Tachykinin NK₁ receptor in the guinea-pig isolated proximal urethra: characterization by receptor selective agonists and antagonists

Carlo Alberto Maggi & Riccardo Patacchini

Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131 Florence, Italy

1 The tachykinin receptor mediating contraction of the guinea-pig isolated proximal urethra has been characterized by use of receptor selective agonists and antagonists. All experiments were performed in the presence of peptidase inhibitors (bestatin, captopril and thiorphan, 1 μ M each) in order to reduce peptide degradation.

2 The natural tachykinins, substance P and neurokinin A produced a concentration-dependent contraction of rings of the proximal urethra which approached the same maximum (about 50% of the response to 80 mM KCl). Substance P (EC₅₀ 155 nM) was slightly (3.6 times) more potent than neurokinin A (EC₅₀ 560 nM).

3 The tachykinin NK₁ receptor selective agonist, [Sar⁹]substance P sulphone (EC₅₀ 62 nM), was slightly more potent than substance P and produced the same maximal response of natural tachykinins. The NK₂ receptor selective agonist, [β Ala⁸] neurokinin A(4–10), was active only at μ M concentrations and its maximal effect did not exceed 20% of that to substance P or neurokinin A. The NK₃ receptor selective agonist, senktide, was ineffective up to 30 μ M.

4 The response to [Sar⁹]substance P sulphone was antagonized in a competitive manner by either ($\pm$)-CP 96,345 (pA₂ 7.75, slope – 1.10) or GR 82,334 (pA₂ 7.31, slope – 1.26), which are selective NK₁ receptor antagonists, while it was unaffected (up to 10 μ M) by MEN 10,376, a selective NK₂ receptor antagonist.

5 The response to 10 μ M [β Ala⁸]neurokinin A (4–10) was abolished by either 0.2 μ M ($\pm$)-CP 96,345 or 1 μ M GR 82,334, suggesting the involvement of NK₁ receptors.

6 Electrical field stimulation (5 and 10 Hz, 0.25 ms, 100 V, trains of 5 s duration) produced tetrodotoxin-sensitive phasic contractions of the urethra which were abolished by atropine plus phentolamine (3 μ M each). Capsaicin (1 μ M) produced a small transient contraction of the urethra which was abolished by ($\pm$)-CP 96,345 (0.1 μ M). ($\pm$)-CP 96,345 did not modify the response to electrical field stimulation.

7 We conclude that tachykinin NK₁ receptors are the main if not the only mediators of the contractile response of guinea-pig proximal urethra to peptides of this family and that this preparation is useful for assessing the affinities of various ligands for the NK₁ receptor. Endogenous tachykinins released from peripheral endings of capsaicin-sensitive primary afferents produce urethral contraction by activating NK₁ receptors.

Keywords: Guinea-pig proximal urethra; tachykinins; tachykinin receptor; receptor antagonists; capsaicin; 'efferent' function of sensory nerves

Introduction

Peptides of the tachykinin family are widely distributed in the urinary tract of various mammalian species, including man. In this region, substance P and neurokinin A are stored in peripheral terminals of primary afferent neurones (capsaicin-sensitive) and, following their release by depolarizing stimuli, exert biological effects (e.g. smooth muscle contraction and plasma protein extravasation) by activating specific receptors located on the membrane of target cells (see Maggi, 1991, for review).

Three distinct types of tachykinin receptors (termed NK₁, NK₂ and NK₃) have been described thus far by pharmacological criteria and subsequently isolated and cloned (see Guard & Watson, 1991; Nakanishi, 1991, for reviews). These receptors are present in the urinary tract (ureter, urinary bladder and urethra) of various species (Maggi, 1991); remarkable species-related variations exist in the types of tachykinin receptor which mediate the same biological function. For instance both NK₁ and NK₂ receptors mediate contraction to tachykinin in the rat and guinea-pig bladder,

while only NK₂ receptors do so in the human or hamster urinary bladder (Maggi, 1991, for review). Tachykinins also produce contraction of the mammalian urethral smooth muscle: in rats both NK₁ and NK₂ receptors are present in the proximal urethra (Maggi *et al.*, 1988) while only NK₂ receptors are present in the human urethra (Parlani *et al.*, 1991). Thus NK₂ receptors are consistently expressed, as mediators of the contractile effect of tachykinins in the lower urinary tract, while the expression of NK₁ receptors on smooth muscle seems more susceptible to species-related variations and/or regional differences. In this study we have characterized the tachykinin receptor mediating contraction of the guinea-pig isolated proximal urethra. Evidence has been obtained, by means of receptor selective agonists and antagonists that only NK₁ receptors are present in this preparation. An attempt was also made to establish whether tachykinins may act as excitatory transmitters in the guinea-pig proximal urethra.

Methods

Male albino guinea-pigs weighing 250–350 g were stunned and bled. The whole urinary bladder and urethra were quickly removed and placed in oxygenated (96% O₂ and 4% CO₂) Krebs solution. A ring of proximal urethra was excised and placed in a 5 ml bath for isolated organs. The ring was suspended between two stainless steel hooks and connected to an isotonic transducer (load 3 mN) for recording of mechanical activity.

Experiments were started after a 90 min equilibration period. KCl (80 mM) was added to the bath and the resulting contraction was used as a reference to express contractile response to tachykinin peptides. Concentration-response curves to natural tachykinins and receptor-selective tachykinin agonists were constructed in a cumulative manner, the next concentration being added when the effect of the preceding one had reached its maximum (within 2–3 min of addition to the bath). Preliminary experiments had shown that the maximal response to substance P (3 μ M) addition as a single concentration to the bath ($55 \pm 7\%$ of KCl maximum) was not significantly different from that recorded at the end of the cumulative concentration-response curve ($49 \pm 6\%$), indicating absence of a significant tachyphylaxis with this protocol of cumulative application of tachykinins. All experiments were performed in the presence of peptidase inhibitors (bestatin, captopril and thiorphan, 1 μ M each, 15 min beforehand).

For studying the effect of tachykinin receptor antagonists, a control curve to the agonist was established first. Then the preparation was thoroughly washed and, 45 min later, the chosen concentration of the antagonist was added to the bath. A new curve to the agonist was performed after a 15 min contact time with the antagonist.

Antagonist potency was evaluated by the Schild plot method (Arunlakshana & Schild, 1959). Since all the antagonists tested provided linear regressions with slopes not significantly different from unity, the antagonists affinity was measured by the constrained plot method (slope constrained to -1) as proposed by Tallarida *et al.* (1979).

Statistical analysis

All data in the text are means $\pm$ s.e. of the mean. Regression analysis was performed by means the least squares method. EC₅₀ and 95% confidence limits (CL) were calculated accordingly.

Drugs

Drugs used were: GR 82,334 [D-Pro⁹[spiro- γ -lactam]Leu¹⁰, Trp¹¹] physalaemin (1–11); Neosystem), bestatin and [Sar⁹]substance P sulphone (Peninsula), atropine HCl (Serva), phentolamine mesylate (Ciba-Geigy), tetrodotoxin (Sankyo), captopril (Squibb) and thiorphan (Sigma). Substance P, neurokinin A, [β -Ala⁸] neurokinin A (4–10), senktide and MEN 10,376 ([Tyr⁵, D-Trp^{6,8,9}, Lys¹⁰] NKA(4–10)) were synthesized at Dept. of Chemistry by conventional solid phase methods. ($\pm$)-CP 96,345 ([[(25,35)-cis-2-(diphenylmethyl)-N-[2-methoxyphenyl)-methyl] - 1-azabicyclo[2.2.2]octan-3-amine]) was synthesized and characterized as described previously (Lecci *et al.*, 1991).

Results

Effect of tachykinin receptor agonists

The guinea-pig isolated proximal urethra was mechanically quiescent. A slowly developing tonic contraction was produced by addition of KCl (80 mM) which was used as a reference response to express the effect of tachykinins and tachykinin-related peptides. The contractile effect of added

Figure 1 Typical tracings showing the contractile response of the guinea-pig isolated proximal urethra to added [Sar⁹]substance P sulphone in the absence (a) and presence (b) of the tachykinin NK₁ receptor antagonist, GR 82,334.

tachykinins in the guinea-pig isolated proximal urethra developed rapidly (Figure 1) and was rapidly reversed upon washout. Reproducible concentration-response curves were obtained at 30 min intervals with washings intervening between them.

Substance P and neurokinin A both produced a concentration-dependent contraction (Figure 2). The contractile response to each concentration of added peptides was fully developed within 2–3 min of their addition to the bath. The maximum response to substance P and neurokinin A averaged 47 ± 5 and $49 \pm 6\%$ of response to KCl ($n = 6$ for each peptide, range 28–89%). Threshold concentrations were 3 nM for substance P and 10 nM for neurokinin A. EC₅₀ values and 95% confidence limits are shown in Table 1 and indicate that substance P is slightly (3.6 times) more potent than neurokinin A in contracting the guinea-pig isolated proximal urethra.

The tachykinin NK₁ receptor selective agonist, [Sar⁹]substance P sulphone, behaved as a full agonist (Figures 1 and 2), producing concentration-dependent contractions (threshold concentration 3 nM) which averaged the same maximum ($50 \pm 3\%$ of the response to KCl) of that produced by natural tachykinins. [Sar⁹]substance P sulphone was slightly (2.5 times) more potent than substance P itself and about 9 times more potent than neurokinin A (Table 1).

The NK₂ receptor selective agonist, [β Ala⁸]neurokinin A(4–10) showed a weak agonist activity at high (μ M) concentrations. At the highest concentration tested (30 μ M) the response to [β Ala⁸]neurokinin A(4–10) did not exceed 15% of that to KCl and averaged $23 \pm 3\%$ of that produced, in the same urethral rings by a maximally effective concentration of substance P (Figure 2, Table 1).

The NK₃ receptor selective agonist, senktide was ineffective up to 30 μ M ($n = 4$, Figure 2, Table 1).

Figure 2 Concentration-dependent contractile response of the guinea-pig isolated proximal urethra to cumulative addition of substance P (O), neurokinin A (□), [Sar⁹]substance P sulphone (●), [βAla⁸]neurokinin A(4–10) (■) or senktide (▲). Each value is mean of 4–8 experiments; s.e.mean shown by vertical lines.

Table 1 EC₅₀ 95% CL values and maximal effect (E_{max}) of natural tachykinins and receptor-selective synthetic agonists in producing contraction of the guinea-pig isolated proximal urethra

Agonist	EC ₅₀ (nM)	95% CL (nM)	E _{max} (% of KCl)
Substance P	155	92–303	47 ± 5
Neurokinin A	560	142–6273	45 ± 6
[Sar ⁹]Substance P sulphone	62	34–144	50 ± 3
[βAla ⁸]Neurokinin A(4–10)	NE	NE	11 ± 4
Senktide	inactive up to 30 μM		

NE = not evaluable. Each value originates from 4–8 experiments.

Effect of receptor selective antagonists

(±)-CP 96,345, GR 82,334 and MEN 10,376 displayed no agonist activity. Both (±)-CP 96,345 (0.2–1 μM, Figure 3) and GR 82,334 (0.5–3 μM, Figure 1 and 3) produced a concentration-dependent rightward shift of the curve to the NK₁ receptor selective agonist [Sar⁹]substance P sulphone without depression of maximal response attainable. Schild's plot analysis revealed the competitive nature of the antagonism (Table 2) and pA₂ values were calculated by the constrained plot method (Tallarida *et al.*, 1979) (Table 2). (±)-CP 96,345 was slightly more potent (0.44 log unit difference in pA₂ values) than GR 82,334. MEN 10,376 (10 μM) failed to affect significantly the concentration-response curve to the NK₁ receptor selective agonist (Figure 3, Table 2).

The question was raised as to whether the small effect produced by [βAla⁸]neurokinin A(4–10) may reflect the existence of a minor population of NK₂ receptors in the preparation. The contraction produced by 10 μM [βAla⁸]neurokinin A(4–10) (11 ± 4% of maximal response to neurokinin A, *n* = 4) however, was abolished by 0.2 μM (±)-CP 96,345 (*n* = 4), indicating its dependence upon activation of NK₁ receptor. Similar results were obtained with 1 μM GR 82,334 as antagonist: the response to 10 μM [βAla⁸]neurokinin A(4–10) (12.5 ± 2% of maximal response to neurokinin A, *n* = 4) was totally abolished in the presence of GR 82,334.

Effect of (±)-CP 96,345 on contractile responses to capsaicin and electrical field stimulation

These experiments aimed to assess whether tachykinins may act as excitatory transmitters in the guinea-pig proximal urethra following activation of intramural nerves by electrical

Figure 3 Effect of (±)-CP 96,345 (a) GR 82,334 (b) or MEN 10,376 (c) on the contractile response of the guinea-pig isolated proximal urethra to the tachykinin NK₁ receptor selective agonist, [Sar⁹]substance P sulphone. In each panel, control curves to the agonist are shown by (O). Concentrations of the antagonists were as follows: (±)-CP 96,345 0.2 μM (●), 0.5 μM (□) and 1 μM (■); GR 82,334: 0.5 μM (●), 1 μM (□) and 3 μM (■); MEN 10,376 10 μM (●). Each value is mean of at least 4 experiments; s.e.mean shown by vertical lines.

Table 2 Affinity of (±)-CP 96,345, GR 82,334 and MEN 10,376 toward contractions produced by the selective NK₁ receptor agonist [Sar⁹] substance P sulphone in the guinea-pig isolated proximal urethra

Antagonist	pA ₂	Slopes of Schild's plot (95% CL)
(±)-CP 96,345	7.75 ± 0.06	- 1.10 (0.38–1.81)
GR 82,334	7.31 ± 0.08	- 1.26 (0.67–1.86)
MEN 10,376	inactive up to 10 μM	

pA₂ values, calculated by the constrained plot method are mean ± s.e.mean of 9–12 experiments.

field stimulation. Electrical stimulation (trains of pulses of 5 s duration, 0.2 ms pulse width, 100 V at a frequency of 5–10 Hz) produced phasic contractions of the urethral rings which were reproducible at 15–20 min intervals. The amplitude of evoked contractions averaged 8 ± 2 and 27 ± 6% of the response to KCl, at 5 and 10 Hz, respectively (*n* = 6). These responses were almost abolished (>95% inhibition) by 1 μM tetrodotoxin, indicating their neural origin. At both frequencies of stimulation the evoked contractions were also abolished by atropine (3 μM) and phentolamine (3 μM) (*n* = 4). Capsaicin (1 μM) produced a small (7.2 ± 1.5% of the res-

ponse to KCl, $n = 5$), tonic-type contraction of the urethra. A second application of capsaicin, 30 min after the first challenge, had no effect, indicating complete desensitization.

($\pm$)-CP 96,345 (0.1 μ M) almost abolished the contractile response to capsaicin, while leaving the response to electrical stimulation (5 Hz) unaffected (Figure 4).

Discussion

By use of receptor selective agonists and antagonists it appears evident that the contractile response of the guinea-pig isolated proximal urethra to tachykinins is exclusively mediated by tachykinin NK₁ receptors. The evidence supporting this statement is as follows: (a) the response to receptor-selective synthetic agonists, among which only [Sar⁹] substance P sulphone, a highly selective NK₁ receptor agonist, produced a response comparable to that of natural tachykinins; (b) antagonism by ($\pm$)-CP-96,345 and GR 82,334 which have been previously characterized as selective NK₁ receptor antagonists (Hagan *et al.*, 1991; Snider *et al.*, 1991); (c) failure of MEN 10,376, a selective NK₂ receptor antagonist (Maggi *et al.*, 1991), to affect the response to [Sar⁹] substance P sulphone and (d) antagonism of the small response to [β Ala⁸]neurokinin A(4–10) by ($\pm$)-CP 96,345 and GR 82,334.

We therefore consider the guinea-pig isolated proximal urethra as a useful 'monoreceptorial' bioassay for the NK₁ tachykinin receptor. In this respect, other preparations with this characteristic have been described, such as the dog carotid artery (D'Orleans-Juste *et al.*, 1986), the guinea-pig vas deferens (Hall & Morton, 1991) the rabbit vena cava and jugular vein (Nantel *et al.*, 1990; 1991). Owing to the recent proposals of the existence of species-related variants of the tachykinin NK₁ receptor vs. NK₁ receptor subtypes (Gitter *et al.*, 1991; Beresford *et al.*, 1991), it is important to compare the activity of various NK₁ receptor antagonists in different preparations from various species. The affinities of ($\pm$)-CP 96,345 and GR 82,334 for the NK₁ receptor in the guinea-pig urethra are very similar to those observed in other preparations from this same species (e.g. guinea-pig ileum and vas deferens). Thus pA_2 values of 8.11 and 7.79 have been determined for ($\pm$)-CP 96,345 in the guinea-pig ileum and vas deferens, respectively (Lecci *et al.*, 1991; Patacchini & Maggi, unpublished data) vs. a pA_2 value of 7.75 measured here in the guinea-pig urethra. Likewise the pA_2 value of GR 82,334 in the guinea-pig ileum (7.64, Hagan *et al.*, 1991) is very close to that reported here (7.31) for the guinea-pig proximal urethra. Overall, the results of these experiments do not

Figure 4 Effect of ($\pm$)-CP 96,345 0.1 μ M (hatched columns) on contraction of the guinea-pig isolated proximal urethra produced by electrical field stimulation (EFS) or capsaicin; control, open columns. Each column is mean of 4 experiments; vertical bars show s.e.mean. Significantly different from control, * $P < 0.05$.

support the idea of heterogeneity of NK₁ receptors in peripheral preparations from guinea-pigs.

Local motor responses ascribable to release of sensory neuropeptides (neurogenic inflammation) have been described as occurring throughout the urinary tract in rats and guinea-pigs (Maggi, 1991 for review). The present findings support the idea that endogenous tachykinins released from peripheral endings of capsaicin-sensitive primary afferents play a role as neurotransmitters by activating NK₁ receptors. The magnitude of the contractile response observed following capsaicin application is small as compared to that produced by exogenous tachykinins or other stimuli (KCl or electrical field stimulation). It may be that other effects produced by tachykinins in the guinea-pig urethra (e.g. plasma protein extravasation) (Abelli *et al.*, 1989) are more important in the general economy of urethral function regulation by sensory nerves in this species. On the other hand, the presence of tachykinin receptors on urethral smooth muscle in this and other species, including man, may indicate a role for locally released tachykinins in the genesis of abnormal urethral contractions (urethral instability) which could take place during chemical or mechanical irritation of primary afferent nerves at this level.

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