A pharmacological study of NK₁ and NK₂ tachykinin receptor characteristics in the rat isolated urinary bladder

¹Judith M. Hall, Joanna M. Flowers & Ian K.M. Morton

Pharmacology Group, Biomedical Sciences Division, King's College London, Manresa Road, London SW3 6LX

1 We have estimated potencies of tachykinin receptor agonist and antagonist analogues in order to determine the recognition characteristics of tachykinin receptors mediating phasic contractile responses of the rat isolated urinary bladder *in vitro*.

2 The NK₁-selective synthetic agonists, substance P methyl ester and GR73632, the synthetic NK₂-selective agonists [β -Ala⁸]-NKA(4-10) and GR64349, and the mammalian tachykinins, neurokinin A and neurokinin B, were assayed relative to substance P and were found to be approximately equipotent. The NK₃-selective agonist, senktide, was inactive (10 μ M).

3 Potencies of all these agonists were not significantly different ($P > 0.05$) when experiments were carried out in the presence of the neutral endopeptidase inhibitor, phosphoramidon, and the kininase II inhibitor, enalaprilat (both 1 μ M).

4 The NK₁-selective antagonist, GR82334, inhibited responses to substance P methyl ester in a competitive manner in the rat urinary bladder and the rat ileum, and also in the guinea-pig ileum. Markedly different pK_B estimates were obtained in the rat bladder (6.38) and rat ileum (6.56) compared to the guinea-pig ileum (7.42). GR82334 (3 μ M) was inactive against responses of the rat bladder to [β -Ala⁸]-NKA(4-10).

5 The NK₁-selective antagonist ($\pm$)-CP-96,345 also inhibited responses of the rat bladder and guinea-pig ileum to substance P methyl ester; however, in the rat bladder at 1 μ M, this antagonist reversibly inhibited responses both to the NK₂-selective agonist [β -Ala⁸]-NKA(4-10) and to the muscarinic agonist carbachol ($P \leq 0.01$), thus showing evidence of some non-selective depressant actions.

6 The NK₂-selective antagonists, MEN10207 and L-659,874, competitively inhibited responses of the rat bladder to the NK₂-selective agonist [β -Ala⁸]-NKA(4-10) giving pK_B estimates of 5.75 and 6.68, respectively. Both antagonists (10 μ M) were inactive against responses to the NK₁-selective agonist substance P methyl ester.

7 These results support the proposal of a mixed population of NK₁ and NK₂ receptors mediating contraction of the rat isolated urinary bladder. The NK₂ receptor is characterized by a relatively low affinity for the NK₂-selective antagonist MEN10207 but a high affinity for L-659,874. The NK₁-mediated responses are inhibited by ($\pm$)-CP-96,345: this compound however, has non-specific depressant effects in the rat bladder at high concentration (1 μ M). In contrast, the NK₁-receptor peptide antagonist GR82334, did not have non-specific depressant effects and competitively inhibited NK₁ responses in the rat bladder and rat ileum with an affinity significantly lower than at the NK₁-receptors in the guinea-pig ileum.

Keywords: Neurokinin; substance P; urinary bladder (rat); tachykinin receptors; neurokinin antagonists; tachykinin; NK₁ receptors; NK₂ receptors; ($\pm$)-CP-96,345, GR82334

Introduction

The currently accepted classification scheme for tachykinin receptors (see, Henry, 1987; Guard & Watson, 1991) identifies three distinct types, NK₁, NK₂ and NK₃, the structural sequences of which have been elucidated (Masu *et al.*, 1987; Yokota *et al.*, 1989; Shigemoto *et al.*, 1990; Nakanishi, 1991). It is now becoming clear that this scheme may need to be refined to take into account species homologues of the same receptor type. In particular, Maggi and co-workers (Maggi *et al.*, 1990) have reported that the NK₂-selective antagonist [Tyr^5 ,D-Trp^{6,8,9},Arg¹⁰]-NKA(4-10) (MEN10207) (Rovero *et al.*, 1990) has high affinity for NK₂ tachykinin receptors in the rabbit pulmonary artery ($pA_2 = 7.65$), but has relatively low affinity for the NK₂ receptors of the hamster trachea ($pA_2 = 5.80$). In contrast, the NK₂-selective hexapeptide antagonist Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH₂ (Maggi *et al.*, 1990) shows a reversed selectivity (pA_2 in hamster trachea 7.67, as compared to 5.42 in the rabbit pulmonary artery). These and other observations, (Buck *et al.*, 1990; van Giersbergen *et al.*, 1991), have led to specula-

tion as to the existence of subtypes or species homologues of NK₂ receptors (Maggi *et al.*, 1990; Patacchini *et al.*, 1991). Very recently, the novel non-peptide NK₁-selective antagonist ($\pm$)-CP-96,345 (Snider *et al.*, 1991) has been reported to have variable affinities at NK₁ receptors when compared between a number of different preparations (Beresford *et al.*, 1991; Gitter *et al.*, 1991; McLean *et al.*, 1991; Rouissi *et al.*, 1991; Snider *et al.*, 1991). It has been suggested that such differences in affinities of antagonists at NK₁ and NK₂ receptors may be related to between-species differences in recognition properties of these tachykinin receptors (Beresford *et al.*, 1991; Gitter *et al.*, 1991; Hall *et al.*, 1992; Watling *et al.*, 1991).

The isolated urinary bladder from a number of species contracts in response to substance P and related tachykinins (Falconieri-Erspamer *et al.*, 1973), and there is evidence suggesting a physiological or pathophysiological role for endogenous tachykinins, released from afferent endings of sensory nerves, in the micturition reflex (Maggi & Meli, 1986; Maggi *et al.*, 1987a). The post-junctional receptors on rat bladder smooth muscle that are involved in the phasic contractile response to tachykinins have been characterised in terms of potencies of agonist analogues in functional studies

¹Author for correspondence.

(Watson *et al.*, 1983; Maggi *et al.*, 1987a,b; 1988) and further defined in radioligand binding studies (Burcher & Buck, 1986; Nimmo *et al.*, 1992). Such studies have indicated the presence of both NK₁ and NK₂ receptors, predominantly on the smooth muscle cells. Although NK₂-selective antagonist ligands have been tested in the rat isolated urinary bladder preparation against responses to substance P and neurokinin A (Maggi *et al.*, 1991), at the present time there have been no reports of quantitative pharmacological analysis of the post-junctional tachykinin receptors using recently available selective ligands.

The aim of this study, therefore, was to investigate further the recognition characteristics of the NK₁ and NK₂ tachykinin receptors mediating phasic contractile responses of the rat isolated urinary bladder, using a range of both naturally occurring agonist and synthetic agonist and antagonist ligands. In particular, the affinities of the NK₁-selective antagonists GR82334 (Hagan *et al.*, 1991a) and (±)-CP-96,345 (Snider *et al.*, 1991), and the NK₂-selective antagonists L-659,874 (McKnight *et al.*, 1988) and MEN10207 (Rovero *et al.*, 1990) were determined. Because the rat urinary bladder is not a monoreceptor system, to obtain reliable antagonist affinity estimates at tachykinin receptors, it was necessary to test these analogues against agonists established as being highly selective for the tachykinin receptor types. The two agonists chosen were, for NK₁ receptors substance P methyl ester (Cascieri *et al.*, 1981; Watson *et al.*, 1983), and for NK₂ receptors, [β-Ala⁸]-NKA(4-10) (Rovero *et al.*, 1989). The antagonist affinities estimated in this study are compared with those obtained from preparations taken from the same and another species, in order to gain evidence regarding possible species-dependent differences in receptor recognition properties.

Preliminary accounts of this work have been communicated to the British Pharmacological Society (Flowers *et al.*, 1991) and the European Neuropeptide Club (Hall *et al.*, 1992).

Methods

General

Male Wistar rats (200–400 g) were killed by stunning and exsanguination. The urinary bladder was excised and superficial fat and connective tissue were removed. The lower third of the bladder was discarded and four preparations of the detrusor muscle (with adhering mucosa) were cut longitudinally from the bladder.

Preparations were mounted under 0.75 g initial tension in 2.5 ml silanised glass organ baths and responses were measured isometrically with Grass FT03B transducers coupled to Grass Model 7E polygraphs or to JJ Instruments Cr452 potentiometric flat-bed recorders (after d.c. amplification).

Experiments were carried out in Krebs solution (composition mM: Na⁺ 140, K⁺ 5.9, Cl⁻ 104.8, H₂PO₄⁻ 1.2, HCO₃⁻ 24.9, Ca²⁺ 2.6, Mg²⁺ 1.15, SO₄²⁻ 1.15, glucose 10), maintained at 37°C and oxygenated with 95% O₂:5% CO₂. Unless stated otherwise, the Krebs solution contained atropine, ibuprofen, mepyramine, cimetidine, guanethidine (all 1 μM) and hexamethonium (10 μM).

In all experiments, preparations were left to equilibrate for 60 min, followed by estimation of the maximal response to carbachol.

Agonist studies

Four preparations were set up in parallel, with two bathed in Krebs solution containing the neutral endopeptidase inhibitor phosphoramidon and the kininase II inhibitor, enalaprilat (both 1 μM), and two in Krebs solution without peptidase inhibitors. A randomised dosing protocol (agonist and con-

centration) was adopted to compare the potencies of tachykinins (1 nM–3 μM) in two series of experiments. In the first series, the NK₁-selective agonists substance P methyl ester and GR73632 were assayed relative to substance P as standard; and in the second series the mammalian tachykinins neurokinin A and neurokinin B were assayed along with the NK₂-selective agonists [β-Ala⁸]-NKA(4-10) and GR64349 also relative to substance P. A 5 min dose-cycle was used for drug concentrations up to 0.1 μM, extended, as necessary, to 10–20 min for drug concentrations greater than 0.1 μM. Preparations were washed as soon as peak phasic responses had been achieved. The NK₃-selective agonist succ-[Asp⁶,Me-Phe⁸]-SP(6-11) (senktide; Wormser *et al.*, 1986) was found to be inactive (10 μM), so was not included in any formal bioassay procedure.

Antagonist studies

A three-point log concentration-response curve was obtained for the agonist under study using serial dosing, which was repeated either in the presence of increasing concentrations of tachykinin receptor antagonist (test) or no antagonist (concurrent time control). A 20 min dose cycle with a 15 min antagonist incubation time was used, and up to four antagonist concentrations were tested in each individual preparation. A 15 min antagonist incubation time was chosen since preliminary studies indicated no change in pK_B estimates when a longer incubation period was used (data not shown). Experiments with MEN10207 and L-659,874 were carried out in the presence of the phosphoramidon and enalaprilat (both 1 μM).

NK₁ antagonist studies

In the rat bladder, the NK₁-selective antagonists GR82334 (0.3–10 μM) and (±)-CP-96,345 (0.1–3 μM) were tested against responses to both substance P methyl ester and neurokinin A. To assess specificity, GR82334 (3 μM) and (±)-CP-96,345 (1 μM) were also tested against responses to submaximal concentrations of [β-Ala⁸]-NKA(4-10) and carbachol. Three control submaximal responses to [β-Ala⁸]-NKA(4-10) and carbachol were obtained, and these were then repeated after a 15 min incubation with antagonist. If inhibition was seen, responses to control doses of agonist were redetermined following a 60 min washout period. For comparison, the antagonists (±)-CP-96,345 (10 nM–0.3 μM) and GR82334 (30 nM–3 μM) were also assayed using Schild plot analysis of antagonism of substance P methyl ester on the longitudinal smooth muscle of the guinea-pig ileum and GR82334 (1–10 μM) in the intact rat ileum (for methods, see Hall & Morton, 1991).

NK₂ antagonist studies

In the rat bladder, the NK₂-selective antagonist MEN10207 (1–10 μM) was tested against responses to [β-Ala⁸]-NKA(4-10). The NK₂-selective antagonist L-659,874 (0.1–10 μM) was tested against responses to [β-Ala⁸]-NKA(4-10) and neurokinin A. Both antagonists (10 μM) were tested for selectivity against responses to submaximal concentrations of substance P methyl ester. Three control submaximal responses to substance P methyl ester were obtained and these were repeated following a 15 min incubation with antagonist.

Source of drugs

Agents were obtained as follows: carbachol, atropine sulphate, ibuprofen and hexamethonium bromide (Sigma, U.K.), mepyramine maleate (May & Baker, U.K.), enalaprilat (Merck, Sharp & Dohme, U.S.A.), cimetidine (Smith, Kline & French, U.K.), guanethidine sulphate (Ciba, U.K.), δ-aminovaleryl-L-Pro⁹,N-MeLeu¹⁰-SP(7-11) (GR73632) and [Lys³,Gly⁸-R-γ-Lactam, Leu⁹]-NKA(3-10) (GR64349) (Glaxo

Group Research, U.K.), [β -Ala⁸]-NKA(4-10) (A. Menarini Pharmaceuticals, Italy; or Bachem, U.K.), [Tyr⁵,D-Trp^{6,8,9},Arg¹⁰]-NKA(4-10) (MEN10207) (A. Menarini Pharmaceuticals, Italy or Peninsula Laboratories, Europe), ($\pm$)-CP-96,345 ([[(2S,3S)-*cis*-2-(diphenylmethyl)-N-[2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine], A. Menarini Pharmaceuticals, Italy), Ac-Leu-Met-Gln-Trp-Phe-Gly-NH₂ (L-659,874) (Cambridge Research Biochemicals, U.K.), [D-Pro⁹[Spiro- γ -Lactam]Leu¹⁰,Trp¹¹]-physalaemin(1-11) (GR82334) (Peninsula Laboratories Europe or Glaxo Group Research), substance P methyl ester and phosphoramidon (Peninsula Laboratories Europe, U.K.). All other peptides were obtained from Bachem, U.K. All salts used were of analytical grade and were obtained from B.D.H., U.K.

Ibuprofen was dissolved in 5% Na₂CO₃. ($\pm$)-CP-96,345, senktide, L-659,874 and neurokinin B were dissolved in DMSO. All other peptides and agents were dissolved in distilled water. Peptides were stored under N₂ at -20°C.

Expression of results and statistical analysis

Contractile responses are expressed as % of maximal response to carbachol, and the estimates are shown as means $\pm$ s.e.mean. Relative activities were calculated from differences in mean pD₂ (-log EC₅₀, 50% carbachol maximum) estimates within each series. Tests for significance were made by Student's *t* test for paired- or two-independent samples as appropriate. The sensitivity of the rat bladder both to substance P methyl ester and to [β -Ala⁸]-NKA(4-10) tended to decrease after construction of the first control curve, but then stabilised for subsequent curves (data not shown). Consequently, individual dose-ratio estimates (*x*) between test and concurrent control preparations where measured, and from these values, individual p*K*_B estimates ($\pm$ s.e.mean) were obtained for competitive interactions from the Gaddum-Schild equation, $pK_B = \log_{10} (x-1) - \log_{10} [A]$, where [A] is the antagonist concentration. Conventional linear regression analysis of Schild plots was used to test for significant departure of the estimated slope (*b*) from a population value (β) of unity slope. The coefficient of linear correlation was used as a measure of goodness-of-fit. Throughout, *n* refers to the number of estimates (p*K*_B), or number of preparations (all other estimates), and in every case represents data obtained from 3–8 animals.

Results

General

Contractile responses of the rat bladder to tachykinins reached a peak phasic response at a rate that depended somewhat on concentration, but was generally within 2 min, after which time preparations were washed until they regained base-line tension.

Agonist studies

Figure 1 shows log concentration-response curves obtained in the two series of experiments in the rat bladder where a range of tachykinins were assayed relative to substance P; in the first series (Figure 1a) for the NK₁-selective agonists; and in the second for the mammalian tachykinins and NK₂-selective agonists (Figure 1b). Mean pD₂ values were estimated at 50% the maximal response to carbachol since maximal responses to individual peptides were not obtained, and these estimates along with activities relative to substance P calculated from these data are shown in Table 1. The peptidase inhibitors phosphoramidon and enalaprilat were without significant effect on agonist pD₂ estimates (*P* > 0.05; see Table 1; Hall *et al.*, 1990).

Figure 1 Mean log concentration-response curves in the rat isolated urinary bladder for agonists active at NK₁ or NK₂ receptors. (a) Shows the NK₁ selective agonists, GR73632 ($\blacktriangle$) and substance P methyl ester ($\blacksquare$); (b) shows the NK₂ selective agonists GR64349 ($\square$) and [β -Ala⁸]-NKA(4-10) ($\blacksquare$), along with neurokinin A ($\blacktriangle$) and neurokinin B ($\blacktriangledown$). Substance P ($\bullet$) was included as an internal standard in both series. Each point expressed as % maximal response to carbachol (CCh) is the mean $\pm$ s.e.mean (vertical bars) for 6 preparations.

NK₁ antagonist studies

In the rat bladder and rat ileum, the NK₁-selective antagonist, GR82334, competitively antagonized responses to substance P methyl ester, and p*K*_B estimates of 6.38 ± 0.13 (*n* = 22) and 6.56 ± 0.09 (*n* = 14) respectively were obtained (see Figure 2a and Table 2). It should be noted that the rat ileum preparation did not appear to offer many advantages as an example of rat NK₁ preparation, in that like the bladder it is not a monoreceptor system, and furthermore then NK₁-receptor-induced contractions were much weaker and rather more variable than in the bladder. GR82334 also competitively antagonised responses to substance P methyl ester in the guinea-pig ileum preparation, but with an affinity that was significantly (*P* < 0.001) higher (p*K*_B = 7.42, Figure 2a) than that obtained in the rat bladder and rat ileum. Over a range of concentrations (0.3–10 μ M) in the bladder, GR82334 showed variable antagonism of responses to neurokinin A (see Table 2). In tests for selectivity in the bladder, GR82334 (3 μ M) was inactive against responses to

Table 1 Activity estimates for neurokinin receptor agonists in the rat urinary bladder

Agonist	Without inhibitors		With inhibitors	
	pD_2 ($\pm$ s.e.mean)	Relative activity	pD_2 ($\pm$ s.e.mean)	
Substance P	6.03 (0.24)	1.00	6.26 (0.12)	
GR73632	6.23 (0.18)	1.59	6.28 (0.22)	
Substance P methyl ester	5.70 (0.29)	0.47	6.07 (0.13)	
Substance P	6.65 (0.30)	1.00	6.39 (0.11)	
Neurokinin A	7.05 (0.17)	2.5	6.79 (0.10)	
Neurokinin B	6.29 (0.15)	0.47	6.08 (0.20)	
GR64349	6.50 (0.20)	0.71	6.59 (0.30)	
[β -Ala ⁸]-NKA(4-10)	6.51 (0.25)	0.72	6.09 (0.23)	

The pD_2 values for each series of experiments, were calculated from $-\log EC_{50}$ estimates in 6 individual preparations: these estimates were unchanged by the presence of the peptidase inhibitors phosphoramidon and enalaprilat (both $1 \mu M$; $P > 0.05$). Activities of agonists relative to substance P (1.00) were calculated from differences in mean pD_2 estimates. The difference in absolute activity of substance P in the two series is due to biological variation, and was not significant ($P > 0.05$).

Figure 2 Schild plot analyses, in the rat urinary bladder, rat ileum, or the guinea-pig ileum, for NK_1 or NK_2 selective antagonists. Throughout, symbols are solid for means (with vertical bars denoting s.e.mean) or open for individual observations: circles are for experiments with rat urinary bladder, triangles for rat ileum, and squares for guinea-pig ileum. b = estimated coefficient of linear regression $\pm$ s.e.mean, P the significance of b (estimated slope) versus β (population slope value = 1.00) as estimated by conventional linear regression analysis of Schild plots.

(a) The NK_1 -selective antagonist, GR82334, against the NK_1 -selective agonist, substance P methyl ester, in the rat bladder ($\bullet$) ($b = 1.04 \pm 0.27$; $P > 0.05$), rat ileum ($\blacktriangle$) ($b = 0.65 \pm 0.22$; $P > 0.05$) or guinea-pig ileum ($\blacksquare$) ($b = 1.09 \pm 0.11$; $P > 0.05$). (b) The NK_2 -selective antagonist, L-659,874 against the NK_2 -selective agonist, [β -Ala⁸]-NKA(4-10) in the rat bladder ($\circ$) ($b = 0.98 \pm 0.06$; $P > 0.05$). (c) The NK_2 -selective antagonist, MEN10207, against [β -Ala⁸]-NKA(4-10) in the rat bladder ($\bullet$) ($b = 0.743 \pm 0.11$; $P > 0.05$). (d) L-659,874 against the non-selective agonist neurokinin A in the rat bladder ($\circ$) ($b = 0.24 \pm 0.07$; $P < 0.001$).

carbachol and [β -Ala⁸]-NKA(4-10) (see Figure 3b and Table 2).

In the rat bladder, ($\pm$)-CP-96,345 was found also to attenuate responses to substance P methyl ester and to neurokinin A (data not shown), and Schild regressions for both agonists were compatible with competition at a single site, yielding pK_B estimates of 6.79 and 6.61, respectively. However, in tests for selectivity in the bladder, ($\pm$)-CP-96,345 ($1 \mu M$) caused a small but significant inhibition ($P \leq 0.01$) of submaximal responses to carbachol and [β -Ala⁸]-NKA(4-10) (see Figure 3a). For these reasons, interpolation from these data is potentially misleading. In the guinea-pig ileum preparation, ($\pm$)-CP-96345 competitively antagonized responses to substance P methyl ester with an affinity ($pK_B = 9.00$) that was significantly higher than its apparent affinity in the bladder ($P < 0.001$) (data not shown).

NK₂ antagonist studies

The NK_2 -selective antagonist, L-659,874, competitively antagonized contractile responses to the NK_2 -selective agonist [β -Ala⁸]-NKA(4-10). These data are displayed as a Schild plot in Figure 2b. A pK_B of 6.68 was calculated (Table 2). L-659,874 ($10 \mu M$) was inactive against responses to the NK_1 -selective agonist substance P methyl ester ($P > 0.05$, Table 2). When tested against the subtype non-selective agonist neurokinin A, L-659,874 (0.1 – $20 \mu M$) attenuated responses, but Schild analysis showed a shallow regression line ($b = 0.24$) that differed significantly from unity ($P < 0.01$) (see Figure 2d), compatible with an action of neurokinin A at a site additional to the NK_2 -receptor.

In the bladder, the NK_2 -selective antagonist MEN10207 competitively antagonized responses to the NK_2 -selective agonist [β -Ala⁸]-NKA(4-10), though with a relatively low affinity ($pK_B = 5.75$; see Figure 2c and Table 2), but was inactive ($10 \mu M$) against contractile responses to substance P methyl ester (Table 2). At $10 \mu M$, MEN10207 showed partial agonist activity (*ca.* 5% CCh maximum; data not shown; see also Maggi *et al.*, 1991).

Table 2 Affinity estimates in the rat isolated urinary bladder for three receptor-selective antagonists when tested against the NK₁-selective agonist substance P methyl ester (SPOMe), the NK₂-selective agonist [β -Ala⁸]-NKA(4-10), and the non-selective agonist neurokinin A (NKA).

Antagonist	SPOMe pK _B	Agonist [β -Ala ⁸]-NKA(4-10) pK _B	NKA pK _B
GR82334	6.38 (0.13;22)	NA (3 μ M;7)	NC† (0.3-10 μ M;11)
MEN10207	NA (10 μ M;5)	5.75 (0.06;9)	NT
L-659,874	NA (10 μ M;5)	6.68 (0.04;9)	NC* (0.1-20 μ M;10)

Estimates of pK_B (with s.e.mean and *n*, in parentheses) are shown only where Schild plot regressions showed no significant departure ($P > 0.05$) from unity slope. NC denotes interaction not competitive with respect to a single site over the range shown in parentheses, as judged from shallow Schild regression, $b = 0.24$ (*), or variable antagonism (†). NA denotes not active (at the concentration shown in parentheses), NT denotes not tested.

Discussion

The present analysis, using selective agonists provides evidence of both NK₁ and the NK₂ receptor types, in the rat isolated urinary bladder. The lack of appreciable activity of the highly NK₃-selective agonist senktide (Wormser *et al.*, 1986) discounts any major contribution from the NK₃ receptor type under the conditions of this study (see also, Maggi *et al.*, 1988; Pietra *et al.*, 1990). Both the neutral endopeptidase inhibitor phosphoramidon and the kininase II inhibitor enalaprilat were without significant effect on agonist potencies, suggesting that peptide breakdown had not distorted agonist activity estimates. We have previously shown aminopeptidase inhibition with bestatin (100 μ M) to be without effect on agonist potencies in this preparation (Hall *et al.*, 1990).

The data obtained with tachykinin-receptor antagonists, in general conform to these conclusions, since both NK₁- and NK₂-selective antagonists competitively blocked responses to their respective receptor-selective agonists. Affinities of antagonists for both the NK₁ and the NK₂ sites differed appreciably from those found in some other preparations (see below).

NK₁ receptor characteristics

The NK₁-selective agonists, substance P methyl ester and GR73632, were both active in the rat urinary bladder, confirming earlier reports (Maggi *et al.*, 1988; Pietra *et al.*, 1990). Both these agonists are established as selective for the NK₁ receptor type, over the NK₂ and NK₃ types, as determined in monoreceptor systems (Hagan *et al.*, 1989; 1991b; Hall & Morton, 1991). These results therefore confirm the presence of NK₁ receptors in the rat urinary bladder.

The characteristics of the NK₁ receptors were further established by use of selective antagonists. Thus, the NK₁-selective antagonist, GR82334, competitively antagonized responses to substance P methyl ester in the rat urinary bladder. The pK_B obtained in the rat bladder (pK_B = 6.38) was similar to that estimated in rat ileum (pK_B = 6.56), and is about one log unit lower than the pK_B estimate against NK₁-mediated contraction in guinea-pig ileum (pK_B = 7.42). This pK_B value obtained in the guinea-pig ileum with GR82334 is in agreement with earlier reports (pK_B = 7.64; Hagan *et al.*, 1991a). These results suggest that GR82334 distinguishes between the NK₁ receptors in these two species. The variable antagonism of responses to neurokinin A by GR82334 is as expected

since this agonist interacts with both NK₁ and NK₂ receptors.

The presence of NK₁ receptors in the bladder appeared to be supported by results with the NK₁-selective non-peptide antagonist ($\pm$)-CP-96,345, which was also much more potent as an antagonist of NK₁ receptors in the guinea-pig ileum as compared to the rat urinary bladder (pK_B estimates of 9.00 and 6.79, respectively). The pK_B estimate we obtained for ($\pm$)-CP-96,345 in the guinea-pig ileum (pK_B = 9.00) is comparable to that reported by other workers (pK_B = 8.89, Beresford *et al.*, 1991; pK_B = 8.11, Lecci *et al.*, 1991). Furthermore, the affinity for ($\pm$)-CP-96,345 in the guinea-pig ileum is similar to its affinity found in some other NK₁ preparations, the rabbit aorta (pK_B = 8.81; Beresford *et al.*, 1991) and rabbit iris (pK_B = 8.85; Hall *et al.*, 1992), the dog carotid artery (pK_B = 8.7, Snider *et al.*, 1991). The relatively low pK_B estimate we obtained in the rat urinary bladder is similar to that determined in another rat preparation, the neonatal rat spinal cord (pK_B = 7.13, Beresford *et al.*, 1991).

Our finding that the affinities for ($\pm$)-CP-96,345 and GR82334 differ significantly in the guinea-pig as compared to the rat preparations, suggests that they both can discriminate between NK₁ receptors in preparations taken from the two species. Indeed, the ability of ($\pm$)-CP-96,345 to distinguish between the NK₁ receptors in a number of different tissues from several species has been demonstrated (Beresford *et al.*, 1991; 1992; Gitter *et al.*, 1991; Snider *et al.*, 1991). However, it should be noted that ($\pm$)-CP-96,345 (unlike GR82334) also inhibited contractile responses to the NK₂-selective agonist, [β -Ala⁸]-NKA(4-10), as well as those to carbachol in the rat bladder, so suggesting a non-specific component to its action as an antagonist; a characteristic also reported in some other preparations (Lecci *et al.*, 1991; Boyle *et al.*, 1991), including the guinea-pig ileum at concentrations (0.5 μ M) in the range of those used in the present study. Furthermore, non-specific effects with both the active (CP-96,345) and inactive (CP-96,344) enantiomers of this compound have been reported (Donnerer *et al.*, 1992; Schmidt *et al.*, 1992), which are apparently related to L-type calcium-channel blockade (Guard & Watling, 1992; Schmidt *et al.*, 1992). The usefulness of this antagonist as an analytical tool in pharmacological studies is therefore limited, though it is clear that it produces little antagonism of responses to substance P methyl ester in the rat bladder at concentrations causing marked antagonism in a number of preparations from other species; e.g. the guinea-pig ileum and trachea, rabbit aorta and iris (Beresford *et al.*, 1991; 1992; Hall *et al.*, 1992).

Figure 3 Tests for selectivity of antagonism by NK₁ antagonists of contractile responses in the rat urinary bladder to submaximal concentrations of carbachol and [β-Ala⁸]-NKA(4-10). In (a) results with (±)-CP-96,345 (1 µM): control (C) pre-antagonist responses (solid column) are normalized to 100% (shown by a dashed-line), and mean test (T) responses (± s.e.mean) after 5 min are shown (cross-hatched columns) followed by post-antagonist re-test (R) after washing for 60 min (open bar). The depression of responses to each of the two agonists was highly significant ($P \leq 0.01$; paired *t* test). In (b) a similar test series with GR82334 (3 µM) is shown, where there was no significant change in the magnitude of contractile response ($P > 0.05$; paired *t* tests).

NK₂ receptor characteristics

The NK₂ selective agonists, GR64349 and [β-Ala⁸]-NKA(4-10), were both potent spasmogens in the rat urinary bladder;

the results with GR64349 confirm a previous report (Pietra *et al.*, 1990). Both agonists are established as selective for the NK₂ receptor type, being weakly inactive at NK₁ and NK₃ receptors (Hagan *et al.*, 1989; 1991b; Hall & Morton, 1991). Further evidence for the presence of NK₂ receptors in the rat urinary bladder was provided by the observation that the NK₂-selective antagonist, MEN10207, competitively antagonized responses to [β-Ala⁸]-NKA(4-10). The relatively low affinity estimate ($pK_B = 5.75$) we obtained for this antagonist, suggests that the receptors in the rat urinary bladder resemble those in the hamster trachea preparation where this analogue has a reported pA_2 of 5.80 (Maggi *et al.*, 1990), rather than the receptors in the rabbit pulmonary artery where it has a higher affinity ($pA_2 = 7.65$, Maggi *et al.*, 1990). The NK₂ selective antagonist, L-659,874 also competitively antagonized responses to the NK₂-selective agonist, [β-Ala⁸]-NKA(4-10), yielding a pK_B estimate of 6.68; a value similar to that reported for this antagonist by McKnight *et al.* (1988) in the rat vas deferens ($pA_2 = 6.8$).

In experiments designed to show dual receptor interaction by neurokinin A in the rat bladder, L-659,874 antagonized responses to neurokinin A with a shallow Schild regression, as might be expected were this agonist also to interact with NK₁ receptors. This observation may therefore be taken to provide further evidence for the presence of both NK₁ and NK₂ receptors in the rat bladder.

In conclusion, the results from the present study support the proposal that both NK₁ and NK₂ receptors are involved in the contractile response of the rat isolated urinary bladder preparation to tachykinins. These data are in agreement with information gained using molecular biology techniques, where expression of mRNA for both NK₁ and NK₂ receptors has been shown in rat preparations including the urinary bladder (Hershey *et al.*, 1991; Ohkubo & Nakanishi, 1991; Takeda & Krause, 1991). Pharmacologically, the NK₂-receptor type is relatively resistant to MEN10207 whilst being relatively sensitive to L-659,874. NK₁-receptor mediated responses in the rat bladder and ileum are antagonized by GR82334, albeit with a significantly lower affinity compared with the guinea-pig ileum. Thus, such data provide evidence of heterogeneity within both the NK₁- and NK₂-receptor classes in different preparations. This heterogeneity may, at least in part, prove to be attributable to inter-species differences, as might be anticipated from the between-species variations demonstrated in known primary structural sequences of tachykinin receptors. As an analytical tool, GR82334 appears valuable for the further study of NK₁ receptor heterogeneity, in as much as it has reasonably high affinity without the undesirable effects of some antagonists, in particular the non-specific depressant effects observed with CP-96,345.

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