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Enhancement of neurokinin A-induced smooth muscle contraction in human urinary bladder by mucosal removal and phosphoramidon: relationship to peptidase inhibition

Fiona J. Warner [a], Fei Shang [a], Richard J. Millard [b], Elizabeth Burcher [a,*]

[a] *Department of Physiology and Pharmacology, University of New South Wales, Sydney NSW 2052, Australia*
[b] *Department of Urology, Prince Henry and Prince of Wales Hospitals, University of New South Wales, Sydney, Australia*

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Abstract

Neurokinin A (NKA) is potent in contracting the human detrusor muscle. Here, we have investigated whether these contractile responses are influenced by the presence of the mucosa, by the peptidase inhibitor phosphoramidon or by possible modulators, prostaglandins and nitric oxide. Contractile responses to neurokinin A were unaffected by indomethacin or *N*-ω-nitro-L-arginine, but were significantly reduced in strips containing mucosa. Phosphoramidon, an inhibitor of neutral endopeptidase 24.11 (neprilysin, CD10), was ineffective at 10 μM, but at 100 μM, significant increase in the maximum response was achieved by neurokinin A in detrusor strips with and without mucosa. In immunohistochemical studies, neutral endopeptidase immunoreactivity occurred in peripheral nerve trunks in the detrusor and in a fibrous meshwork in the subepithelial lamina propria. Our data indicate that neutral endopeptidase is present in bladder mucosa and detrusor, and support the concept that this metalloprotease and/or related enzymes are important in regulating the actions of tachykinins.

Keywords: Urinary bladder; Tachykinin; Neurokinin A; Phosphoramidon; Neutral endopeptidase; Urothelium

1. Introduction

Nerves containing substance P and neurokinin A have been identified in the urinary bladder of many animal species (Sharkey et al., 1983; Saban et al., 1992). Tachykinins are expressed in lumbosacral dorsal root ganglion sensory neurons that project to the rat bladder as thin myelinated A-delta or unmyelinated C-fibres (Maggi and Meli, 1988). In the majority of these neurons, neurokinin A is co-expressed with substance P and calcitonin gene-related peptide (De Groat, 1987; Keast and de Groat, 1992). In small laboratory animals, sensory neuropeptides can be depleted from primary afferent neurons using the neurotoxin capsaicin (see Maggi, 1995).

In human urinary bladder, tachykinin immunoreactive nerves are present in the subepithelial layer, usually surrounding intramural ganglia and around blood vessels (Gu et al., 1984; Crowe et al., 1991; Wakabayashi et al., 1993; Smet et al., 1997). However, these nerves are sparsely distributed and very rarely project to the smooth muscle bundles of the detrusor (Smet et al., 1997). Intra-vesical installation of capsaicin and resiniferatoxin, a compound whose actions are similar to those of capsaicin, has been used as a treatment in patients with urge incontinence and detrusor hyperreflexia (Maggi et al., 1989; Fowler et al., 1994). These findings have suggested that tachykinins have a role as neurotransmitters and regulators of afferent and possibly efferent components of the micturition reflex (Maggi and Meli, 1988; Maggi, 1991).

Neurokinin A is the most potent tachykinin in contracting the smooth muscle of the urinary bladder, via tachykinin NK_2 receptors, in most animal species (Maggi et al., 1987; Saban et al., 1992; Mussap et al., 1996) as well as humans (Dion et al., 1988; Zeng et al., 1995). Radioligand binding and autoradiographic studies have demonstrated tachykinin NK_2 binding sites, but not NK_1 binding sites, on detrusor muscle (Zeng et al., 1995; Burcher et al., 2000). Although the action of neurokinin A to contract detrusor smooth muscle via the tachykinin NK_2 receptor is thought to be direct, few studies have rigorously examined this question in the adult human

* Corresponding author. Tel.: +61-2-9385-2562; fax: +61-2-9385-1059.
E-mail address: E.Burcher@unsw.edu.au (E. Burcher).

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bladder. Removal of the mucosa and/or inhibitors of the enzyme neutral endopeptidase 24.11 (neprilysin) potentiated contractile responses to tachykinins in the guinea pig (Maggi et al., 1987) and dog (Saban et al., 1992; Mussap et al., 1996) isolated bladder. Neutral endopeptidase is a cell surface metalloprotease which cleaves a number of peptides at the N-terminal side of hydrophobic amino acids residues; in addition to tachykinins, it also metabolises enkephalins and atrial natriuretic peptide (for review, see Turner et al., 2001). Here, we considered whether the contractile responses of human detrusor might be influenced by the presence of the mucosa, neutral endopeptidase and by possible modulators such as prostaglandins and nitric oxide. The source of these modulators was also of interest, as to whether they originated from muscle, mucosa or both regions.

2. Materials and methods

2.1. Human bladder specimens

Specimens from the dome of macroscopically normal human urinary bladders were collected from 11 patients of both sexes, aged 62–82 years. Tissue (~ 5–10 g) was obtained from bladder removed due to carcinoma ($n=7$ patients) and small specimens (10 × 5 mm) of detrusor were also collected during radical prostatectomy ($n=4$). All specimens were taken from patients who had not undergone radiotherapy or chemotherapy or showed signs of obstruction. Collection of tissue was approved by the University of New South Wales Human Ethics Committee and was in accordance with the recommendations of the Declarations of Helsinki and Tokyo.

Human bladder specimens were used in functional studies within 4 h of collection from surgery. For immunohistochemical and histological studies, pieces of bladder (7 × 5 mm) from cystectomy patients, with all layers intact, were placed in ornithine carbamyl transferase (O.C.T) mounting solution (TissueTek™) contained within small aluminium moulds. These tissue blocks were then 'snap' frozen in liquid nitrogen and stored at − 70 °C until use.

2.2. Isolated smooth muscle studies

Strips of detrusor (5 × 3 mm) were prepared by removing the serosa, submucosa and mucosa. For investigation of the effects of mucosa on the contractile response of neurokinin A, adjacent strips from the same patient were cut from the full thickness of the bladder: these contained detrusor, submucosa and mucosa, but not serosa. Strips were suspended in 2 ml organ baths containing carbogenated Krebs–Henseleit solution at 37 °C (Zeng et al., 1995). Changes in tension were measured isometrically and recorded using the computer program, Polygraph (University of New South Wales, Australia). After tissue equilibration and at the end of all experiments, a maximum tissue response was achieved using carbachol (100 μM). The contractile response of detrusor strips to carbachol was not significantly affected by the removal of mucosa or by enzyme inhibitor phosphoramidon. Concentration–response curves to neurokinin A were constructed by discrete additions of agonist in 60-min cycles. At the plateau of contraction for each concentration (4–6 min contact time), the bath was washed out with fresh Krebs–Henseleit solution at 37 °C. All responses were calculated as a percentage of the maximum response to carbachol achieved in that strip. Data were expressed as the mean ± SEM.

2.3. Factors affecting contractile responses to neurokinin A

Concentration–response curves to neurokinin A were constructed by discrete additions, in tissue with and without mucosa from the same patient (paired design). In addition, concentration–response curves of neurokinin A were also constructed in detrusor strips (from the same patient) with and without mucosa in the presence of the cyclo-oxygenase inhibitor, indomethacin (4 μM), the nitric oxide synthase (NOS) inhibitor, *N*-ω-nitro-L-arginine (L-NNA, 100 μM) or the neutral endopeptidase inhibitor, phosphoramidon (10 and 100 μM). Each inhibitor was pre-incubated with the tissue 20 min prior to application of neurokinin A. Responses obtained were compared to those in detrusor strips without mucosa and in the absence of any inhibitor. Statistical analysis was performed using two-way analysis of variance (ANOVA).

2.4. Immunohistochemical studies

Frozen sections (15 μm) of human bladder were fixed in chloroform/acetone (50:50 v/v) for 7 min at − 20 °C, then rinsed in 0.1 M phosphate buffered saline and incubated in Tris buffer saline containing 2% horse serum and 0.4% Triton X-100 (TBS-TX, 0.1 M, pH 7.6). The primary antibody was a mouse anti-CD10 monoclonal antibody, clone SS2/36, which does not cross react with human endothelin converting enzyme-1 (Korth et al., 1999). Sections were incubated with primary antibody (1:800) in 0.1 M TBS-TX, (pH 7.6) containing 2% horse serum overnight at room temperature, and subsequently rinsed three times with 0.1 M TBS-TX. Following this, sections were incubated with secondary antibody, horse anti-mouse immunoglobulin (1:200) in 0.1 M TBS-TX (pH 7.6), containing 2% horse serum for 2 h. Adjacent sections were stained for the neuronal marker, protein gene product 9.5 (PGP 13C4 monoclonal antibody, 1:500), as described above. Sections were processed using the avidin–biotin complex method, and mounted in DePex. Negative controls were processed in the absence of the primary antibody. Adjacent histological sections were stained with haematoxylin and eosin.

2.5. Materials

Neurokinin A was obtained from Auspep (Australia) and Chiron Mimotopes (Australia). Stock solutions were pre-

pared in 0.01 M acetic acid with β-mercaptoethanol (5% v/v) and stored at −20 °C. Phosphoramidon, indomethacin, L-NNA and horse serum were purchased from Sigma (Australia). Mouse anti-human monoclonal antibodies CD10 (SS2/36) and PGP 9.5 (13C4) were purchased from DAKO (Australia) and Ultraclone (UK), respectively. Anti-mouse immunoglobulin was purchased from Immuno Diagnostics (Australia), avidin–biotin complex reagent from Vector Laboratories (USA) and O.C.T. mounting compound (TissueTek™) from Bayer Health Care (Australia). All other reagents were of analytical grade.

3. Results

3.1. Contractile responses to NKA

NKA produced concentration-dependent contractile responses of strips of human detrusor, with a maximum response less than half of that produced by carbachol. Responses to NKA were obtained in strips without mucosa ($pD_2 = 7.1 \pm 0.11$, $E_{max} = 46 \pm 3.4\%$, $n = 6$) and in paired strips containing mucosa ($pD_2 = 7.0 \pm 0.09$, $E_{max} = 40 \pm 2.3\%$, $n = 6$) (Fig. 1A). A small but significant decrease was attributable to the presence of the mucosa ($P < 0.05$, two-way ANOVA).

Indomethacin and L-NNA did not significantly alter the potency or maximum response of NKA, in detrusor strips both with and without mucosa (Fig. 1B,C). In addition, no relaxation in response to indomethacin or L-NNA was observed. Phosphoramidon (10 μM) had no effect on the contractile response to NKA in detrusor strips (with and without mucosa) (Fig. 2A). However, in the presence of a higher concentration of phosphoramidon (100 μM), there was a shift to the left in the concentration response curve of NKA and a significant increase in the maximum response in detrusor strips, both with and without mucosa ($P < 0.05$–0.001, two-way ANOVA) (Fig. 2B).

3.2. Localisation of neutral endopeptidase-like immunoreactivity

Strong immunoreactivity for neutral endopeptidase was associated with large peripheral nerve trunks running through the detrusor (Fig. 3A,B) and with the lamina propria under the urothelium (Fig. 3C,D), in all six specimens studied. Since many structures stained by the CD10 antibody appeared very similar to nerve fibres, additional experiments were carried out ($n = 4$) whereby adjacent sections were stained for neutral endopeptidase and PGP immunoreactivity. In the nerve trunks, the CD10-immunopositive nerve fibres represented about 25% of the total number of nerve fibres stained in adjacent sections with PGP (not shown).

The staining in the lamina propria was examined under higher power. Immunoreactivity for neutral endopeptidase

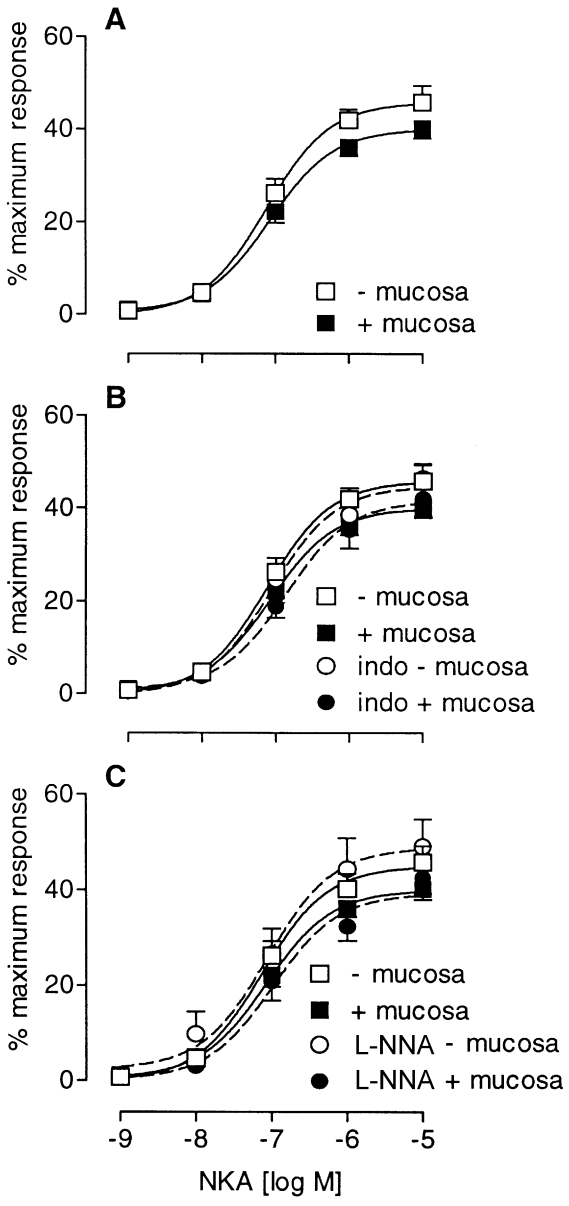

Fig. 1. Concentration–response curves to NKA in human detrusor (A) with and without mucosa; (B) with and without mucosa in the presence of indomethacin (4 μM); (C) with and without mucosa in the presence of L-NNA (100 μM). Data points represent mean ± SEM from six individual patients, expressed as a percentage of the contraction obtained from a maximal concentration of carbachol (100 μM). A significant difference was observed between concentration–response curves to NKA in the absence and presence of mucosa ($P < 0.05$, two-way ANOVA).

appeared as an extensive network of fibrous appearance, which included some varicose fibres (Fig. 3E). In the superficial lamina propria, these formed a fine meshwork surrounding small structures, 6–8 μm in size, possibly microvessels (Fig. 3E). This meshwork was also stained by PGP in adjacent sections, although larger varicose fibres were also seen and the PGP-immunoreactive fibres extended deeper into the lamina propria (not shown). Individual neuron-like structures (Fig. 3F) and small "knots" of

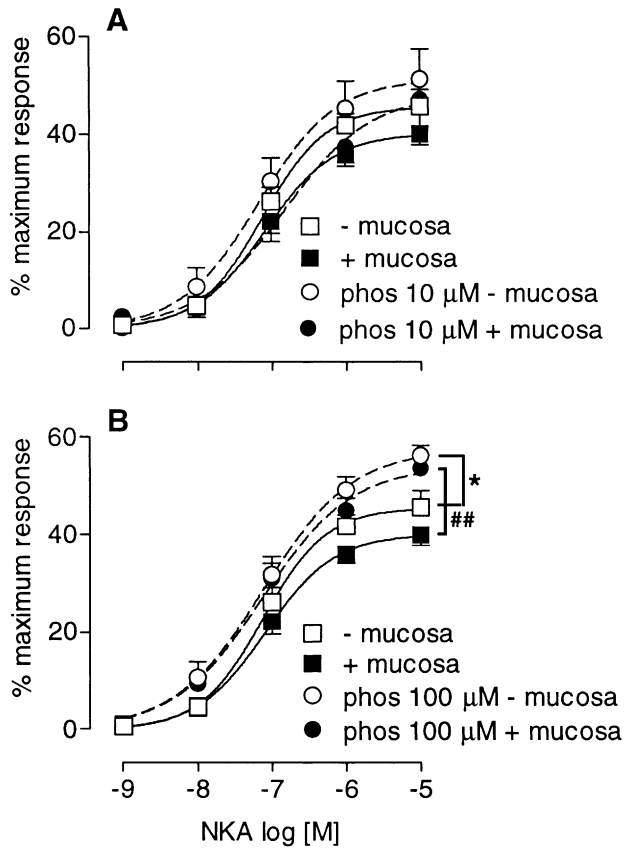

Fig. 2. Concentration–response curves to NKA in human detrusor strips with and without mucosa in the presence of (A) phosphoramidon (10 μM); (B) phosphoramidon (100 μM). Data points represent mean ± SEM from six to seven individual patients expressed as a percentage of the contraction obtained from a maximal dose of carbachol (100 μM). In the presence of phosphoramidon 100 μM, a significant difference was observed between strips with (##, $P<0.001$, two-way ANOVA) and without mucosa (*, $P<0.05$, two-way ANOVA).

intensely staining non-varicose fibres (Fig. 3G) in the lamina propria were also neutral endopeptidase-immunoreactive.

Control sections incubated without CD10 antibody showed no localisation of neutral endopeptidase-immunoreactivity in the lamina propria. Weak staining in detrusor muscle and in the walls of blood vessels was found to be nonspecific.

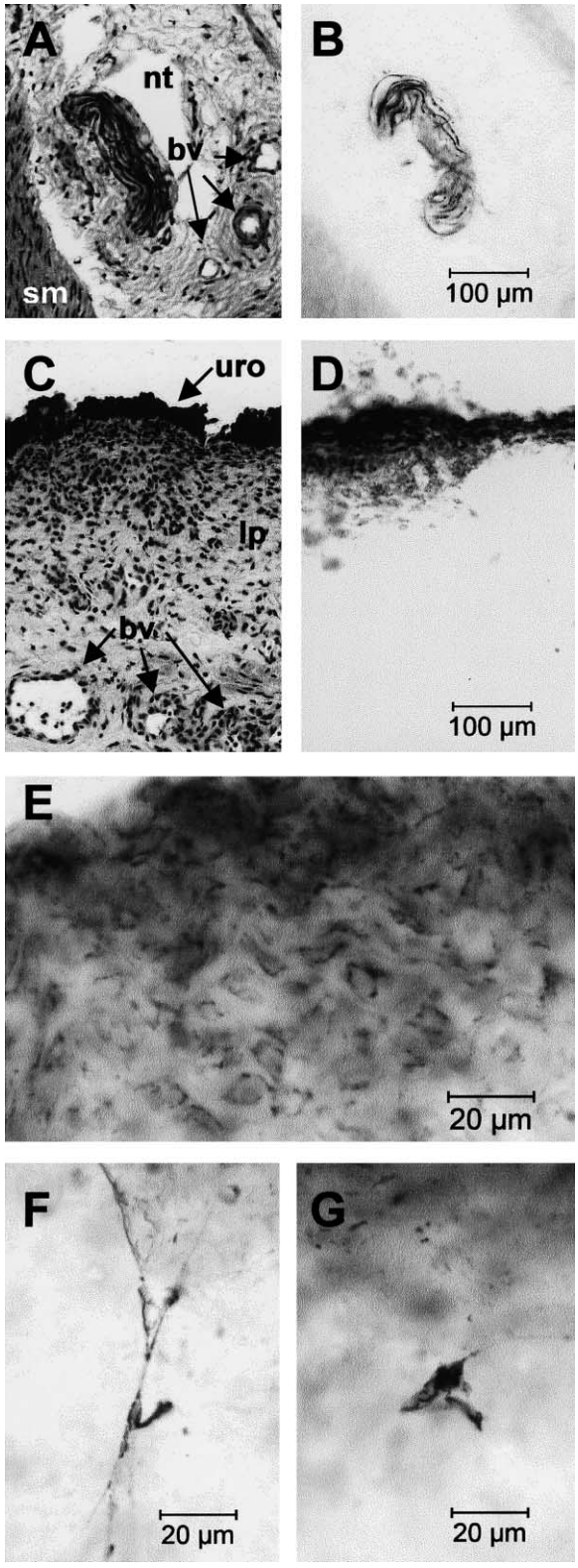

Fig. 3. (A,C) Low power photomicrographs stained with haematoxylin and eosin to demonstrate tissue morphology, with (B,D) adjacent sections illustrating neutral endopeptidase-like immunoreactivity in sections of human bladder. (B) Dense immunoreactivity was observed in individual nerve fibres in peripheral nerve trunks, running in connective tissue between smooth muscle bundles within the detrusor. (D) A network of immunoreactivity was seen on and underneath the urothelium (C,D). No neutral endopeptidase immunoreactivity was observed on smooth muscle or blood vessels. (E–G) High power photomicrographs showing neutral endopeptidase-like immunoreactivity in subepithelial lamina propria. (E) Dense meshwork of varicose fibres forming loops around structures of size 6–8 μm, (F) individual long fibre and (G) a dense "basket" of non-varicose fibres deeper in lamina propria. bv, blood vessel; nt, nerve trunk; sm, smooth muscle; lp, lamina propria; uro, urothelium.

4. Discussion

Tachykinins are potent in contracting human detrusor smooth muscle in vitro (Dion et al., 1988; Maggi et al., 1987; Zeng et al., 1995). The localisation of neurokinin A and substance P in afferent pathways that innervate the urinary bladder of many species including humans (Crowe et al., 1991; Smet et al., 1997) has led to the suggestion that these peptides may have a role in micturition. Several studies support this concept: for instance, substance P and neurokinin A released from the peripheral terminals of sensory neurons affect smooth muscle contraction, vascular tone and permeability (Kalbfleisch and Daniel, 1987; Maggi and Meli, 1988). However, despite the contractile potency of exogenous neurokinin A, the hypothesis that it plays an important role as an efferent neurotransmitter has not been supported by our recent studies in isolated human detrusor from child (Werkström et al., 2000) and adult (Moore et al., 1999).

In human bladder the contractile responses to neurokinin A were moderately, but significantly, enhanced in the absence of the mucosa. In the guinea pig (Maggi et al., 1987) and dog urinary bladder (Saban et al., 1992), contractile responses to tachykinins were markedly enhanced in the absence of mucosa. This effect might be due to the release of modulatory agents, such as nitric oxide and/or prostaglandins, or to enzymatic removal of tachykinins.

Nitric oxide (NO) has been proposed to function as an inhibitory neurotransmitter in the human urinary bladder (Smet et al., 1996). Nitric oxide synthase (NOS) exists in several isoforms. In the epithelium, NO can be generated via inducible NOS, (Cook et al., 1994). Nerves immunoreactive for neural NOS are abundant in the bladder neck and urethra, but are also found in the detrusor of the human bladder (Smet et al., 1996). Although nerve-stimulated release of NO relaxes smooth muscle of the human urethra (Leone et al., 1994) and sheep bladder neck (Thornbury et al., 1992), there is evidence both for (James et al., 1993) and against (Andersson and Persson, 1993) nerve-mediated NO smooth muscle relaxation in human detrusor. In the present study utilising tissue from the bladder dome, inhibition of NOS by L-NNA did not alter neurokinin A-induced detrusor contraction, indicating that release of NO has little or no role in modulating contractile responses to neurokinin A.

In mammalian urinary bladders, prostaglandins are thought to maintain bladder tone and contribute to the process of micturition (Khan et al., 1998). Prostaglandins and thromboxane produce concentration-dependent contraction of human detrusor strips (Bultitude et al., 1976; Palea et al., 1998) and indomethacin, which inhibits prostaglandin formation, relaxes the human detrusor in vitro (Abrams et al., 1979). However, indomethacin did not relax preparations in the present experiments. Tachykinins are able to induce release of various prostaglandins, as shown, for example, in the hamster urinary bladder (Tramontana et al., 2000). However, in the present study, indomethacin did not alter neurokinin A-induced contractions, even in strips containing mucosa. This lack of effect of indomethacin was also observed in child (Zeng and Burcher, 1996) and dog detrusor muscle (Mussap et al., 1996), suggesting species differences in the ability of tachykinins to induce prostanoid release in the detrusor.

Peptidases, particularly neutral endopeptidase, are important in limiting the actions of tachykinins released from primary afferent nociceptive neurons (Turner et al., 2001). This concept has been examined for the airways, in particular, where removal of the neutral endopeptidase-enriched respiratory epithelium and the presence of peptidase inhibitors markedly enhance contractile responses to tachykinins (Black et al., 1988; Devillier et al., 1988). It appears that the localisation of neutral endopeptidase has not been investigated in the human urinary bladder. In this study, phosphoramidon at 100 μM significantly potentiated the maximum response of neurokinin A in detrusor strips in the absence and especially in the presence of the mucosa. This suggests that neutral endopeptidase or very similar enzyme is present in both mucosa and detrusor of the human bladder. In parallel binding studies with human detrusor membranes, specific binding of the radioligand [^{125}I]iodohistidyl^{1} neurokinin A was enhanced threefold in the presence of 1 mM phosphoramidon (Warner et al., submitted for publication). In guinea pig bladder, thiorphan, another inhibitor of neutral endopeptidase, also enhanced responses of tachykinins in the presence and absence of mucosa, with the greatest effect in strips with mucosa (Maggi et al., 1990). In dog urinary bladder, phosphoramidon enhanced responses to tachykinins in detrusor strips without mucosa, suggesting the presence of neutral endopeptidase in the detrusor, as well as in the mucosa (Saban et al., 1992; Mussap et al., 1996).

The presence of immunoreactivity for neutral endopeptidase (discussed below), as well as the potentiation by phosphoramidon, initially suggests involvement of neutral endopeptidase in limiting responses to neurokinin A in human bladder. However, the effective concentration of phosphoramidon was very high relative to its recorded affinity ($K_i = 2$ nM) for neutral endopeptidase and it is known that this agent also inhibits other related enzymes, including endothelin converting enzyme-1 (EC 3.4.24.71) ($IC_{50} = 1$ μM; Turner and Tanzawa, 1997). Endothelin converting enzyme-1 is also a type II integral membrane protein and a member of the zinc metalloprotease family; it has been localised in human urinary bladder urothelium, but with a different staining pattern compared with that of neutral endopeptidase (Korth et al., 1999). In the hydrolysis of substance P, both enzymes display similar K_m (30–90 μM) values, although neutral endopeptidase has over a 320-fold higher rate (Matsas et al., 1984; Johnson et al., 1999). Thus it is very likely that endothelin converting enzyme-1 is also capable of degrading neurokinin A.

The presence of neutral endopeptidase in both mucosa and detrusor was confirmed in our immunohistochemical studies, which showed localisation of immunoreactivity

under the urothelium, as well as in large nerve bundles running through the detrusor. It is probable that neutral endopeptidase was associated with small nerve fibres in the subepithelium, since very similar PGP-immunoreactivity was also observed under the urothelium, here as well as in earlier studies in human bladder (Smet et al., 1996, 1997). Thus neutral endopeptidase is likely to be involved in limiting peptidergic actions and/or neurotransmission in the human bladder, although its relative importance in metabolising tachykinins, compared with other peptides, is not clear. Single varicose nerve fibres immunoreactive for neurokinin A and substance P occur in the subepithelium of human bladder (Smet et al., 1997; D.S.H. Lam and E. Burcher, unpublished data), although the pattern does not resemble the neutral endopeptidase-immunoreactive ''meshwork'' seen here. It may be of relevance that in autoradiographic studies in human bladder, tachykinin NK_1 binding sites occurred on small arteries, arterioles and post-capillary venules in the lamina propria, but not detrusor smooth muscle (Burcher et al., 2000), in support of a possible role for substance P in control of blood flow and vascular permeability suggested by previous functional studies (for review, see Maggi, 1995). Thus subepithelial neutral endopeptidase may be of physiological relevance in metabolising substance P and limiting its presumed action of enhanced vascular permeability and role in neurogenic inflammation (for review, see Maggi, 1995).

Neutral endopeptidase immunoreactivity appears to be associated with only a subset of bladder nerve fibres, since the network of nerve fibres deeper in the lamina propria and the majority of nerve cell bodies stained by PGP were not neutral endopeptidase-immunoreactive. Mapping neutral endopeptidase immunoreactivity in the central nervous system has revealed excellent matching with neurofilament protein immunoreactivity and good matching with substance P- and Leu-enkephalin immunoreactivity, with localisation to Schwann cell membranes around dorsal root ganglion cells and fibres (Matsas et al., 1986). It should also be noted that neutral endopeptidase is expressed by other cell types and structures outside the nervous system, such as lymphocytes, glomeruli and collecting tubules, and may have diverse physiological and pathophysiological roles (Turner et al., 2001).

In conclusion, our data show that neurokinin A has only direct effects in contraction of human isolated detrusor muscle. Phosphoramidon-mediated inhibition of type II metalloproteases such as neutral endopeptidase or endothelin converting enzyme-1 enhanced responses in preparations with and without mucosa. Thus enzymatic degradation appears to have a more important role in regulating in the contractile response of neurokinin A in normal bladder than other factors such as NO or prostaglandin formation. Our immunohistochemical and histological studies confirmed the presence of neutral endopeptidase in both muscle and mucosal layers of the bladder whereas previous studies found endothelin converting enzyme-1 in urothelium but not smooth muscle (Korth et al., 1999). Changes in the density of type II metalloproteases could be relevant in bladder disorders such as urge incontinence (Maggi et al., 1989) and detrusor hyperreflexia (Fowler et al., 1994), which may be associated with increased levels of tachykinins and afferent signalling, or in conditions with inflammation of the bladder mucosa.

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