nasal lavage accompanying each Amin. determination except the Amin. measurement prior to challenge, when no lavage was taken in order that the treatment was not washed out of the nasal cavity. This protocol is summarised in figure 5.3.

5.2.4 Data analysis

The dimensions of the nasal airway vary between subjects and also within subjects from day to day and so the data have been normalized by expressing changes in Amin. as a percentage of the baseline control value. The absolute values for the baseline measurements have been given with each set of data. Means are given together with

standard error of the mean (s.e.mean). The appropriate, non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

5.3 Results

5.3.1 Effect of nitric oxide synthase inhibitors on bradykinin- and histamine-induced reductions in nasal airway patency

In order to demonstrate that L-NAME had no vasoconstrictor action of its own, which might affect nasal patency, the vasoconstricting sympathomimetic, ephedrine was used as a positive control. Ephedrine, 2.5μmol, per nostril caused a significant increase , compared with saline, in the minimal cross-sectional area (Amin.) of the unstimulated human nasal airway, whereas L-NAME, 1μmol, alone had no effect (Table 5.1).

Bradykinin and histamine both caused a decrease in the baseline (100%) Amin. in the absence of any other drug (Figure 5.4). Figure 5.4 shows the dose-response curve for the effect of L-NAME or D-NAME on the decrease in Amin. induced by the administration of bradykinin, 300μg, per nostril. Also shown is the dose-response curve for L-NAME and the decrease in A min. caused by histamine, 300μg. L-NAME or D-NAME were administered 30 min. prior to challenge with bradykinin or histamine. L-NAME, 0.1 to 10μmol, dose-dependently inhibited the decrease in Amin. caused by bradykinin (Friedman's test, $p<0.04$) whereas the same doses of L-NAME failed to inhibit the decrease in Amin. caused by histamine, 300μg (Friedman's test, $p>0.2$). D-NAME failed to inhibit the decrease in Amin. caused by bradykinin (Friedman's test, $p>0.2$). Another inhibitor of nitric oxide synthase, N^G-monomethyl-L-arginine (L-NMMA), 1μmol, also inhibited the bradykinin-induced reduction of Amin.(Figure 5.5): the difference being significant ($p<0.05$) when assessed by the Wilcoxon paired, sign-rank test. In contrast, L-NMMA, 10μmol, failed to inhibit the fall in Amin. caused by histamine ($p>0.2$). In the same subjects, this dose of L-NMMA did inhibit the histamine-induced albumin release into the nasal airway (Figure 5.9).

Treatment	Percentage of baseline Amin.
Saline	102.9 ± 3.2
L-NAME	96.4 ± 3.0
ephedrine	124.0 ± 2.6*
lignocaine	95.8±5.2
benzocaine	101.9 ± 4.9

Table 5.1. The effects of saline, L-NAME, 1 μmol, ephedrine, 2.5 μmol, lignocaine, 10 mg, and benzocaine, 10mg on the minimal cross-sectional area (Amin.) of the unstimulated human nasal airway. The data are the means ± s.e.m. from six subjects. The data have been normalized by expressing the Amin., values as a percentage of the baseline value for each subject. The mean ± s.e.m. baseline value for Amin., was 0.55 ± 0.04 cm^2. * indicates as significant difference by the Wilcoxon paired, sign-rank test, between saline and ephedrine ($p<0.05$).

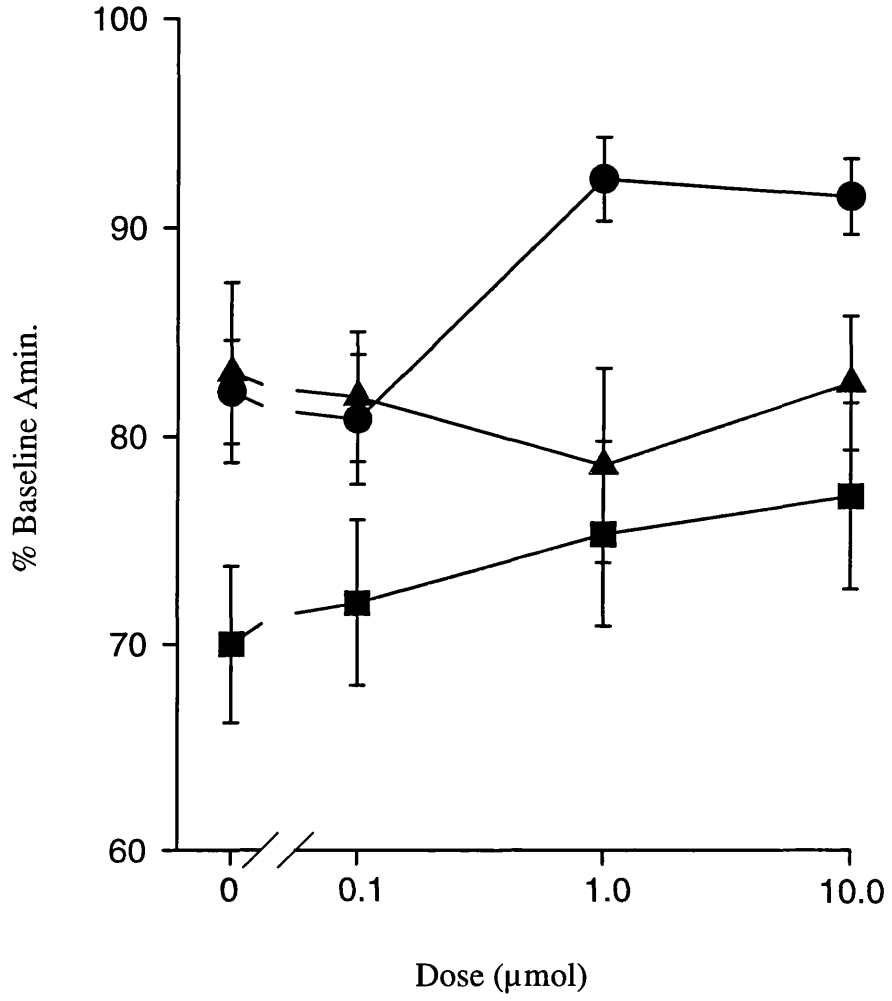

Figure 5.4. Dose-response curve for the effect of N^G-nitro-L-arginine methyl ester (L-NAME) on the reduction of minimal nasal cross-sectional area (Amin.) induced by bradykinin. L-NAME or D-NAME was administered 30 min. prior to bradykinin or histamine and Amin., was measured 10 min. after bradykinin or histamine administration. Bradykinin, 300µg + L-NAME (●); bradykinin, 300µg + D-NAME (▲); histamine, 300µg + L-NAME (■). Data are the means ± s.e.m. from 8 subjects. Changes in Amin., have been normalized by expressing them as a percentage of the baseline for each subject. The mean ± s.e.m. baseline value of Amin., was 0.54 ± 0.02 cm^2.

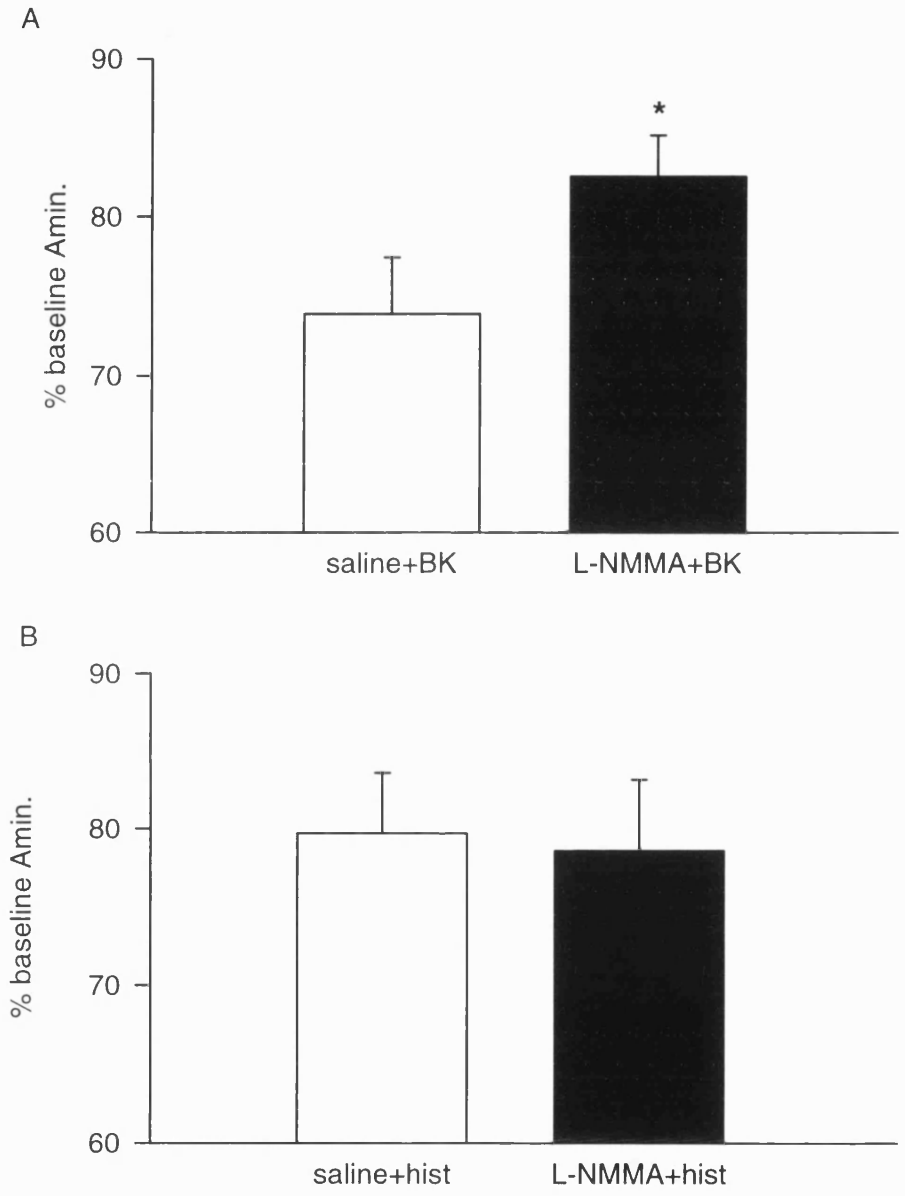

Figure 5.5. Effect of N^G-monomethyl L-arginine (L-NMMA) on bradykinin- and histamine-induced reductions in Amin. L-NMMA, 1μmol antagonises bradykinin-induced reductions in Amin. (graph A). L-NMMA, 10μmol fails to antagonise histamine-induced reductions in Amin. (graph B). The data are means ± s.e.m. from 7 subjects. Changes in Amin. have been normalized by expressing them as a percentage of the baseline Amin. for each subject. The mean ± s.e.m. baseline value of Amin., was 0.54 ± 0.01 cm^2. * indicates a significant difference by the Wilcoxon paired, sign-rank test ($p<0.05$), from the % reduction in Amin. with saline pretreatment.

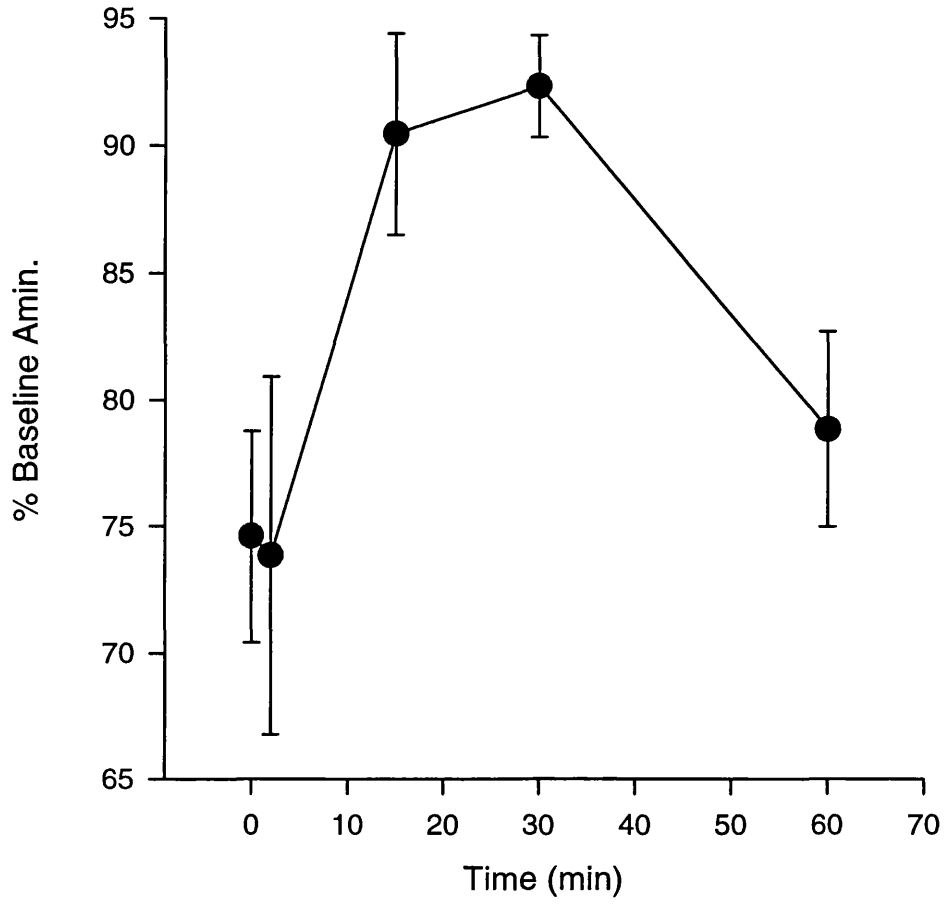

Figure 5.6. Effect of the duration of pretreatment with L-NAME on the reduction of Amin., caused by bradykinin, 300μg. L-NAME, 1μmol, was administered at the times shown prior to the challenge with bradykinin. The data are means ± s.e.m. from 5 subjects. Changes in Amin. have been normalized by expressing them as a percentage of the baseline Amin. for each subject. The mean ± s.e.m. baseline value of Amin., was 0.60 ± 0.09 cm^2. * indicates a significant difference by the Wilcoxon paired, sign-rank test ($p<0.05$), from the value at zero time when L-NAME and bradykinin were given simultaneously.

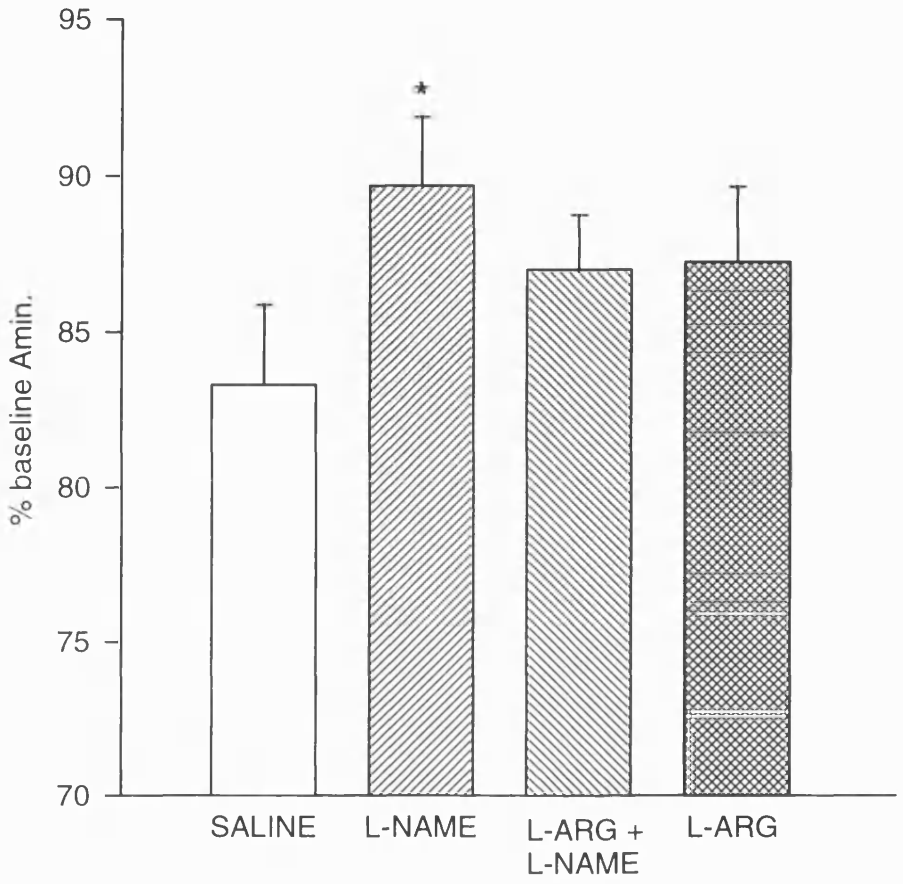

Figure 5.7. Effect of L-arginine, 30µmol, on the effect of L-NAME, 1µmol, on bradykinin-induced reductions in the patency of the nasal cavity (Amin.). The data are the means ± s.e.m. from 10 subjects. The mean ± s.e.m. baseline value of Amin., was 0.53 ± 0.01 cm^2 * indicates a significant difference between the values for bradykinin alone and the values in the presence of L-NAME ($p<0.05$). The test of significance applied was the Wilcoxon paired, sign-rank test.

Figure 5.6 shows the effect of the duration of pretreatment with L-NAME on the reduction in Amin. induced by bradykinin. The effect of L-NAME was significant when it was administered 15 and 30 min. prior to bradykinin ($p<0.05$, assessed by the Wilcoxon paired, sign-rank test) and the magnitude of the effect of L-NAME decreased with longer pretreatment time.

To test the specificity of L-NAME, we attempted to reverse its effect on the reduction in Amin. induced by bradykinin by adding the nitric oxide synthase substrate, L-arginine. L-NAME, 1μmol, significantly inhibited the reduction in nasal patency induced by bradykinin, 300μg ($p<0.05$, assessed by the Wilcoxon paired, sign-rank test). Addition of L-arginine, 30μmol, together with L-NAME, 1μmol, failed to reverse the inhibitory action of L-NAME on the reduction in Amin. induced by bradykinin, $p<0.1$, assessed by the Wilcoxon paired, sign-rank test (Figure 5.7).

5.3.2 Effect of inhibitors of nitric oxide synthase on bradykinin- and histamine-induced extravasation of albumin into the nasal lavage

In addition to studying the action of L-NAME on nasal blockage induced by bradykinin and histamine, the effect of L-NAME on plasma extravasation induced by these substances was examined. Figure 5.8 shows that L-NAME, given 30 min. prior to challenge with bradykinin 300μg, or histamine, 300μg, reduced the extravasation of albumin into nasal lavage fluid, in a dose-dependent manner (Friedman's test, $p<0.05$). D-NAME, when administered in the same manner, did not reduce the albumin extravasation by induced by either bradykinin or histamine (Friedman's test, $p>0.1$). Compared with saline, L-NAME, 10μmol, alone did not cause an increase in the release of albumin into the nasal cavity. The albumin release after saline was 0.1 ± 0.1 mg/dl (mean ± s.e.m., n=5) and after L-NAME, 10μmol it was 0.1 ± 0.1mg/dl. This experiment would only detect an increase of albumin release by L-NAME, since the albumin release following saline is at the lower limit of detection of the assay used.

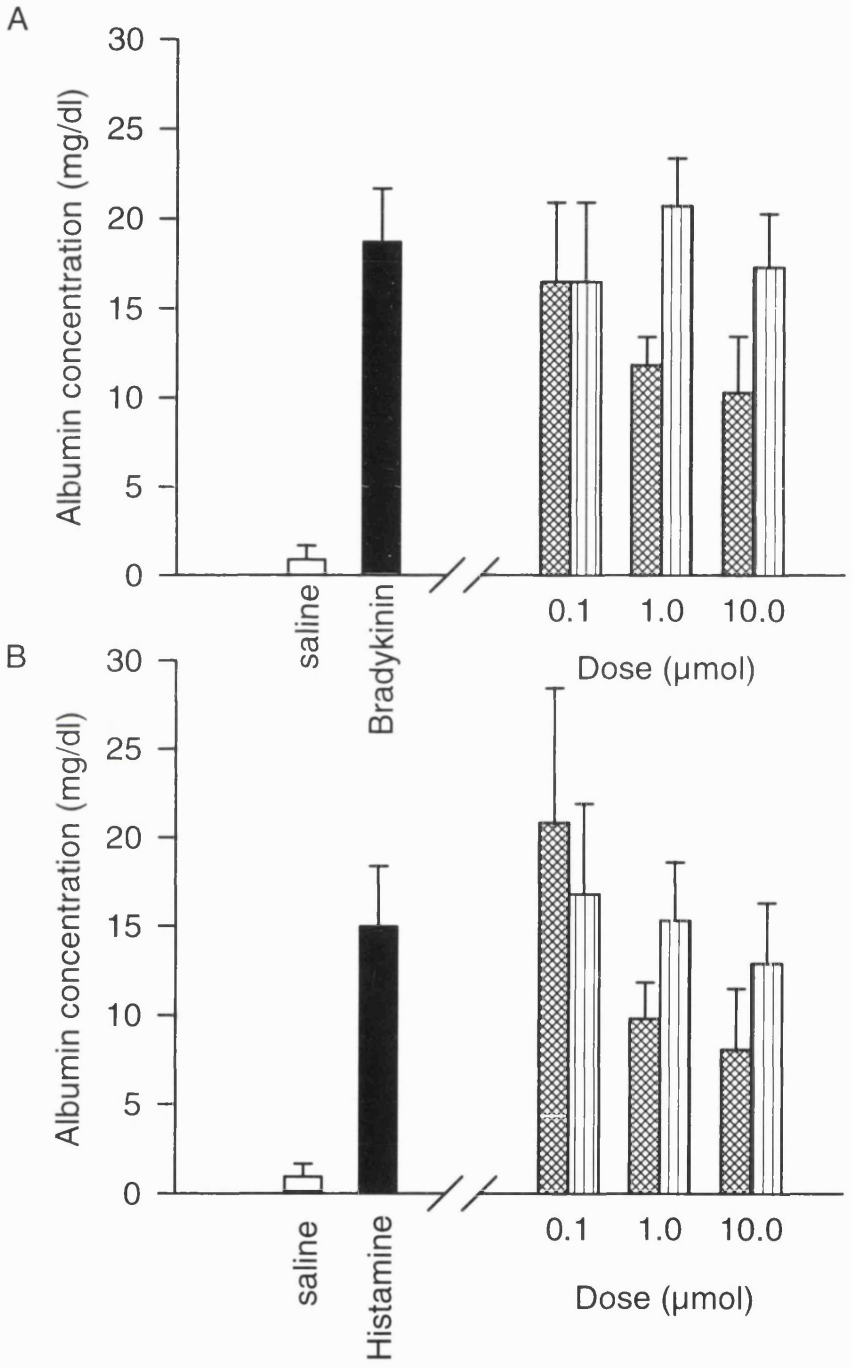

Figure 5.8. Dose-response relationship for the effect of L-NAME and D-NAME on bradykinin- and histamine-induced increases in the albumin concentration in nasal lavage. Figure A presents data from experiments using bradykinin, 300µg, and B from experiments using histamine, 300µg. □ saline; ■ bradykinin, 300µg, or histamine, 300µg; ▩ bradykinin, 300µg + L-NAME or histamine, 300µg + L-NAME; ▥ bradykinin, 300µg + D-NAME or histamine, 300µg + D-NAME. The data are the means ± s.e.m. from 10 subjects.

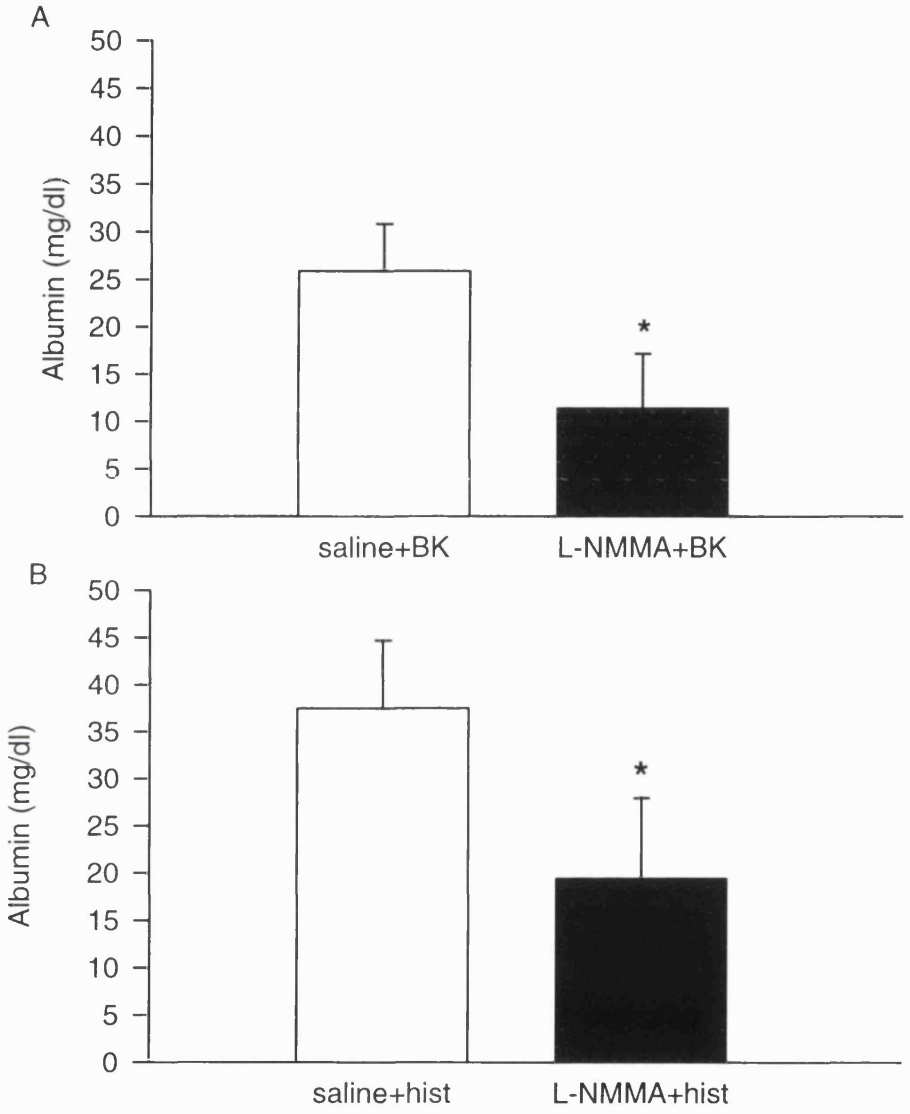

Figure 5.9. Effect of N^G-monomethyl L-arginine (L-NMMA) on bradykinin- and histamine-induced increases in albumin content of the nasal lavage. L-NMMA, 1μmol antagonises bradykinin-induced albumin extravasation into the nasal lavage (graph A). L-NMMA, 10μmol antagonises histamine-induced albumin extravasation into the nasal lavage (graph B). The data are means ± s.e.m. from 7 subjects. * indicates a significant difference by the Wilcoxon paired, sign-rank test ($p<0.05$).

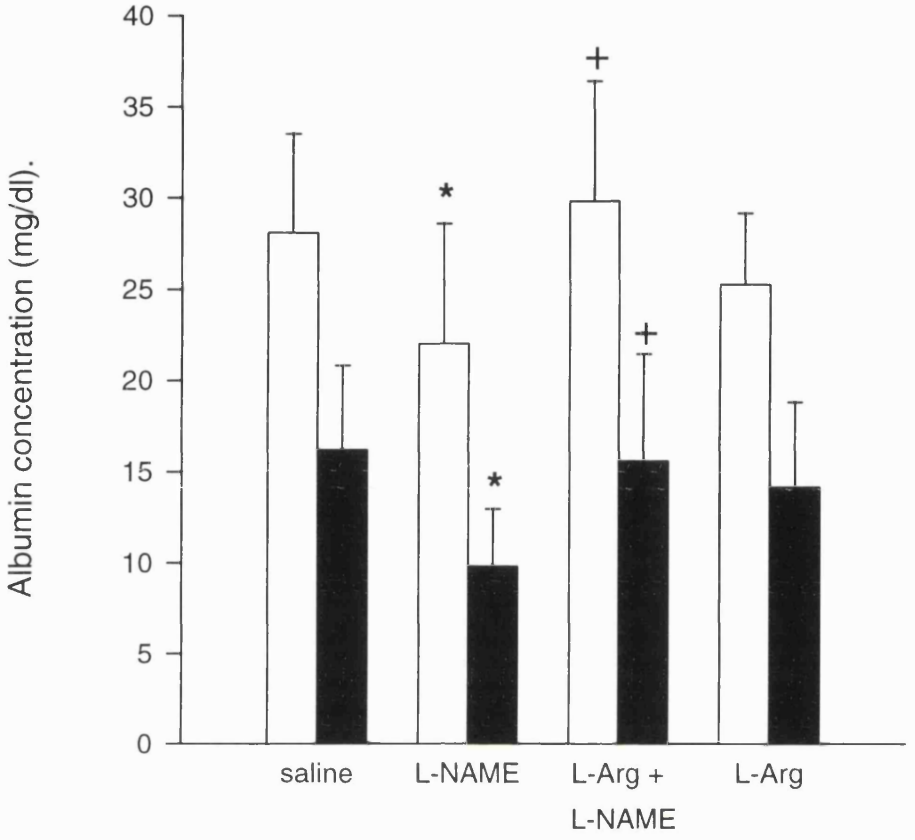

Figure 5.10. Reversal by L-arginine, 30μmol, of the effect of L-NAME, 1μmol, on bradykinin- or histamine-induced increases in albumin concentration in nasal lavage. □ bradykinin, 300μg, ■ histamine, 300μg. The data are the means ± s.e.m. from 10 subjects. * indicates a significant difference between the values for histamine or bradykinin alone and the values in the presence of L-NAME ($p<0.03$). + indicates a significant difference between the values in the presence of L-NAME and the values in the presence of L-NAME + L-arginine ($p<0.03$). In both cases, the test of significance applied was the Wilcoxon paired, sign-rank test.

Figure 5.9 shows that bradykinin and histamine-induced extravasation of albumin into the nasal lavage fluid was also significantly reduced by L-NMMA, 1 and 10μmol respectively ($p<0.05$, assessed by the Wilcoxon paired, sign-rank test).

To test the specificity of L-NAME, we attempted to reverse its effect on albumin release into the nasal cavity by adding the nitric oxide synthase substrate, L-arginine. Addition of L-arginine, 30μmol, together with L-NAME, 1μmol, reversed the inhibitory action of L-NAME on the increased albumin extravasation induced by both bradykinin and histamine, $p<0.03$, assessed by the Wilcoxon paired, sign-rank test (Figure 5.10). L-arginine alone was without effect on albumin extravasation (Figure 5.10)

5.3.3 Effect of local anaesthetics on bradykinin- and histamine- induced reductions in nasal airway patency and albumin extravasation into the nasal lavage

To examine the possible involvement of nerves in the nasal responses to histamine and bradykinin, we studied the responses to these substances after local anaesthesia. The doses of lignocaine and benzocaine were selected on the basis of their relative potencies and the dose of lignocaine which is used clinically to achieve nasal anaesthesia. Both drugs abolished sensation to tactile stimuli applied inside the nostril. Application of lignocaine, 10mg, or benzocaine, 10mg, alone to the nasal airway did not change Amin. ($p>0.2$), assessed by Wilcoxon paired, sign-rank test), although the vasoconstrictor, ephedrine, 2.5μmol, significantly increased Amin. (Table 1). Pretreatment of the nasal airway with lignocaine, 10mg, or benzocaine, 10mg, failed to affect the reduction in Amin. or the albumin extravasation, induced by either bradykinin, 300μg, or histamine 300μg, $p>0.1$, assessed by Wilcoxon paired, sign-rank test (Table 5.2).

	Percentage of baseline Amin.		Albumin concentration (mg/dl)
Bradykinin, 300 μg +lignocaine	79.2 ± 4.7		25.6 ± 5.4
Bradykinin, 300 μg + saline	78.5 ± 4.7		22.3 ± 6.1
Bradykinin, 300μg + benzocaine	79.0±6.1		14.8±6.1
Bradykinin, 300μg + saline	82.1 ± 5.8		16.4 ± 4.2
Histamine, 300 μg + lignocaine	80.1±5.1		19.1±5.8
Histamine, 300 μg + saline	75.1 ± 3.6		18.9 ± 4.7
Histamine, 300μg + benzocaine	82.4±5.2		19.2±3.8
Histamine, 300μg + saline	79.3±4.9		21.0±4.7

Table 5.2. The effect of lignocaine, 10 mg, or benzocaine, 10mg, on the histamine- and bradykinin-induced reduction of minimal nasal cross-sectional area (Amin.) and increased albumin concentration in nasal lavage. The data are the means ± s.e.m. from 5 subjects. To normalize the data, Amin., values are expressed as a percentage of the baseline value for each subject. The mean ± s.e.m. baseline value for Amin. was 0.48 ± 0.03 cm^2.

5.3.4 Effect of inhibitors of nitric oxide synthase on antigen - induced reductions in nasal airway patency and albumin extravasation into the nasal lavage

Figure 5.11 shows that house-dust mite antigen, in allergic subjects, caused a reduction of (Amin). L-NAME, 1μmol per nostril, given 30 min. prior to antigen, inhibited the antigen-induced reduction of Amin.($p<0.02$, assessed by the Wilcoxon, paired, sign-rank test). Challenge with house-dust mite antigen failed to increase the extravasation of albumin into the nasal lavage, baseline albumin lavage concentration = 2.8 ± 0.7mg/dl, post-challenge albumin lavage concentration = 1.2 ± 0.9 mg/dl.

Figure 5.12 shows that mixed grass pollen antigen, in allergic subjects, caused a reduction of nasal airway patency (Amin) and an increase in the extravasation of serum albumin into the nasal lavage. L-NAME, 1μmol per nostril, given 30 min prior to antigen, inhibited the antigen-induced leakage of albumin into the nasal lavage ($p<0.02$ assessed by the Wilcoxon, paired, sign-rank test) but did not inhibit the antigen-induced reduction of Amin. ($p>0.4$), assessed by the Wilcoxon, paired, sign-rank test).

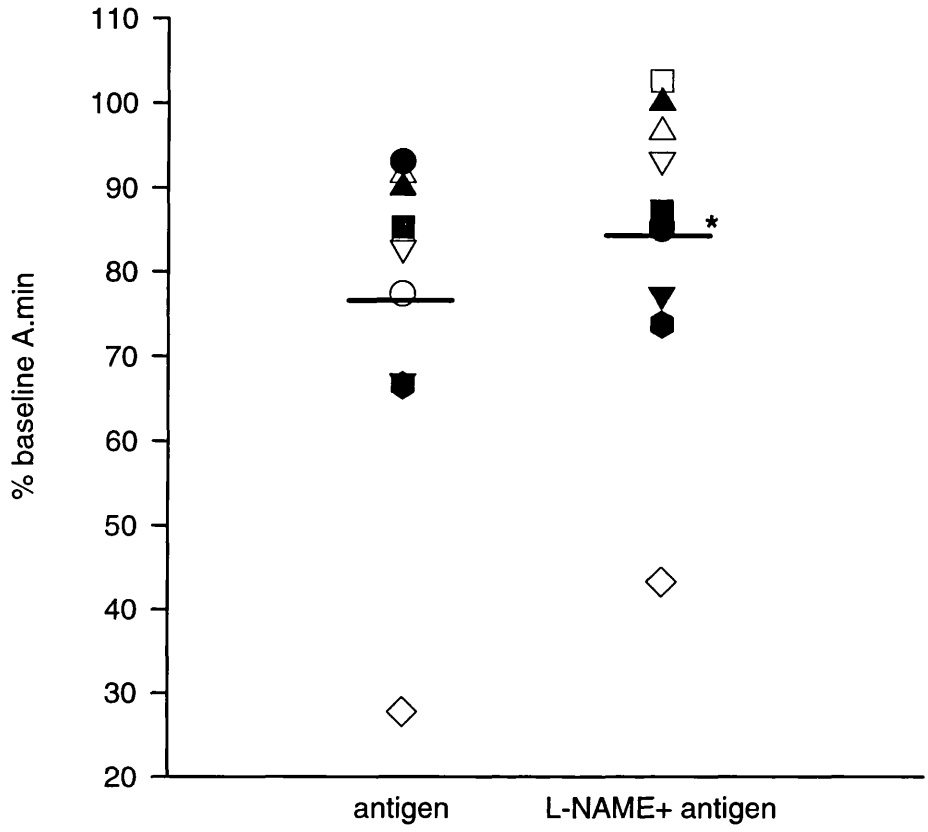

Figure 5.11. The effect of either saline or L-NAME, 1μmol per nostril, given 30 min. prior to antigen challenge (500u house-dust mite per nostril) on the change in nasal patency (Amin). Each symbol represents a separate subject and the horizontal bars indicate the mean values. Data are presented as percentage change from the baseline Amin. The mean ± s.e.m. baseline value of Amin., was 0.46 ± 0.02 cm^2 . * indicates a significant difference compared with antigen ($p<0.05$), analysed by the paired, Wilcoxon sign-rank test.

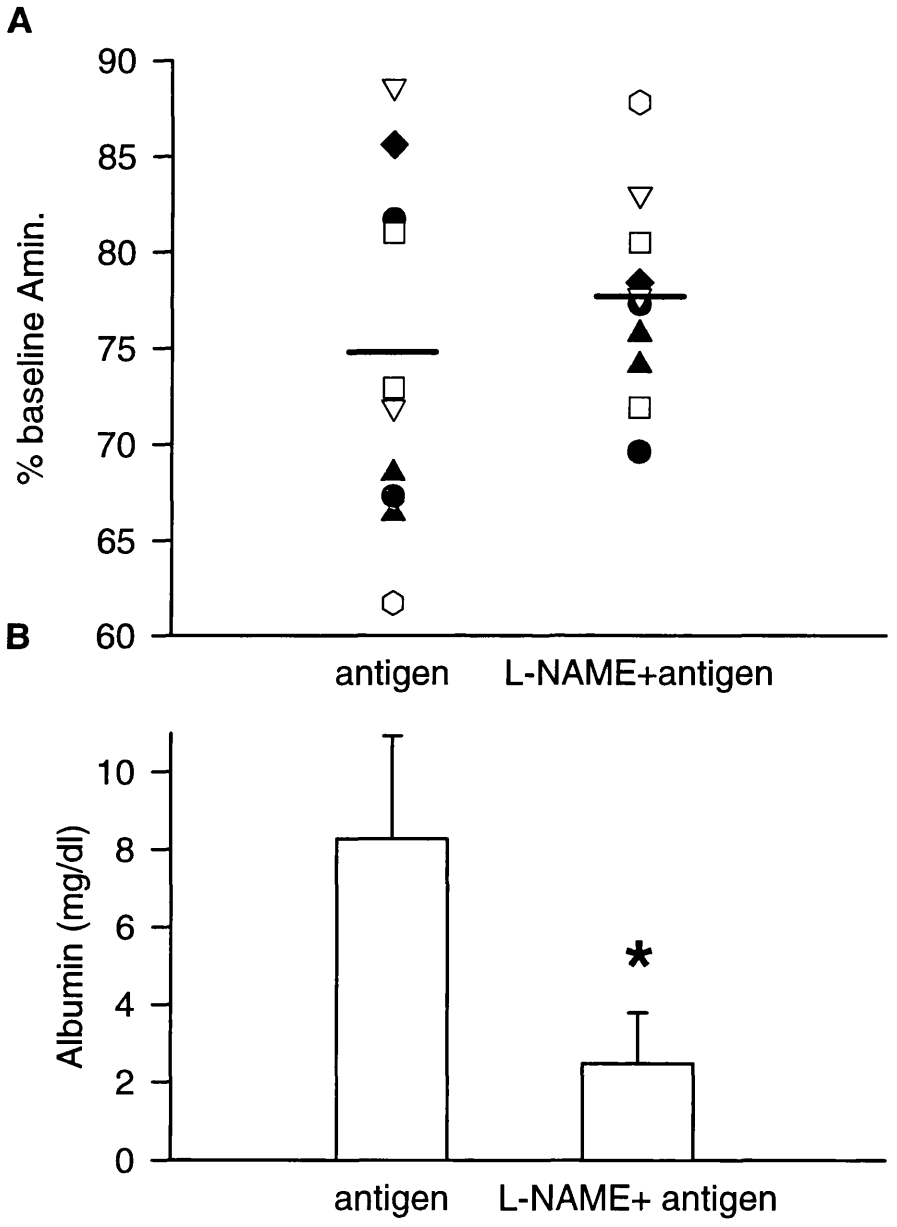

Figure 5.12. The effect of either saline or L-NAME, 1μmol per nostril, given 30 min. prior to antigen challenge (1000 u mixed grass pollens per nostril) on (a) the change in nasal patency (Amin) and (b) the change in albumin release into the nasal airway. In (a) each symbol represents a separate subject and the horizontal bars indicate the mean values which were not significantly different. Data are presented as percentage change from the baseline Amin. The mean ± s.e.m. baseline value of Amin., was 0.56 ± 0.03 cm^2 In (b) the data are given as mean ± s.e.mean albumin concentrations in nasal lavage from the same 10 subjects. * indicates a significant difference compared with antigen ($p<0.02$), analysed by the paired, Wilcoxon sign-rank test.

5.4 Discussion

The data presented in this chapter demonstrate that both bradykinin and histamine cause an increase in the extravasation of human serum albumin into nasal lavage fluid, which is interpreted as an increase in vascular permeability of the nasal mucosal vessels induced by these agents. Extravasation of albumin into the nasal cavity requires an increase in vascular permeability but, in addition, an increase in blood flow into the permeabilized vascular bed will increase the extravasation. For both histamine and bradykinin, the increase in plasma extravasation that they induce was inhibited by the inhibitor of nitric oxide synthase, L-NAME and this inhibition was not observed with D-NAME, indicating that there was a selective effect of L-NAME. L-NAME has been shown to be a low affinity muscarinic receptor antagonist (Buxton *et al.*, 1993) and therefore the inhibition of albumin extravasation could be due to an anticholinergic effect. Previous studies report that the muscarinic receptor antagonists, ipratropium bromide and atropine, have no effect on bradykinin-induced and histamine-induced albumin release, respectively (Rajakulasingam *et al.*, 1992a; Raphael *et al.*, 1989). To provide further evidence that the effect of L-NAME is due to inhibition of nitric oxide synthase, another nitric oxide synthase inhibitor, L-NMMA, was used and it also inhibited bradykinin and histamine-induced albumin extravasation. L-NMMA has been shown to have no affinity for the muscarinic receptor (Buxton *et al.*, 1993). Furthermore, the inhibitory effect of L-NAME on plasma extravasation following histamine or bradykinin was reversed by adding an excess of the substrate for nitric oxide synthase, L-arginine. Together, these data lead to the conclusion that histamine- or bradykinin-induced increase in plasma extravasation into the nasal airway mucosa is secondary, at least in part, to the formation of nitric oxide. It should be noted that inhibition of the basal nitric oxide generation could possibly account for the ability of nitric oxide synthase inhibitors to antagonise bradykinin and histamine-induced increases in albumin extravasation. The release of albumin into the nasal cavity at rest is at the lowest level for detection by the albumin radial immunoassay. Therefore, any reduction in basal albumin release which may be caused by the nitric oxide synthase inhibitors is unmeasurable.

It has been reported that a high dose of the endothelial-independent nitric oxide donor, nitroprusside, inhibits the plasma extravastion induced by nasal challenge with histamine (Greiff *et al.*, 1995). The authors conclude that nitric oxide production inhibits the increase in nasal vascular permeability induced by histamine; a conclusion which contradicts the data presented in this chapter and contradicts the established role of nitric oxide as an agent which increases vascular permeability. However, closer inspection of the data reported by Greiff *et al.*, suggests an alternative explanation which is entirely consistent with the results presented in this chapter. Grieff *et al.* (1995) report that nitroprusside administered to the human nasal cavity at high dose causes nasal congestion and an increase in plasma extravasation. Therefore, nitric oxide causes nasal blockage and an increase in vascular permeability. High concentrations of nitric oxide donors, such as nitroprusside, inhibit the endothelial-dependent generation of nitric oxide via the large amount of nitric oxide formed by these donors having an inhibitory effect on the activity of nitric oxide synthase (Moncada *et al.*, 1991; Buga *et al.*, 1993). Hence, the inhibition of histamine-induced plasma extravasation reported by Grieff *et al.* probably reflects nitroprusside-induced inhibition of the nitric oxide synthase present in the nasal mucosa, a conclusion which is entirely consistent with the data presented in this chapter.

In addition to their actions on nasal vascular permeability, both histamine and bradykinin cause a decrease in the minimal cross-sectional area (Amin.) of the nasal airway, which reflects a decrease in nasal patency, or, in other words, increased nasal blockage. It has been previously shown that a reduction in Amin. caused by histamine or bradykinin is accompanied by an increase in nasal airway resistance (Austin & Foreman, 1994b). In comparison with the vasoconstrictor ephedrine, L-NAME, at a concentration which did affect the responses to bradykinin, did not, by itself, cause an increase in Amin. which suggests that L-NAME is not acting as a vasoconstrictor in the unstimulated nasal airway. It also suggests that nitric oxide is not involved in the regulation of vascular tone in the unstimulated nasal airway. The nasal blockage induced by bradykinin was reversed by L-NAME, but not by D-NAME which we interpret as evidence for a role for nitric oxide formation, at least part, in the nasal blockage caused by bradykinin. Interestingly, histamine-induced nasal blockage was

not influenced by L-NAME, or L-NMMA, which implies that histamine induces nasal blockage by a mechanism different from that for bradykinin and in which nitric oxide is either not involved or is generated by an enzyme which is more resistant to the inhibitors we have used.

One possible explanation for the failure of L-NAME to inhibit histamine-induced reductions in nasal patency, but inhibit increases in vascular permeability, may relate to differences in the type of histamine receptor involved in the two nasal responses. It has been reported that histamine mediates nasal blockage via activation of H_1 and H_2 receptors (Havas *et al.*, 1986), whereas the increase in the permeability of the nasal vessels is mediated by only the H_1 receptor (Raphael *et al.*, 1989). In various tissues, it has been reported that H_1 receptor activation stimulates the production of nitric oxide, but H_2 receptor activation does not (Kelm *et al.*, 1993; Laight *et al.*, 1995). The inability of L-NAME to inhibit histamine-induced reductions in nasal airway patency may therefore reflect the contribution of H_2 receptors to this response.

The nasal blockage induced by bradykinin, although significantly attenuated by L-NAME, is not completely abolished even with the highest dose of L-NAME. This is consistent with observations in other tissues (Cowan & Cohen, 1992; Weldon *et al.*,1995) that a mechanism other than nitric oxide stimulation of cGMP production may account for a substantial proportion of bradykinin-induced vasodilatation. In the nasal airways, aspirin pretreatment has no significant effect on bradykinin-induced albumin or TAME-esterase increases in the lavage, or symptom-scores reported by the subjects (Churchill *et al.*, 1991). Thus, products of the cyclooxygenase pathway appear not to mediate the nasal actions of bradykinin. In the human nasal airway, bradykinin-induced nasal blockage is partly mediated by histamine release (see chapter 6). As histamine-induced reductions in nasal patency are not inhibited by the doses of L-NAME used, the L-NAME resistant component of bradykinin-induced nasal blockage may by mediated by histamine. Alternatively it may be due to a nitric oxide-cGMP independent mechanism which has not yet been characterised in other tissues. Bradykinin causes the release of a diffusible endothelium-derived hyperpolarizing factor (EDHF), which can induce hyperpolarisation and relaxation of

vascular smooth muscle in the human coronary artery (Mombouli & Vanhoutte, 1995). The exact nature of EDHF is unknown, but it appears to cause hyperpolarisation and relaxation of vascular smooth muscle by activating potassium channels. The vascular relaxations mediated by EDHF are not antagonised by inhibitors of nitric oxide synthase or cyclooxygenase (Mombouli & Vanhoutte, 1995b). EDHF generation could contribute to the L-NAME resistant component of the reduction in nasal patency induced by bradykinin.

The inhibition of bradykinin-induced reductions in nasal patency by L-NAME is not significantly reversed by adding excess L-arginine. The dose of L-arginine used reversed the L-NAME inhibition of bradykinin- and histamine- induced increases in albumin extravasation into the nasal cavity. The failure of L-arginine to reverse the effect of L-NAME on bradykinin-induced reductions in Amin. may be explained by the dose used being too low, L-arginine failing to gain access to the enzyme or the inhibition by L-NAME being the result of its activity as a muscarinic receptor antagonist. This last explanation is unlikely as L-NMMA, a nitric oxide synthase inhibitor with no affinity for the muscarinic receptor, also inhibits bradykinin-induced reductions in nasal patency and previous studies have shown that bradykinin-induced increases in nasal airway resistance are unaffected by pretreatment with the muscarinic receptor antagonist, ipratropium bromide (Rajakulasingam *et al.*, 1992a).

Changes in nasal airway patency result from the operation of several mechanisms. The nasal airway contains cavernous sinusoids analogous to the erectile tissue of the penis, and an increase in blood flow into these sinusoids produces a rapid reduction of the volume of the nasal cavity with resultant reduction in air flow. In addition, serous and mucous secretion into the nasal cavity reduce its patency. Both bradykinin and histamine induce this secretion (Baraniuk *et al.*,1990b; Raphael *et al.*, 1989) and differences in their potencies may reflect the inability of L-NAME to attenuate histamine-induced nasal blockage. As already discussed the inability of L-NAME to attenuate histamine-induced reductions in the patency of the nasal airway may reflect the contribution of the histamine H_2 receptor. The patency of the nasal cavity is also reduced by swelling of the nasal mucosa resulting from increased microvascular

permeability and increased blood flow. The data show that for both bradykinin and histamine the increased plasma extravasation in the nasal mucosa is nitric oxide-mediated. However, the actions on plasma extravasation are not a sole explanation for the increase in nasal blockage induced by these agents since nasal blockage induced by bradykinin involves nitric oxide formation while that induced by histamine does not.

In addition to the presence of endothelial and inducible nitric oxide synthase being reported in the human nasal mucosa, neuronal nitric oxide synthase is also present in the nerves of the human nasal mucosa (Kulkarni *et al.*,1994). In the canine nasal mucosa, *in vitro*, nitric oxide mediates non-adrenergic, non-cholinergic vasodilatation (Watanabe *et al.*, 1995). However, the application of the local anaesthetics, lignocaine and benzocaine, to the human nasal mucosa fails to inhibit nasal blockage or albumin extravasation induced by either histamine or bradykinin. This is consistent with other studies which have failed to show any role for nerves in the nasal blockage and increase in vascular permeability induced by bradykinin or histamine. It appears, therefore, that nasal blockage and plasma extravasation in response to histamine and bradykinin does not involve activation of nerves.

The reduction in the patency of the human nasal airway and the extravasation of plasma albumin caused by bradykinin is mediated, in part, by the release of nitric oxide. Histamine-induced nasal blockage appears not to involve the generation of nitric oxide whereas its action on plasma extravasation does. Both L-NAME and L-NMMA inhibit all the isoforms of nitric oxide synthase and therefore the location and type of nitric oxide synthase involved in the nasal actions of bradykinin and histamine remains to be determined. As local anaesthetics do not affect the nasal blockage or plasma extravasation induced by bradykinin or histamine, neuronal nitric oxide synthase appears not to be involved in the response. The development of selective nitric oxide synthase inhibitors suitable for use in humans is required to determine the isoform of nitric oxide synthase involved in the responses of the human nasal airway.

Chapter 3 presents data showing that the reduction in nasal airway patency induced by house dust mite challenge in sensitive atopic subjects is significantly antagonised by the bradykinin B_2 receptor antagonist, icatibant. L-NAME pretreatment produces a small, but significant reduction in the nasal blockage induced by house dust mite challenge of subjects with perennial allergic rhinitis. This is consistent with bradykinin producing a reduction in nasal patency via nitric oxide generation. Following house-dust mite challenge, there is an increase in nitric oxide generation in the nasal cavity (Garrelds *et al.*, 1995), which further supports a role for nitric oxide in house-dust mite induced nasal allergy. As with previous studies (Austin *et al.*, 1994), house dust mite challenge failed to increase the albumin extravasation into the nasal cavity. The reduction of nasal airway patency induced by challenge with grass pollen in subjects with seasonal allergic rhinitis is not mediated by bradykinin as icatibant does not antagonise grass-induced nasal blockage (see chapter 3). A number of studies also demonstrate that a range of chemically different histamine H_1 receptor antagonists fail to antagonise the increase in nasal airway resistance induced by grass pollen when measured quantitatively by rhinomanometry (Corrado *et al.*, 1987; Hamilton *et al.*, 1994; Thomas *et al.*, 1992). This chapter shows that L-NAME, 1μmol, also fails significantly to inhibit the reduction in nasal patency induced by grass pollen challenge in sensitive, atopic subjects. Whether a higher dose of L-NAME would antagonise this nasal blockage remains undetermined. Leukotrienes have recently been implicated in grass-induced nasal blockage as 5-lipoxygenase inhibits reduce grass pollen induced nasal blockage (Howarth *et al.*, 1995). In the future, it would be interesting to investigate the effect of nitric oxide synthase inhibitors on leukotriene-induced reductions in nasal patency.

The increase in vascular permeability induced by grass pollen is antagonised by histamine H_1 receptor antagonists (Shin *et al.*, 1992; Naclerio *et al.*, 1990). This increase in albumin extravasation is not antagonised by pretreatment with the bradykinin B_2 receptor antagonist, icatibant, indicating bradykinin is not a mediator of grass pollen-induced increases in nasal vascular permeability (see chapter 3). Consistent with histamine mediating the albumin extravasation induced by challenge with grass pollen, L-NAME significantly inhibits the albumin release induced by grass

challenge as it does with albumin released by histamine challenge. Studies investigating the effect of D-NAME, L-NMMA and L-arginine on the reduction in nasal patency and albumin extravasation induced by antigen were precluded by the limited availability of atopic subjects. However, as the effects of L-NAME on antigen challenge are consistent with its effect on the nasal actions of bradykinin and histamine, it is reasonable to assume that the actions of L-NAME on experimentally-induced seasonal and perennial allergic rhinitis are a result of inhibition of nitric oxide synthase. Also, as atropine is reported to have no effect on antigen-induced albumin release or nasal congestion (Naclerio & Baroody, 1995), the low affinity of L-NAME for the muscarinic receptor is unlikely to contribute to the effects of L-NAME on antigen challenge. Therefore, the reduction in nasal patency induced by house dust mite challenge and the increase in vascular permeability induced by grass pollen challenge, in subjects with perennial and seasonal allergic rhinitis respectively, are dependent upon the generation of nitric oxide.

Summary

- The effects of inhibitors of nitric oxide synthase and local anaesthetics were studied on changes in human nasal airway patency and albumin extravasation in response to bradykinin, histamine and antigen, *in vivo*.

- Compared with the action of the vasoconstrictor, ephedrine, 2.5 μmol, N^G-nitro-L-arginine methyl ester (L-NAME), 1 μmol, alone did not change the resting value of the minimal cross-sectional area (Amin.) of the human nasal airway. L-NAME, 0.1 to 10 μmol, produced a dose-related inhibition of the reduction in Amin. caused by bradykinin, 300 μg. N^G-monomethyl-L-arginine (L-NMMA), 1 μmol, similarly reduced the effect of bradykinin, 300 μg, on Amin., but N^G-nitro-D-arginine methyl ester (D-NAME), had no effect. L-NAME, 0.1 to 10 μmol or L-NMMA, 10μmol failed to inhibit the effect of histamine, 300 μg, on Amin.

- The inhibition by L-NAME, 1 μmol, of the action of bradykinin, 300 μg, on Amin. was maximal between 15 and 30 min after pretreatment with L-NAME.

- L-NAME, 1 and 10 μmol, inhibited the extravasation of albumin into the nasal cavity induced by bradykinin, 300 μg, and also by histamine, 300 μg. L-NMMA, 1 and 10μmol, respectively, also inhibited the extravasation of albumin into the nasal cavity induced by bradykinin, 300 μg, and by histamine, 300 μg. D-NAME, 1 and 10 μmol, had no effect on the extravasation of albumin in response to bradykinin or histamine.

- L-arginine, 30 μmol, reversed the effect of L-NAME, 1 μmol, on the bradykinin- and histamine-induced albumin extravasation into the nasal airway.

- Local anaesthesia of the nasal airway with lignocaine, 10 mg, or benzocaine, 10mg, failed to inhibit the reduction in Amin. or the albumin extravasation induced by either bradykinin, 300 μg, and histamine, 300 μg.

- In conclusion, the extravasation of plasma albumin caused by bradykinin and by histamine involves the generation of nitric oxide. The nasal blockage induced by bradykinin involves nitric oxide generation but the nasal blockage induced by histamine does not.

- In addition, L-NAME, 1μmol, inhibited the increase in albumin extravasation induced by grass pollen antigen challenge of subjects with seasonal allergic rhinitis. The reduction in the patency of the nasal airway, induced by grass pollen antigen challenge, was unaffected by pretreatment with L-NAME. The reduction in the patency of the nasal airway induced by challenging subjects with perennial allergic rhinitis with house dust mite antigen is significantly inhibited by pretreatment with L-NAME, 1μmol. Therefore, the reduction in nasal airway patency induced by house dust mite antigen and the albumin extravasation induced by grass pollen antigen are partly mediated by the production of nitric oxide.

CHAPTER 6

THE CONTRIBUTION OF HISTAMINE TO THE ACTION OF BRADYKININ IN THE HUMAN NASAL AIRWAY

6.1 Introduction

Nasal challenge of allergic individuals with allergen results in the appearance of kinins in nasal lavage fluid (Proud *et al.*, 1983). Such kinin generation correlates with the onset of symptoms and with the presence of other inflammatory mediators, particularly those derived from mast cells, such as histamine and prostaglandin D_2. Intranasal administration of bradykinin results in rhinorrhoea and nasal blockage (Proud *et al.*, 1988; Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a) and these effects have been attributed to a direct, receptor-mediated action, probably involving the B_2 kinin receptor (Rajakulasingam *et al.*, 1991; Austin & Foreman, 1994a). However, kinins may also have indirect effects, and receptor-independent mechanisms have been proposed for the actions of kinins in guinea-pig trachea (Mizrahi *et al.*, 1982) and rat peritoneal mast cells (Devillier *et al.*, 1988).

Bradykinin and kinin analogues release histamine from rodent mast cells. This effect appears not to be receptor-mediated since kinin antagonists also stimulate histamine release (Devillier *et al.*, 1988). Positive charges at the N-terminal end of the amino acid sequence appear to be crucial for histamine release (Devillier *et al.*, 1989). The histamine release is not a result of cytotoxicity and is dose-dependently inhibited by pertussis toxin or benzalkonium chloride (Bueb *et al.*, 1990b). Bradykinin stimulates the GTPase activity of purified G-proteins and, therefore, it has been proposed that bradykinin releases histamine by direct activation of a pertussis-toxin sensitive G-protein (Mousli *et al.*, 1991). Analogues of bradykinin have also been shown to release histamine from human skin mast cells (Cohan *et al.*, 1991).

The role of histamine in bradykinin-induced nasal blockage and plasma extravasation is controversial. Rajakulasingam *et al.* (1992) reported that an orally administered H_1 receptor antagonist, terfenadine, produced no effect on bradykinin-induced increases

in nasal airway resistance, rhinorhoea, nasal pain or the albumin level in the nasal lavage. However, Austin (1994) showed that two chemically different H_1 receptor antagonists, terfenadine and cetirizine, both attenuate bradykinin-induced nasal blockage as measured by active posterior rhinomanometry. Bradykinin-induced increases in nasal vascular permeability, quantified by lavage albumin levels, were also attenuated by cetirizine. These studies were not placebo controlled or double-blind. Bradykinin challenge does not, however, increase the release of histamine into the nasal lavage (Austin, 1994).

The studies described in this chapter determine whether the histamine H_1 receptor antagonist, cetirizine, antagonises bradykinin-induced nasal blockage as measured by acoustic rhinometry in a double-blind, placebo controlled study. Furthermore, the ability of bradykinin to release histamine from *ex vivo* human nasal tissue was investigated

6.2 Experimental protocol

6.2.1 Protocol for human nasal challenge experiments

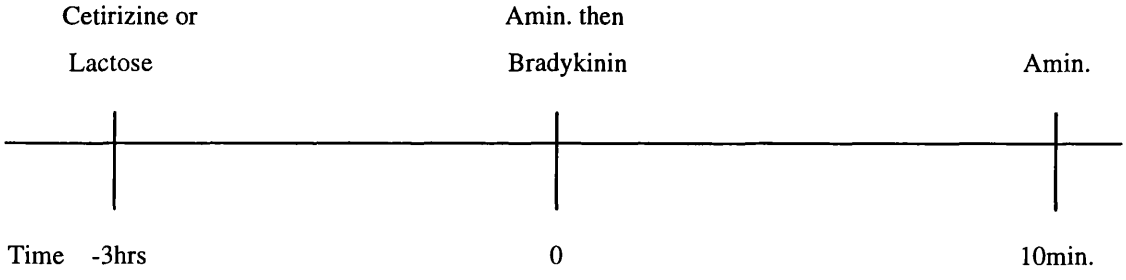

Figure 6.1. Protocol for investigating the effect of cetrizine on bradykinin-induced reductions in the patency of the human nasal airway.

A double-blind, cross-over study of the effect of cetirizine on the action of bradykinin on the minimal nasal cross-sectional area (Amin.) was performed. Subjects were given either placebo (lactose) or cetirizine (10mg), orally. The first treatment received was determined randomly. Three hours later the minimum cross-sectional area of the nasal cavity (Amin.) was determined, then the subjects were challenged with bradykinin,

100μg. Ten minutes after challenge the Amin. was redetermined. On a different day the protocol was repeated with subjects receiving the treatment (placebo or cetirizine) not administered previously. An identical study was performed with bradykinin, 300μg. There were at least three days between treatments. The protocol is summarised in figure 6.1.

6.2.2 Protocol for histamine release experiments from human nasal tissue

Human nasal tissue was obtained from turbinectomy operations; the inferior turbinate bone being removed to relieve chronic nasal obstruction. Soft tissue was dissected free from the turbinate bone, weighed and chopped finely with scissors. The tissue fragments were incubated in 100ml of Tyrode solution containing collagenase type 4, 40mg together with bovine serum albumin, 100mg, for 1 hour at 37°C with gentle stirring (Pearce *et al.*, 1987). The tissue was then expressed through a syringe and the cell suspension filtered through gauze to remove coarse fragments. The cell suspension was centrifuged at 200g for 5 minutes at 4^0C and then resuspended in 20ml: this process being repeated three times to wash the cells. The cells were finally resuspended in the volume required to give the necessary number of samples for the experiment. The yield of samples was about 10 per g wet weight of tissue and each sample contained approximately 2 nmols of histamine.

Aliquots of cell suspension of 450μl were warmed to 37°C for 5 minutes and then the stimuli of histamine release were added, at a concentration needed to give the desired final concentration, in a volume of 50μl: control samples receiving 50μl of Tyrode solution. Incubation was continued for 10 minutes and then 1.5ml of ice-cold Tyrode solution was added to stop any continuing reaction. The cells were centrifuged at 200g for 5 minutes at 4^0C and the supernatants retained for histamine assay. The pellets were resuspended in 2ml of Tyrode solution and boiled for 5 minutes to release the residual cell histamine. Residual and released histamine were analysed by the flurometric method on an autoanalyser (Evans *et al.*, 1973) together with histamine standards.

6.2.3 Data analysis

Changes in Amin. are expressed as a percentage of the baseline (pre-bradykinin challenge) Amin. Histamine release from human nasal tissue is expressed as a percentage of the total cell histamine for each sample.

All data is expressed as mean ± s.e.m. The data is statistically analysed by the non-parametric Wilcoxon sign-rank test or unpaired student t-test.

6.3 Results

6.3.1 The effect of cetirizine on bradykinin-induced decreases in nasal patency

The data from the double-blind, randomised, cross-over experiment to demonstrate the effect of cetirizine on the response to bradykinin are shown in Figure 6.2. Bradykinin, 100μg and 300μg caused a significant (Wilcoxon paired test, n=5, $p<0.05$) decrease in the minimum cross-sectional area of the nasal cavity (Amin.). Cetirizine significantly reduced the decrease in Amin. induced by bradykinin, 300μg (Wilcoxon paired test, n=5, $p<0.05$), but failed to significantly reduce the decrease in Amin. induced by bradykinin, 100μg (Wilcoxon paired test, n=5, $p>0.05$).

6.3.2 The effect of bradykinin on histamine release from ex vivo human nasal tissue

Bradykinin induced a significant (unpaired t-test, $p<0.05$) release of histamine from human isolated nasal mast cells at concentrations of 33 and 100μM (Figure 6.3). Histamine release induced by anti-IgE and calcium ionophore A23187 were incorporated as positive controls (unpaired t-test, $p<0.05$).

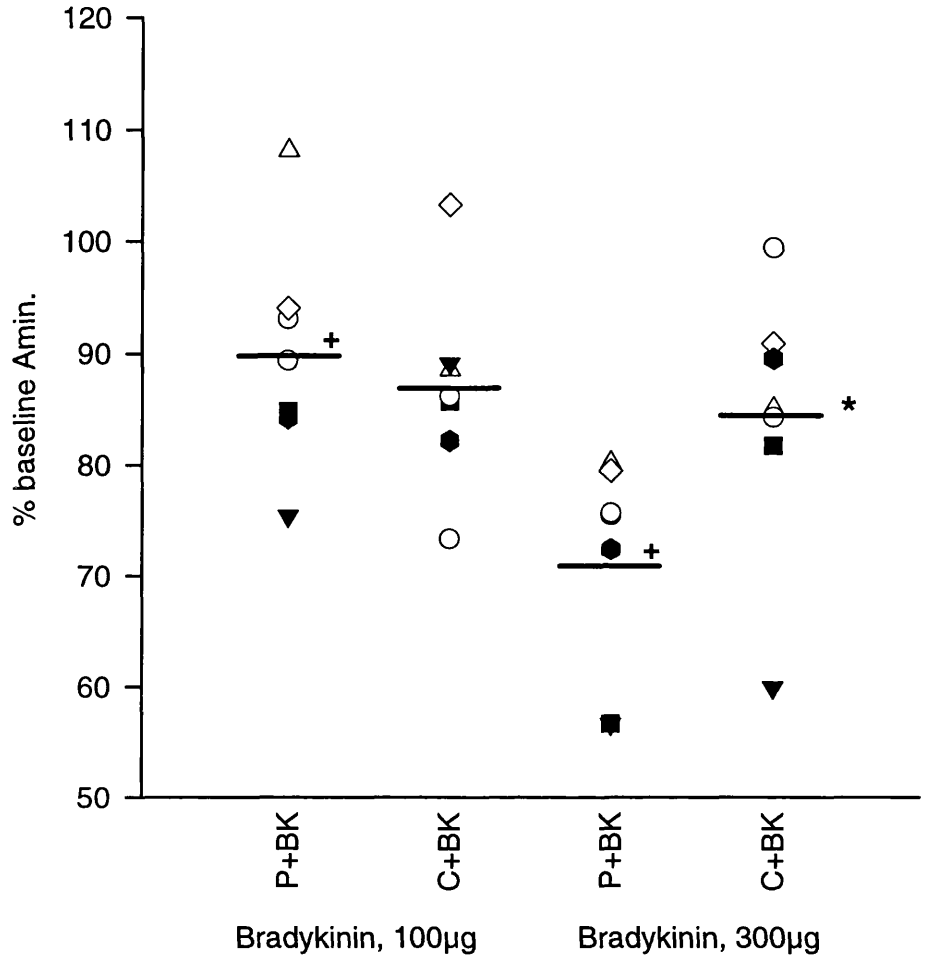

Figure 6.2. Effect of cetirizine (C) or placebo (P) on the change in minimal cross-sectional area (Amin.) of the nasal cavity induced by bradykinin (BK). The data are from two randomised, double-blind, cross-over studies: one where bradykinin, 100µg was used and the other for bradykinin, 300µg. Five subjects participated in each study. The baseline Amin. values were: 0.34cm^2 for placebo and bradykinin, 100µg; 0.59cm^2 for cetirizine and bradykinin, 100µg; 0.60cm^2 for placebo and bradykinin, 300µg; 0.49cm^2 for cetirizine and bradykinin, 300µg. Each symbol represents a different subject and the horizontal bars are the means. * indicates a statistically significant difference between placebo and cetirizine (Wilcoxon paired test, $p<0.05$) + indicates a significant difference between bradykinin and baseline (saline) (Wilcoxon paired test, $p<0.05$).

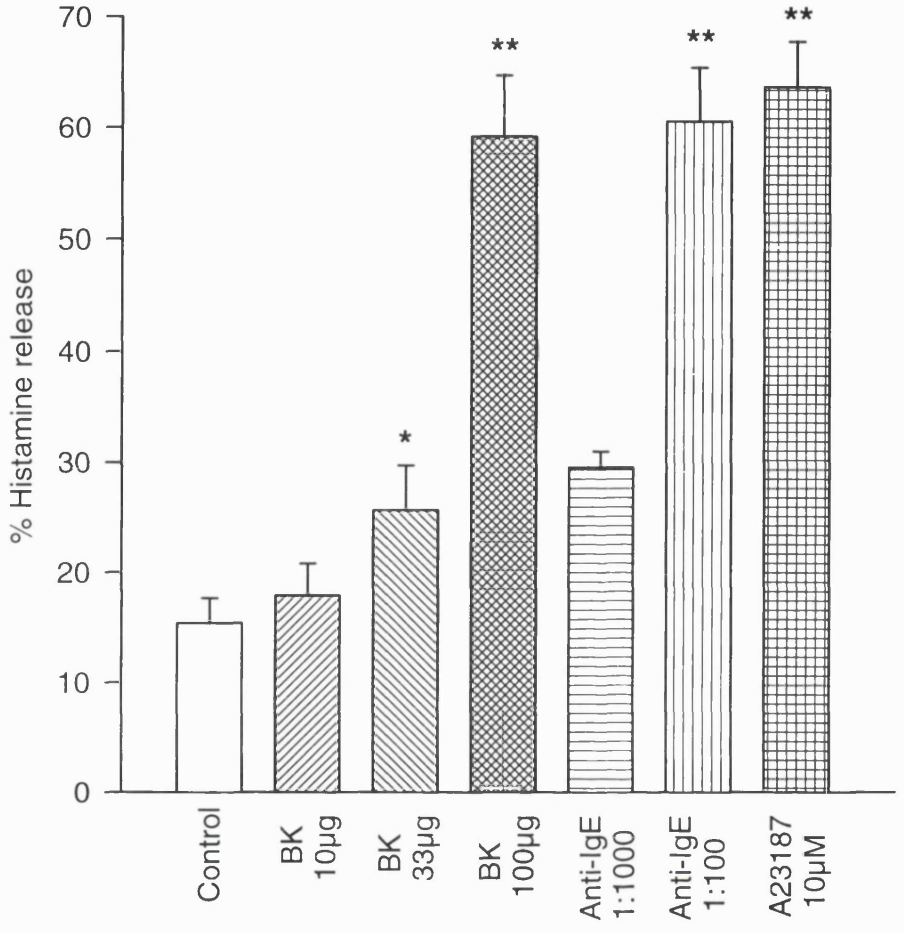

Figure 6.3. Histamine release from human isolated nasal cells *in vitro* by bradykinin (BK), anti-IgE and the calcium ionophore, A 23187. Data are the means ± s.e.mean from 3 different tissues with triplicate samples. * ($p<0.05$) and ** ($p<0.01$) indicate statistically significant differences from the unstimulated (control) release by unpaired t-test.

6.4 Discussion

As previously demonstrated, intranasally administered bradykinin induces a reduction of nasal patency. Nasal patency is also reduced by administering histamine intranasally, an effect which is antagonised by histamine H_1 receptor antagonists (Britton *et al.*, 1978; Austin & Foreman, 1994b; Horak *et al.*, 1993; Braunstein *et al.*, 1992) and H_2 receptor antagonists (Havas *et al.*, 1986). As already discussed, bradykinin stimulates histamine release from rodent mast cells (Ishizaka *et al.*, 1985; Devillier *et al.*, 1988).

This double-blind, placebo-controlled study shows that the effect on nasal patency of bradykinin, 300μg, is inhibited by the H_1 receptor antagonist of histamine, cetirizine, used at a standard therapeutic dose. The same effect is observed when nasal airway resistance is assessed by active posterior rhinomanometry in the place of acoustic rhinometry (Austin, 1994). This previous study demonstrated that both cetirizine and terfenadine antagonise the increases in nasal airway resistance induced by bradykinin at 500 and 1000μg. The increase in symptoms associated with bradykinin administration, 1000μg, are also partially inhibited by the H_1-receptor antagonist, cetirizine (Austin, 1994). Furthermore, cetirizine inhibited the release of albumin into the nasal cavity caused by bradykinin, 1000μg (Austin, 1994). It should be noted that the studies by Austin (1994) were not double-blind or placebo controlled, but the results in this chapter corroborate the earlier findings. The data suggest, therefore, that bradykinin, at higher doses, may produce some of its nasal actions by the release of histamine, presumably from nasal mast cells or basophils. Histamine increases nasal airway resistance partly by an action on H_1 receptors in the nasal airway (Britton *et al.*, 1978) and this effect can be blocked by various H_1-receptor antagonists, including terfenadine and cetirizine (Austin & Foreman, 1994b; Horak *et al.*, 1993; Braunstein *et al.*, 1992). However, in a previous study of the involvement of histamine in bradykinin-induced effects in the human nasal airway, terfenadine had no effect on bradykinin-induced increases in nasal airway resistance (Rajakulasingam *et al.*, 1992a). The discrepancy between that study and this one is difficult to explain, but we have shown that the part played by histamine is dependent on the dose of bradykinin.

Also, terfenadine is less potent than cetirizine (Campoli-Richards *et al.*, 1990). This study shows that bradykinin-induced reductions in nasal patency can be partially inhibited by cetirizine pretreatment. An H_1 receptor-independent effect of cetirizine is unlikely as two chemically unrelated histamine H_1 receptor antagonists, terfenadine and cetirizine, both antagonise bradykinin-induced increases in nasal airway resistance (Austin, 1994).

Bradykinin causes histamine release from rat peritoneal and mouse mast cells *in vitro* (Johnson *et al.*, 1973; Ishizaka *et al.*, 1985) . It does not, however, activate human blood basophils or human lung mast cells *in vitro* (Lawrence *et al.*, 1989), but there is indirect evidence that it causes histamine release from human skin mast cells (Li Kam Wa *et al.*, 1992). It has been shown that bradykinin-induced paw oedema in the mouse is completely inhibited by the H_1-antagonist, mepyramine (Wiseman *et al.*, 1994), and that histamine antagonists decrease bradykinin-evoked vascular permeability in rabbit skin (Marceau *et al.*, 1981). In human skin, there is evidence that the flare response to intradermal bradykinin injection is mediated by histamine (Wallengren & Hakanson, 1992), as mepyramine and compound 48/80 significantly reduce the bradykinin-induced flare. However, other studies have failed to replicate this finding (Polosa *et al.*, 1993).

The action of the histamine antagonists on the nasal effects of bradykinin suggest that bradykinin may release histamine in the human nose. However, even at the highest dose of bradykinin given, there is no detectable increase of histamine in the nasal lavage fluid (Austin, 1994). The discrepancy between the effect of H_1 receptor blockade on bradykinin-induced changes in nasal airway resistance and the lack of histamine release into nasal lavage, could be explained by the clearance, into the venous circulation, of histamine released from tissue mast cells or basophils. To test this explanation, the action of bradykinin on histamine release from cells isolated from human nasal tissue was studied. Bradykinin caused a significant release of histamine from the suspension of dispersed human nasal tissue at 33μM and 100μM concentrations. Therefore, *in vitro,* bradykinin releases histamine from human nasal tissue, but only at relatively high concentrations. This is consistent with cetirizine only

inhibiting bradykinin-induced decreases in nasal patency at the higher bradykinin doses. However, as the concentration of bradykinin at the nasal mast cells *in vivo* is unknown, it is only possible to speculate as to the relationship between the histamine release observed in *ex vivo* nasal tissue and the actions of cetirizine on bradykinin-induced nasal blockage. The mechanism by which bradykinin causes histamine release from human nasal tissue is undetermined. The effects of bradykinin receptor antagonists on histamine release will reveal whether this release is receptor-mediated, or receptor-independent as in rodent mast cells. Another possible mechanism by which bradykinin may stimulate histamine release in human nasal tissue is via c-fibre activation and release of neuropeptides, as substance P has been demonstrated to release histamine from cultured human nasal mucosal cells *in vitro* (Schierhorn *et al.*, 1995). However, this mechanism is improbable as the concentration of substance P needed to release histamine is unlikely to be achieved using *ex vivo* dispersed nasal tissue and *in vivo* nasal challenge with a range of doses of substance P appears to cause nasal blockage by a histamine-independent mechanism (Braunstein *et al.*, 1994).

These results indicate that bradykinin releases histamine from human nasal tissue and this plays a role in bradykinin-induced nasal blockage, at least at the higher doses of bradykinin. The previous failure to show increased histamine concentrations in the nasal wash following bradykinin challenge may be explained by the venous circulation removing the released histamine before it enters the nasal cavity.

Summary

- Bradykinin caused a dose-dependent reduction in the patency of the human nasal airway. Pretreatment with cetirizine (10mg orally) inhibited the reduction in nasal airway patency induced by bradykinin, 300μg, but not by bradykinin, 100μg.

- Isolated human nasal cells released histamine in response to bradykinin, 33 and 100μM, anti-IgE and calcium ionophore, A23187.

- In conclusion, the actions of bradykinin in the human nasal airway are, in part, accounted for by the release of histamine. Histamine only appears to contribute to the nasal actions of bradykinin at the higher range of bradykinin dose.

CHAPTER 7

THE CONTRIBUTION OF KININS TO PLATELET ACTIVATING FACTOR-INDUCED HYPERRESPONSIVENESS OF THE HUMAN NASAL AIRWAY

7.1 Introduction

Administration of platelet activating factor (PAF) into the human nasal airway induces a non-specific nasal hyperresponsiveness which is one of the features of allergic rhinitis. The response to histamine, bradykinin and grass pollen antigen is significantly enhanced if the nasal cavity is pretreated with PAF (Andersson & Pipkorn, 1988; Austin & Foreman, 1993). Nasal challenge with PAF causes an increase in the numbers of eosinophils in the nasal cavity (Klementsson & Andersson, 1992; Tedeschi *et al.*, 1994b) and this nasal eosinophillia is accompanied by release of eosinophil cationic protein (ECP) (Austin & Foreman, 1993; Tedeschi *et al.*, 1994a), a product of eosinophil activation. The time-course of PAF-induced hyperresponsiveness is associated with an increase in ECP in the nasal lavage fluid; implicating eosinophil activation in the development of PAF-induced hyperresponsiveness (Austin & Foreman, 1993). Eosinophil activation causes the release of the toxic protein, major basic protein (MBP), which is capable of causing hyperresponsiveness of the lower airways in both primates and rodents (Gundel *et al.*, 1991).

Recently, it has been reported that MBP-induced hyperresponiveness in the lower airway of the rat is dependent upon the generation of bradykinin (Coyle *et al.*, 1995). Intratracheal administration of MBP increased the production of kinins in the rat lower airways and the airway hyperresponsiveness induced by MBP was inhibited by the bradykinin B_2 receptor antagonist, NPC 17713 (Coyle *et al.*, 1995).

Therefore, this chapter investigates the contribution of kinins to the human nasal airway hyperresponsiveness induced by platelet activating factor.

7.2 Experimental protocol

Normal, healthy, non-atopic human subjects were used in all the studies presented in this chapter.

7.2.1 The effect of icatibant on platelet activating factor-induced nasal hyperresponsiveness

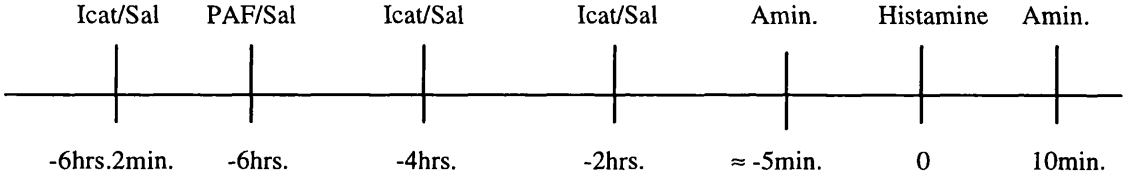

Figure 7.1. Protocol for investigating the effect of icatibant on PAF-induced nasal hyperresponsiveness by measuring the reduction in nasal patency induced by histamine. Icat = icatibant, Sal = saline.

The study measured the change in the patency of the human nasal airway induced by nasal challenge with histamine. By intranasal administration, each subject received either icatibant, 200μg, or saline (see figure 7.1). As the duration of action of icatibant as a bradykinin antagonist in the human nasal airway is only approximately 2 hours (see figure 3.6), subjects received either saline or icatibant again 2 and 4 hours after the initial administration. Two minutes after the first treatment with icatibant or saline, subjects received either PAF, 60μg or saline. Six hours after challenge with PAF or saline subjects were challenged with histamine, 200μg. Previous studies have demonstrated a significant increase in the responsiveness of the human nasal airway six hours after challenge with PAF, 60μg (Austin & Foreman, 1993). Immediately prior to, and 10min. after histamine challenge, the subject's minimum nasal cross-

sectional area (Amin.) was measured using acoustic rhinometry. The protocol is summarised in figure 7.1.

Using the same protocol as above, the effect of icatibant on PAF-induced hyperresponsiveness was again studied, but the nasal challenge agent was substance P instead of histamine. The minimum nasal cross-sectional area was measured immediately prior to challenge with substance P, 33μg, and 2, 5 and 10min. after challenge. The dose of 33μg of substance P was chosen as previous studies show that this dose produces an increase in the resistance of the nasal airway approximately equal to the increase induced by histamine, 200μg (Lurie *et al.*, 1994).

In both the studies with histamine and substance P, each subject received all the four combinations of treatment, i.e.:

- Icatibant, 200μg, (three administrations) and PAF, 60μg, (one administration)
- Icatibant, 200μg, (three administrations) and saline (one administration).
- Saline (three administrations) and PAF, 60μg (one administration).
- Saline (three administrations) and saline (one administration).

Each combination was administered on separate occasions at least two days apart. Previous studies show that the nasal hyperresponsiveness induced by PAF persists less than 48 hours (Austin, 1994). The order of treatments was determined randomly.

7.2.2 The effect of PAF on kinin generation in the human nasal airway

Subjects received either PAF, 60μg, or saline, delivered intranasally. Five minutes prior to treatment three nasal lavages were performed: the first two were discarded, and the third lavage retained to form the baseline measurement. Nasal lavages were also performed 2, 4 and 6 hours after treatment with saline or PAF. This protocol is summarised in figure 7.2.

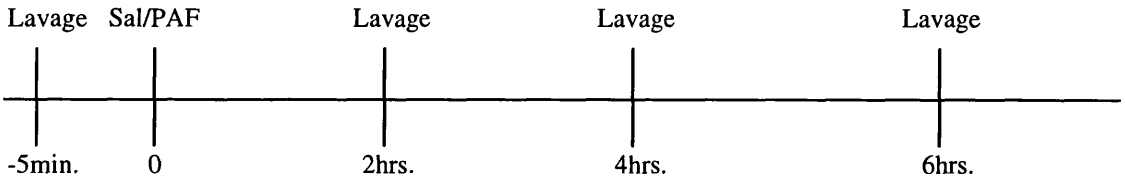

Figure 7.2. Protocol for investigating the effect of PAF on kinin generation in the human nasal airway. Sal = saline.

Each subject received both saline and PAF on separate occasions at least 2 days apart. The order of treatments was determined randomly.

The nasal lavages were assayed for their kinin content. EDTA, 40mM was added and the lavages were stored on ice, then at -70^{0}C until analysis (Proud *et al.*, 1983). The kinin content of the nasal lavage was determined using a commercially available bradykinin radioimmunoassay kit, the antiserum used for this assay was raised in the rabbit. [^{125}I]-bradykinin was used as the radioisotope and the range of sensitivity of this assay was 1-128 pg of kinin per tube. The assay has equal sensitivity for bradykinin and kallidin.

7.2.3 Data analysis

The dimensions of the nasal airway vary between subjects and also within subjects from day to day and so the data have been normalized by expressing changes in Amin. as a percentage of the baseline control value. The absolute values for the baseline measurements have been given with each set of data. Means are given together with standard error of the mean (s.e.mean). The appropriate, non-parametric statistical test is given with each data set. A value of $p<0.05$ is taken as significant.

7.3 Results

7.3.1 The effect of icatibant on platelet activating factor-induced nasal hyperresponsiveness

Figure 7.3 shows that histamine, 200μg, produced a significant reduction in the minimal cross-sectional area (Amin.) of the human nasal airway when the airway is pretreated with only saline ($p<0.05$, analysed by the Wilcoxon sign-rank test). This reduction in Amin. was not significantly different from the reduction in Amin. induced by histamine with icatibant, 200μg and saline pretreatment ($p>0.05$, analysed by the Wilcoxon sign-rank test). The reduction in Amin. induced by histamine, with saline only pretreatment, was significantly less than the reduction induced by histamine with saline and PAF, 60μg, pretreatment ($p<0.05$, analysed by the Wilcoxon sign-rank test). The histamine-induced reduction in Amin., after saline and PAF pretreatment, was significantly greater than the reduction following pretreatment with icatibant, 200μg, and PAF, 60μg ($p<0.02$, analysed by the Wilcoxon sign-rank test).

The data presented in figure 7.4 represents the reduction in Amin. induced by substance P, 33μg, with only saline pretreatment. Substance P, 33μg, caused a significant reduction in Amin. at 2 and 5 min. after administration ($p<0.05$, analysed by the Wilcoxon sign-rank test). Ten minutes after substance P challenge, there was no significant reduction in Amin. ($p>0.07$, analysed by the Wilcoxon sign-rank test). The data presented in figure 7.5 represents the percentage of baseline Amin. 2, 5 and 10min. after substance P, 33μg, challenge, with various pretreatments. Figure 7.5 shows that the reductions in Amin. induced by substance P following pretreatment with saline and PAF, 60μg; icatibant, 200μg, and PAF 60μg; and saline and icatibant were not significantly different to the reduction in Amin. induced by substance P with only saline pretreatment (for all treatments $p>0.1$, analysed by the Wilcoxon sign-rank test).

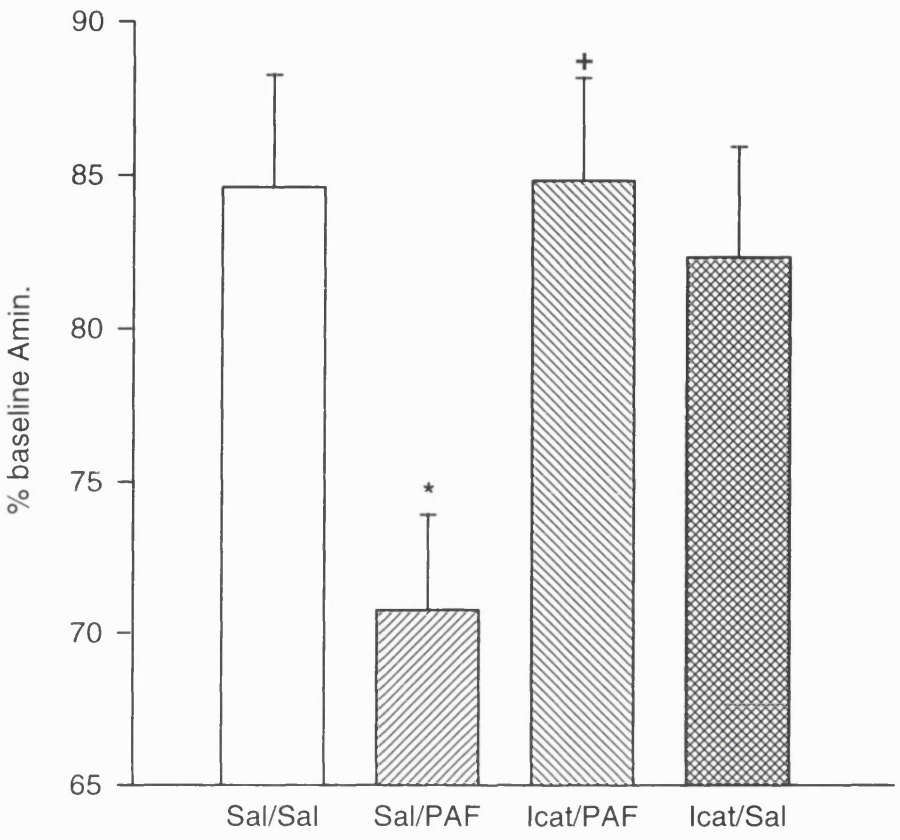

Figure 7.3. The percentage change in the minimum cross-sectional of the nasal airway (Amin.) after challenge with histamine, 200µg. Amin. was measured immediately prior to and 10min. after challenge. The nasal cavity was pretreated with saline (Sal), icatibant (icat), 200µg and/or PAF, 60µg, as described in the experimental protocol. Data are means from 8 subjects. Vertical bars represent s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.52±0.01 cm^2. * = significant increase in the histamine-induced % change in Amin. following saline/PAF pretreatment ($p<0.05$, analysed by the Wilcoxon sign-rank test). + = significant difference in the histamine-induced % change in Amin. when pretreatment with saline/PAF is compared to icatibant/PAF ($p<0.02$, analysed by the Wilcoxon sign-rank test).

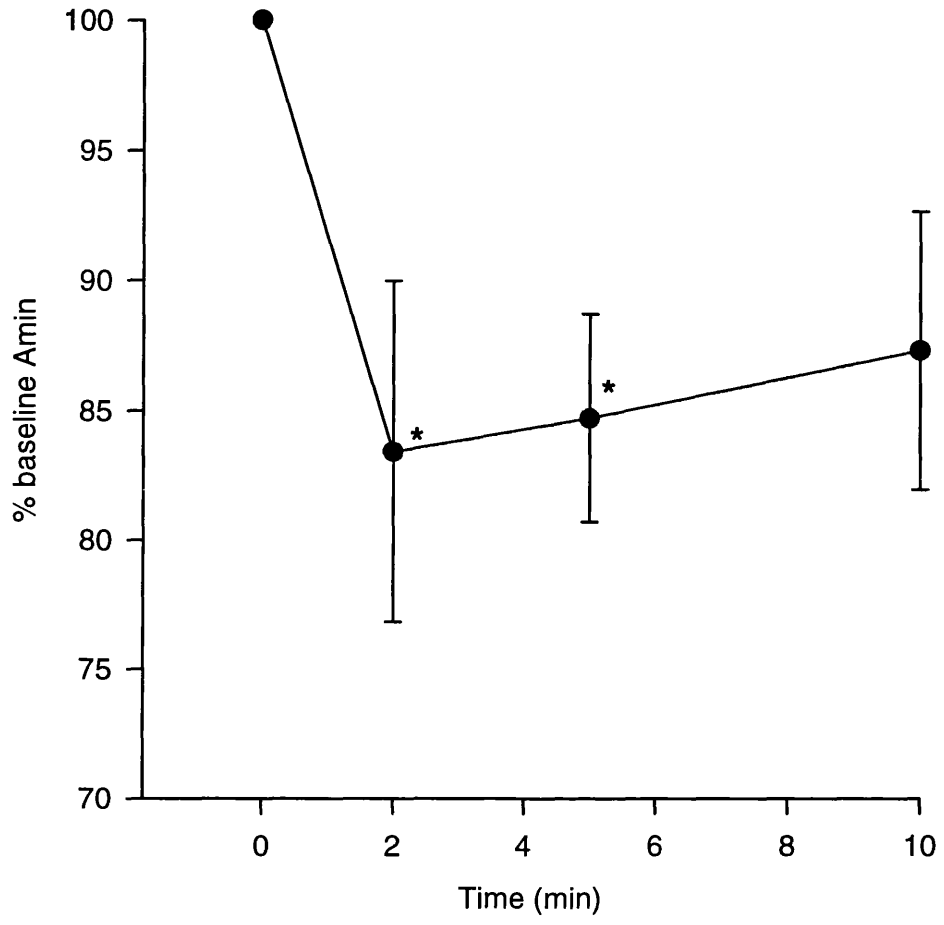

Figure 7.4. The percentage change in the minimum cross-sectional of the nasal airway (Amin.) at various times after challenge with substance P, 33μg. The nasal cavity was pretreated with only saline at the times indicated in the experimental protocol. Data are means from 8 subjects. Vertical bars represent s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.56±0.02 cm^2. *= $p<0.05$, analysed by the Wilcoxon sign-rank test.

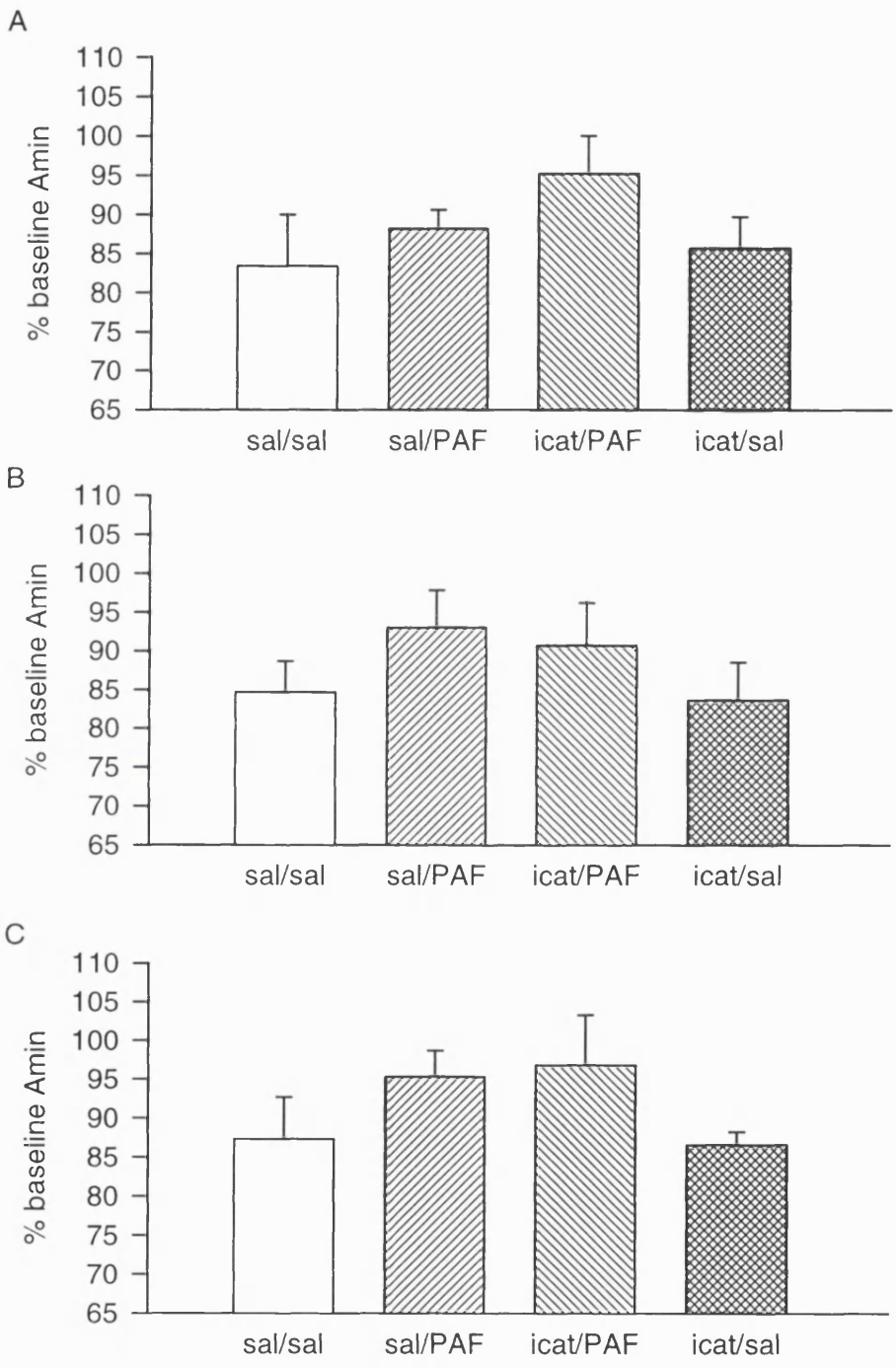

Figure 7.5. The percentage change in the minimum cross-sectional of the nasal airway (Amin.) after challenge with substance P, 33µg. Amin. was measured 2min. (graph A), 5min. (graph B) and 10min. (graph C) after challenge. The nasal cavity was pretreated with saline (Sal), icatibant (icat), 200µg and/or PAF, 60µg, as described in the experimental protocol. Data are means from 8 subjects. Vertical bars represent s.e.mean. Changes in Amin. have been normalised by expressing them as a percentage of baseline for each subject. The mean ± s.e.mean baseline value for Amin. was 0.51±0.01 cm^2.

7.3.2 The effect of PAF on kinin generation in the human nasal airway

Two hours following nasal challenge with PAF, 60μg, there was a significant increase in the kinin content of the nasal lavage fluid compared with the baseline kinin level (figure 7.6) ($p<0.05$, analysed by the Wilcoxon sign-rank test). PAF did not cause a significant increase in the kinin content of the nasal lavage fluid taken 4 and 6 hours after challenge ($p>0.05$, analysed by the Wilcoxon sign-rank test). Nasal challenge with saline did not significantly increase the kinin content of the nasal lavage fluid (for all lavages, $p>0.1$, analysed by the Wilcoxon sign-rank test).

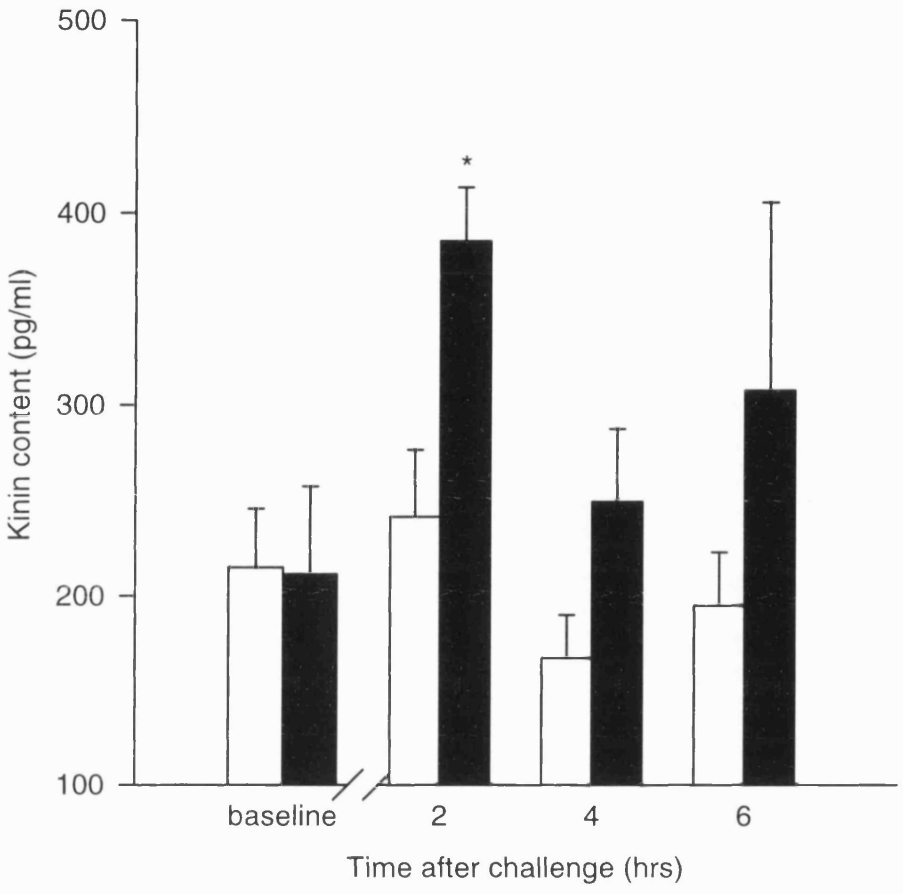

Figure 7.6. The effect of PAF on the kinin concentration in the nasal lavage fluid. The nasal airway was challenged with either PAF, 60µg (filled bars) or saline (open bars) and the airway was lavaged 2, 4 and 6 hours after challenge. Also shown is the baseline concentration of kinin in the lavage performed prior to challenge . Data are means from 8 subjects. Vertical bars represent s.e.mean. *= $p<0.05$, analysed by the Wilcoxon sign-rank test.

7.4 Discussion

In this chapter it has been shown again that intranasal administration of histamine produces a reduction in the patency of the human nasal airway. This is consistent with the data presented in previous chapters and the results reported by other authors (Doyle et al., 1990; Austin & Foreman, 1994b). Pretreatment of the nasal airway with icatibant, a bradykinin B_2 receptor antagonist, had no significant effect on the nasal response to histamine challenge. Therefore, the reduction in the patency of the nasal airway induced by histamine is not mediated by the activation of bradykinin B_2 receptors. Pretreatment of the nasal airway with platelet activating factor (PAF) significantly enhanced the reduction in nasal airway patency induced by histamine. Previous studies have reported that PAF enhanced the increase in the resistance of the human nasal airway induced by histamine and bradykinin (Austin & Foreman, 1993). This study measured nasal airway congestion by rhinomanometry. In the present chapter, the ability of PAF to enhance the nasal blockage induced by histamine challenge was confirmed by using acoustic rhinometry to measure the patency of the nasal airway.

The PAF-induced increase in the response of the nasal airway (nasal hyperresponsiveness) to histamine was abolished by pretreatment with icatibant. This suggests that kinin generation is required for PAF to enhance the responsiveness of the human nasal airway. A role for kinins in the actions of PAF in the human nasal airway is further supported by the demonstration that nasal challenge with PAF significantly increased the kinin content of the nasal lavage fluid.

Austin & Foreman (1993) proposed that the PAF-induced hyperresponsiveness of the human nasal airway is non-specific as responses to histamine and bradykinin were enhanced. In addition to using histamine in this study, the effect of icatibant and PAF on the nasal actions of substance P was also investigated. Obviously, bradykinin could not be used as the nasal challenge agent in this study as icatibant, a bradykinin B_2 receptor antagonist, was also being used. With saline only pretreatment, two and five minutes after challenge with substance P, 33μg, there was a significant reduction in

the patency of the nasal airway. However, ten minutes after challenge the reduction in nasal airway patency was not significant. This is consistent with the results of Lurie *et al.* (1994), who reported that the increase in nasal airway resistance induced by substance P, 33μg, was only significant for the first six minutes after challenge.

Interestingly, pretreatment of the nasal airway with PAF did not enhance the response to substance P. Therefore, the hyperresponsiveness induced by PAF is not totally non-specific. Klementsson & Andersson (1992) also reported data indicating that PAF-induced nasal airway hyperresponsiveness is specific for certain agents: PAF pretreatment did not generate hyperresponsiveness when methacholine-induced nasal secretion was measured. It should be noted that this study by Klementsson & Andersson used subjects with seasonal allergic rhinitis, whereas the data presented in this chapter is from experiments using normal, non-atopic subjects. The explanation for why only certain agents are affected by PAF is unknown. It may be because histamine and substance P produce their effects in the nose through different signal transduction mechanisms, or substance P and histamine may produce nasal blockage by acting on different structures in the nasal mucosa. Alternatively, the effect of PAF on substance P-induced reductions in nasal airway patency may only become apparent when higher doses of PAF are used.

Coyle *et al.* (1995) reported that major basic protein, a toxic protein released by activated eosinophils, produced hyperresponsiveness of the rat lower airways by a bradykinin-dependent mechanism. The ability of icatibant to inhibit PAF-induced nasal airway hyperresponsiveness may be explained by the following model. Intranasal administration of PAF causes an increase in the number of eosinophils in the nasal mucosa (Klementsson & Andersson, 1992; Tedeschi *et al.*, 1994b) and subsequent activation of these cells (Austin & Foreman, 1993; Tedeschi *et al.*, 1994a). The substances released during eosinophil activation, for example major basic protein, have been reported to produce lower airway hyperresponsiveness in a number of species (Gundel *et al.*, 1991) and activate plasma kallikrein, so generate kinins (Venge *et al.*, 1979). Therefore, the recruitment and activation of eosinophils, which results

from nasal challenge with PAF, may produce kinins which, as in the lower airways of the rat, are essential for the development of airway hyperresponsiveness.

There are, of course, alternative models to explain the kinin dependency of PAF-induced nasal airway hyperresponsiveness. The increase in the kinin content of the nasal lavage fluid following nasal challenge with PAF, and the kinin-dependency of PAF-induced nasal hyperresponsiveness, could result from an action of PAF which is independent of eosinophils. Therefore, the hyperresponsiveness induced by eosinophil major basic protein in the rat lower airways and by PAF in the human nasal airway could be independent effects which are both dependent on the generation of kinins. In fact, challenging the rat lower airways with eosinophil major basic protein (Coyle *et al.*, 1995) could lead to the generation of PAF, which then may account for the kinin-dependent hyperresponsiveness. These hypothese are highly speculative, especially as there are no published reports of PAF mediating its actions through bradykinin generation (Sakomoto *et al.*, 1992). However, there is, as already discussed, strong evidence that nasal challenge with PAF increases the number of activated eosinophils in the human nasal mucosa and that the products of eosinophil activation produce airway hyperresponsiveness dependent on the generation of kinins. Therefore, the hypothesis that eosinophils mediate the kinin-dependent nasal hyperresponsiveness induced by PAF seems the most plausible.

The role kinins play in the development of airway hyperresponsiveness is unknown. Fox *et al.* (1996) reported that bradykinin produced sensitisation of the C-fibres in the guinea-pig trachea. The authors concluded that this mechanism may mediate the development of the cough induced by angiotensin-converting enzyme inhibitors, as this cough is antagonised by icatibant. It is possible that the kinins generated following PAF application could sensitise the C-fibres of the human nasal airway. As a result of sensitisation, the C-fibres may then contribute to the actions of histamine in the nasal airway, resulting in an increase in the nasal response to histamine challenge. It should be noted, however, that chapter 5 reported data suggesting that nerves do not mediate the nasal response to histamine in the absence of PAF pretreatment. There is evidence that nerves play a role in the airway hyperresponsiveness induced by both

PAF and cationic proteins. In rabbits, lower airway hyperresponsiveness induced by PAF is inhibited by capsaicin pretreatment (Spina *et al.*, 1991). In the rat lower airways, cationic protein-induced hyperresponsiveness is inhibited by neurokinin NK-1 receptor antagonists or capsaicin pretreatment (Coyle *et al.*, 1994). This indicates that nerves play a central role in airway hyperresponsiveness, although it is important to note that data from experiments on other species may not be directly transferable to man. Whether, following exposure to kinins, C-fibres are sensitised and contribute nasal airway hyperresponsiveness is a target for future research.

Further studies are required to clarify the role of kinins in the development of human nasal airway hyperresponsiveness. For example, Coyle *et al.* (1995) demonstrated that, although kinin generation is essential for major basic protein-induced hyperresponsiveness, bradykinin challenge of the rat lower airways does not produce hyperresponsiveness. The effect of bradykinin pretreatment on the response to histamine needs to be investigated in the human nasal airway. Also, the effect of nasal challenge with exogenous major basic protein has not been reported. Major basic protein is released into the nasal cavity following antigen challenge of subjects with allergic rhinitis (Togias *et al.*, 1988; Bascom *et al.*, 1989) and this protein produces hyperresponsiveness of the lower airways in rodents and primates (Gundel *et al.*, 1991). Hence, it is important that the effects of nasal challenge with major basic protein are investigated, especially with regard to nasal airway hyperresponsiveness and the role of kinins. Leggieri *et al.* (1991) reported that PAF, at a dose of 157μg, produced an increase in the resistance of the human nasal airway. In light of the data presented in this chapter showing that nasal administration of PAF generates kinins, it would be interesting to investigate the effect of icatibant on PAF-induced nasal airway blockage.

Summary

- Nasal challenge with histamine, 200μg, in non-atopic human subjects induced a reduction in the patency of the nasal airway.

- The reduction in the patency of the nasal airway induced by histamine challenge was enhanced by pretreatment with platelet activating factor (PAF), 60μg.

- Pretreatment with icatibant, 200μg, inhibited the PAF-induced nasal airway hyperresponsiveness to histamine challenge.

- Substance P, 33μg, caused a significant reduction in the patency of the human nasal airway.

- Pretreatment with PAF, 60μg, did not enhance the nasal response to substance P.

- Nasal challenge with PAF, 60μg, produced a significant increase in the kinin content of the nasal lavage fluid.

- In conclusion, intranasal administration of PAF causes the generation of kinins in the nasal airway. The generation of kinins is essential for the development of nasal airway hyperresponsiveness.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE AIMS

The aim of this study was to investigate the characteristics of the bradykinin receptor in the human nasal airway and its role in allergic rhinitis. The previous chapters demonstrated that significant, reproducible data can be obtained from studies performed *in vivo* on human volunteers and *in vitro* on human nasal tissue removed at routine surgery.

Intranasal application of bradykinin produces a reduction in the patency of the human nasal airway and an increase in the plasma extravasation into the nasal cavity. These effects are mediated by two mechanisms: bradykinin B_2 receptor activation and the release of histamine. The data presented in chapters 3 and 4 demonstrated that the human nasal bradykinin receptor represents a B_2 receptor type and not an example of the purported B_3 receptor. The evidence for the existence of a B_3 receptor in the guinea-pig lower airways is partly based on the inability of certain bradykinin B_2 receptor antagonists, for example NPC 567, to antagonise the actions of bradykinin (Farmer *et al.*, 1989). However, the evidence for a bradykinin B_3 receptor is equivocal. A number of studies have failed to detect the presence of a B_3 receptor in the guinea pig lower airways (Pruneau *et al.*, 1994; Trifilieff *et al.*, 1994; Scherrer *et al.*, 1995). Also, the cloning and expression of the rodent and human bradykinin B_2 receptor has demonstrated significant differences in their pharmacology. In particular, cloned B_2 receptors from different species have different affinities for the antagonist, NPC 567 (Hess *et al.*, 1994; Davis *et al.*, 1995). As antagonists such as NPC 567 have formed the basis of reported B_2 receptor heterogeneity, species differences in the evolution of the B_2 receptor gene may account for the reports.

Chapter 3 demonstrates that NPC 567 is inactive in the human nasal airway *in vivo*. In the absence of any *in vitro* binding data, this *in vivo* data would support the existence of a B_3 receptor in the human nasal airway. However, the binding data presented in chapter 4 demonstrated that NPC 567 binds to the human nasal bradykinin receptor with an affinity characteristic of the human bradykinin B_2 receptor. It is important to

note that, while data from binding studies is valuable, the binding site may not constitute a functional receptor. Therefore, it is always necessary, if possible, to compare data from binding studies with *in vivo* data. The potency ratios of the bradykinin B_2 receptor antagonists, icatibant and [1-adamantane acetyl-D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin, calculated in this project from both *in vivo* and binding studies, are in close agreement. This provides strong evidence that the binding site characterised by *in vitro* radioligand binding studies represents a functional bradykinin receptor. The explanation for the failure of NPC 567 to antagonise the effects of bradykinin *in vivo* is unclear. But the inactivity of NPC 567 *in vivo* demonstrates that, while binding studies provide important data on the affinity of a ligand for a receptor, a number of other factors are equally as important in determining the activity of a substance *in vivo*.

Bradykinin, at a dose of 300μg and above, produces its effects in the human nasal airway via histamine release (chapter 6 and Austin, 1994). There are still a number of unanswered questions regarding bradykinin-induced histamine release from human nasal tissue. For example, is this release receptor-independent, as is the case with bradykinin-induced histamine release from rodent mast cells. This can easily be determined by investigating whether bradykinin receptor antagonists release histamine from isolated nasal tissue. Future studies should include a full characterisation of histamine release from human nasal tissue. The binding and histamine release data presented in this project have demonstrated that human nasal tissue collected from operation can be effectively used for experimentation. If this can be used to culture human nasal tissue, this cultured tissue would provide the opportunity for many more experiments. For instance, cultured human nasal tissue could be used to study the effect of bradykinin on glandular secretion, a parameter not studied in this project.

The effects of bradykinin, histamine and antigen in the human nasal airway are mediated by the generation of nitric oxide. Therefore, inhibitors of nitric oxide synthase may provide a new clinical treatment for allergic rhinitis. There are still questions about the role of nitric oxide to be answered. For example, which isoform of nitric oxide synthase is responsible for the generation of nitric oxide in the human

nasal airway? To resolve this question selective inhibitors of the isoforms of nitric oxide synthase are required. These selective inhibitors must be suitable for use in humans *in vivo*. It appears not to be neuronal nitric oxide synthase that is involved in the nasal response to bradykinin as local anaesthetics have no effect on the actions of bradykinin. This is consistent with other studies demonstrating no role for nerve activation in the human nasal actions of bradykinin. In the nasal mucosa of the guinea-pig and rat, nerves mediate the increase in vascular permeability induced by nasal challenge with bradykinin (Ricciardolo *et al.*, 1994; Bertrand *et al.*, 1993). The difference in the role of nerves in the human and rat nasal mucosa demonstrates that although studies in other animals often provide valuable results, the data is not always directly transferable to human beings.

Chapter 3 presented data investigating the effect of the bradykinin B_2 receptor antagonist, icatibant, on the nasal response to challenge with antigen. This data provides some very important observations in relation to clinical treatment of allergic rhinitis. At present, seasonal and perennial allergic rhinitis are considered to be the same disease, but with allergy to different antigens. However, the role of bradykinin is different in the two conditions. While the nasal blockage resulting from house-dust mite-induced perennial allergic rhinitis is antagonised by icatibant, the effects of nasal challenge with grass pollen antigen, in subjects with seasonal allergic rhinitis, are not antagonised by the doses of icatibant used. This indicates a role for bradykinin in perennial allergic rhinitis, but kinins appear not to play a role in seasonal rhinitis. Therefore, bradykinin B_2 receptor antagonists may provide a new clinical treatment for perennial allergic rhinitis. As discussed in chapter 3, the difference in the sensitivity to icatibant may be a result of house dust mite antigens possessing the ability to generate bradykinin from kininogen. Cytokine-induced upregulation of bradykinin B_2 receptors, as has been demonstrated in human synovial cells (Bathon *et al.*, 1992), may also contribute to the central role for kinins in perennial allergic rhinitis. However, as cytokines have been demonstrated to be generated in seasonal allergic rhinitis (Howarth, 1995), if cytokine-induced receptor upregulation does contribute to the central role for kinins in perennial rhinitis, then the lack of a role for kinins in seasonal rhinitis must be explained. Another difference in the pathology of

perennial and seasonal allergic rhinitis is that grass pollen antigen challenge produced an increase in the albumin released into the nasal cavity, whereas house dust mite antigen challenge did not. This is interpreted as a difference in the vascular responses to antigen challenge in the two conditions. Subjects with perennial allergic rhinitis release significantly less albumin into the nasal cavity following bradykinin challenge, when compared to non-atopic controls (Baraniuk *et al.*, 1994b). This is further evidence that in perennial allergic rhinitis, the nasal vascular responses are altered.

Patients presenting with seasonal allergic rhinitis have symptoms of nasal airway congestion, rhinorrhoea, sneezing and pruritus of the nose and eyes. However patients presenting with perennial allergic rhinitis mainly complain only of nasal congestion (Dr. G.K. Scadding - personal communication). The symptoms of seasonal allergic rhinitis, with the exception of the nasal airway congestion, are effectively treated with histamine H_1 receptor antagonists; implicating histamine as the agent producing the symptoms. The lack of rhinorrhoea, sneezing and pruritus in perennial allergic rhinitis could reflect a lack of histamine action in this condition. There is an important difference between perennial and seasonal allergic rhinitis in the exposure to antigen. While in seasonal allergic rhinitis, antigen exposure only occurs at certain times in the year, the antigen exposure in perennial allergic rhinitis is constant all year round. This difference in antigen exposure could lead to the differences in the pathophysiology of seasonal and perennial allergic rhinitis. For example, constant antigen exposure could lead to depletion of mast cell histamine or a desensitization of nasal histamine receptors. This may explain the lack of histamine-induced symptoms in perennial allergic rhinitis. Receptor desensitization, resulting from the constant mediator release possibly induced by perennial antigen exposure, may explain the reduced nasal vascular responses present in subjects with perennial rhinitis. Obviously this theory is speculative, but future studies should investigate the differences in pathology between the two types of allergic rhinitis. For example, the histamine content of the nasal lavage fluid should be compared following challenge with house dust mite and grass pollen antigens. Also, the characteristics of mast cells from subjects with perennial and seasonal allergic rhinitis could be compared *in vitro*, particularly with regard to histamine content and the ability of the cells to release histamine following

stimulation. Autoradiography could be used with *ex vivo* human nasal tissue to investigate whether there are differences in receptor numbers in seasonal and perennial rhinitis. To conclude, it is clear that seasonal and perennial allergic rhinitis have distinct differences in their pathology. Therefore, it is very important that the treatments used clinically reflect these differences.

Platelet activating factor (PAF) induces hyperresponsiveness of the human nasal airway. It is still unclear whether release of PAF in allergic rhinitis is involved in the generation of the hyperresponsiveness that is typical of the late response to antigen challenge. More fundamentally, the question of whether PAF-induced hyperresponsiveness is receptor-mediated is unknown. Therefore, it is very important that future studies investigate the effect of potent PAF receptor antagonists on the nasal effects of PAF challenge. If the nasal effects of PAF are receptor-mediated, then the effect of PAF receptor antagonists on antigen challenge can be studied, particularly with regard to the development of the late response. Chapter 7 demonstrates that the generation of kinins is critical for the development of PAF-induced nasal airway hyperresponsiveness. The mechanism of this kinin-dependent hyperresponsiveness is unknown and is an obvious target for future studies.

From the data presented in this project, kinins appear to have an important role in perennial allergic rhinitis. This project has also elucidated some of the characteristics of the bradykinin receptor in the human nasal airway. These data may be valuable in the future development of clinical treatment for allergic rhinitis.

CHAPTER 9

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