Typical and atypical NK₁ tachykinin receptor characteristics in the rabbit isolated iris sphincter

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1 A contraction of the rabbit isolated iris sphincter smooth muscle follows activation of either tachykinin NK₁ or NK₃ receptors. We have here characterized the pharmacological activity profiles of various tachykinin receptor agonists considered to have NK₁-receptor-preferring activity in this preparation.

2 Two groups of NK₁-receptor-preferring agonists could be distinguished in terms of a common pharmacological profile. The first group (Group 1) included [Glp⁶,L-Pro⁹]-SP(6-11) (septide), [Glp⁶]-SP(6-11), substance P methyl ester, δ -aminovaleryl-[L-Pro⁹, N-MeLeu¹⁰]-SP(7-11) (GR73632), and [Apa⁹⁻¹⁰]-SP. The second group (Group 2) included [Pro⁹]-SP, substance P, physalaemin and ranamargin.

3 Under control conditions, the responses to Group 1 agonists were relatively fast in offset (time for reversal of maximal responses, 11.2–18.2 min), and were antagonized by NK₁-receptor-selective antagonists (range of pK_B estimates vs various agonists; GR82334, 7.1–8.2; ($\pm$)-CP-96,345, 8.9–9.5; RP67580, 7.0–7.4). Following incubation of the tissue with phenoxybenzamine (20 μ M, 10 min), the affinity of GR82334, tested against the Group 1 agonists, substance P methyl ester and septide, was not significantly different ($P < 0.05$; $n = 7$ –18) to that determined in untreated tissues (substance P methyl ester pK_B 7.5 $\pm$ 0.1 and 7.2 $\pm$ 0.2, respectively; septide 7.7 $\pm$ 0.2 and 7.9 $\pm$ 0.2, respectively). Further, response offset times (5.0–8.5 min) were little reduced as compared to those observed in untreated tissues.

4 Under control conditions, the response to Group 2 agonists was markedly slow in offset (times for reversal of maximal responses, 51.4–70.4 min), and was not attenuated significantly by the NK₁-receptor-selective antagonists GR82334 (1 μ M), ($\pm$)-CP-96,345 (0.1 μ M) or RP67580 (1 μ M). In contrast, after phenoxybenzamine pretreatment, responses to Group 2 agonists reversed rapidly (times for reversal of maximal responses, 13.1–24.2 min), and were now antagonized by GR82334 (pK_B estimates, 6.4–7.1).

5 The responses to the NK₃-receptor-selective agonist Succ-[Asp⁶,Me-Phe⁸]-SP(6-11) (senktide) were relatively fast in offset (time for reversal of maximal response was 18.6 $\pm$ 1.7 min) and were not inhibited by GR82334 (10 μ M; $n = 5$). The contractile response resulting from co-application of the Group 1 agonist, septide together with senktide, did not exhibit prolonged response offset kinetics.

6 Assuming simple competition at equilibrium, these data from the rabbit iris smooth muscle could be explained either by interaction of the various ligands with two separately-existing NK₁ receptor-subtypes or -isoforms; or alternatively by a preferential interaction of the two agonist groups with different binding domains on a common NK₁ receptor.

Keywords: Tachykinin receptors; iris sphincter; substance P; NK₁ antagonist; GR82334; phenoxybenzamine; neurokinin; ($\pm$)-CP-96,345; RP67580; receptor subtypes

Introduction

Stimulation both of NK₁ and NK₃ receptors, causes contraction in the rabbit isolated iris sphincter preparation (Hall *et al.*, 1991; 1993b; Håkanson *et al.*, 1991; see Hall, 1994). However, in our preliminary studies we identified profound differences in the pharmacological characteristics of certain tachykinins and analogues considered to act preferentially at NK₁ receptors in this preparation (Hall *et al.*, 1991; 1993c). Thus, the contractile responses to several NK₁-receptor-preferring tachykinins including substance P methyl ester, [Glp⁶,L-Pro⁹]-SP(6-11) (septide), and δ -aminovaleryl-[L-Pro⁹, N-MeLeu¹⁰]-SP(7-11) (GR73632) reverse rapidly (<20 min; see Hall *et al.*, 1991; 1994) and are readily inhibited by NK₁ receptor antagonists with affinities typical of NK₁ receptors in rabbit preparations (Hall *et al.*, 1994). In contrast, other agonist ligands including substance P and physalaemin, show 'atypical' responses since these are maintained for very long periods (>1 h) and, further, are relatively resistant to blockade by NK₁ receptor antagonists (Muramatsu *et al.*, 1987; Too *et al.*, 1988; Hall *et al.*, 1994). These contrasting washout-kinetics of tachykinins and analogues seem not to

result from differences in the metabolic stability of the peptides (Hall *et al.*, 1991), nor from the differential release of other mediators (Too *et al.*, 1988). We have previously referred to the former group of agonists with 'typical' responses as Group 1, and those with 'atypical' responses as Group 2 (Hall *et al.*, 1994): this convention will be adopted here for simplicity of description, and the division will be re-examined in the Discussion.

There are reports of responses to substance P being mediated through activation of atypical tachykinin receptors in other preparations (Petitet *et al.*, 1992). Indeed there are proposals both for the existence of distinct subtypes of NK₁ receptors, and for inter-species variants or homologues (see Hall *et al.*, 1993a; Maggi *et al.*, 1993). In view of these issues, it would be of the greatest pharmacological significance if subtypes of NK₁ receptor could be demonstrated within a single tissue from a given animal species. The aim of our study, therefore, was to investigate whether NK₁-receptor-preferring agonists interact with two different NK₁ receptor subtypes in rabbit iris sphincter, or if other reasons can be advanced to explain the profoundly different pharmacological profiles of these groups of NK₁-receptor-preferring agonists. To this end, we have compared the con-

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tractile responses of a number of NK₁-receptor-preferring agonists with respect to their washout-kinetics, sensitivity to inhibition by the NK₁-receptor selective antagonists GR82334 (Hagan *et al.*, 1991); (±)-CP-96,345 (Snider *et al.*, 1991); RP67580 (Garret *et al.*, 1991; see Maggi *et al.*, 1993); and modification of these characteristics by pretreatment with phenoxybenzamine. There are previous reports of phenoxybenzamine pretreatment showing agonist-dependent effects with certain tachykinins in various preparations, including the rabbit iris (Too *et al.*, 1988; Hall *et al.*, 1993c; 1994).

Preliminary accounts of this work have been communicated to the British Pharmacological Society (Hall *et al.*, 1994) and to the International Symposium on Substance P and Related Peptides, Shizuoka, Japan (Hall *et al.*, 1993c).

Methods

Tissue preparation and recording arrangements

Male New Zealand albino rabbits (2.5–3.0 kg) were killed by an overdose of i.v. pentobarbitone sodium (Sagatal). The eyes were enucleated immediately after death and opened by an incision 2–3 mm dorsal to the limbus, followed by excision of the iris from the ciliary margin. The sphincter pupillae muscles were dissected free of dilator muscle and mounted intact on stainless steel tissue-holders in 2.5 ml silanised glass organ baths in Krebs solution at 37°C. The preparations were attached to isometric Grass FT03B force-displacement transducers under an initial resting tension of 150 mg. The mechanical activity was recorded on Grass model 7E polygraphs. The composition of the Krebs solution was (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. The Krebs solution was maintained at pH 7.4 by constant bubbling with 95% O₂:5% CO₂, and contained hexamethonium (100 µM), mepyramine, cimetidine, guanethidine and ibuprofen (all 1 µM), and atropine (1 µM) (except in experiments with phenoxybenzamine since effective phenoxybenzamine treatment was estimated in terms of muscarinic blockade, where atropine treatment would preclude this).

Tachykinin receptor antagonist studies

An equilibration period of 60 min was allowed. In all experiments, paired irises from the same animal were used. Full cumulative concentration-response curves were obtained for agonists (see Results), either in the absence (concurrent time control preparations), or the presence (test preparations), of GR82334 (1, 3.2 or 10 µM), RP67580 (1, 3.2 or 10 µM) or (±)-CP-96,345 (32, 100 or 320 nM), with up to four agonists applied to each preparation in a random order. In experiments with substance P, physalaemin, ranamargarin and [Pro⁹]-SP, the prolonged washout of responses did not allow determination of more than one concentration-response curve in a single preparation at the end of the experiment (see Results). With GR82334, additional experiments were carried out as above, but in preparations pretreated with phenoxybenzamine (20 µM, 10 min application followed by washing every 5 min for 20 min; see Too *et al.*, 1988). In these experiments, carbachol was included as an independent test for effective (muscarinic) receptor alkylation, which was feasible since in these experiments atropine was omitted from the medium. Antagonist incubation time was 5 min, except in some additional experiments with GR82334 against responses to substance P; where in testing for possible non-equilibrium conditions, the antagonist incubation time was increased to 30 min.

Co-administration of septide and senktide

The time course was investigated of the composite response following co-administration *ca.* EC₅₀ concentrations of both

the NK₁-receptor-selective agonist septide (1–3 nM) and the NK₃-receptor-selective agonist senktide (1–3 nM).

Washout of responses to agonists

In preparations where tachykinin receptor antagonists had not been used, the rates of offset of responses to tachykinins were estimated. After cumulative application of doses to reach a maximum response, the organ bath was thoroughly washed out and this was repeated at 5 min intervals until tension had resumed the pre-dose level. Because of their very slow offset rates, estimates for Group 2 agonists were made only at the end of experiments: other agonists were applied in a random order.

Source of agents used

Agents were obtained as follows: Sagatal (RMB Animal Health Ltd., U.K.), carbamylcholine chloride (carbachol), hexamethonium bromide, ibuprofen, (Sigma, U.K.), mepyramine maleate (May & Baker, U.K.), guanethidine sulphate (CIBA, U.K.), substance P, substance P methyl ester (Bachem, U.K.), [Glp⁶]-SP(6-11), physalaemin, Succ-[Asp⁶, Me-Phe⁸]-SP(6-11) (senktide), ranamargarin (Peninsula Laboratories, Europe), δ-aminovaleryl-L-Pro⁹, N-MeLeu¹⁰-SP(7-11) (GR73632), and [D-Pro⁹][Spiro-γ-lactam]Leu¹⁰, Trp¹¹]-physalaemin(1-11) (GR82334) (Peninsula Laboratories Europe; or Glaxo Group Research, U.K.), phenoxybenzamine (Smith, Kline & French, U.K.), [Glp⁶, L-Pro⁹]-SP(6-11) (septide, gift Dr B. Williams, Merck, Sharp and Dohme, U.K.), [Pro⁹]-SP(1-11) (Neosystems, France), [Apa⁹⁻¹⁰]-SP(1-11) (Apa = aminopentanoic acid) (gift Dr S. Lavielle, Université Pierre et Marie Curie, Paris, France), (3αR, 7αR)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl]-perhydroisoin-dol-4-one (RP67580); gift Dr C. Garret (Rhône-Poulenc Rorer, France), [(2s,3s)-cis-2-(diphenylmethyl)-N-(2-iodo-phenyl)-methyl]-1-azabicyclo-[2,2,2]octan-3-amine ((±)-CP-96,345); gift Dr C.A. Maggi (A. Menarini Pharmaceuticals, Italy). All salts were of analytical grade and were obtained from B.D.H., U.K.

Senktide and (±)-CP-96,345 were dissolved in dimethylsulphoxide, RP67580 was dissolved in 0.05 M HCl, [Glp⁶]-SP(6-11) was dissolved in formic acid, and ibuprofen was dissolved in 5% Na₂CO₃. All other agents, and all dilutions, were made up in distilled water. Peptides and non-peptide tachykinin receptor antagonists were stored at -20°C. Phenoxybenzamine was dissolved and diluted in 0.05 M hydrochloric acid (see Too *et al.*, 1988). Dimethylsulphoxide and hydrochloric acid (0.05 M) (administered in volumes used in experiments), did not effect basal tension *per se* or the concentration-response curve to neurokinin A (*n* = 4 each, data not shown).

Expression of results and statistical analysis

For estimation of time of reversal of agonist responses, the times to reach 75%, 50%, 25% and 0% of maximal agonist-induced tension were measured from individual traces, and from these values the arithmetic mean and s.e.mean were calculated.

In all other experiments, responses were calculated as % maximal response to carbachol (obtained with 1–10 mM carbachol, in view of the presence of atropine in the Krebs medium) in individual preparations, determined at the start of experiments. Data from individual experiments were combined and analysed in two ways. To allow conventional graphical display, responses were calculated for each concentration of test agonist in individual experiments, and plotted as mean responses ± s.e.mean. For the purposes of obtaining objective slope and EC₅₀ estimates, individual agonist concentration-response relationships were analysed by determining the least-squares fit of data to the logistic function $y = y_{\max} \frac{A^p}{(A^p + A_{50}^p)}$; where *y* is the response (expressed in

terms of maximum responses to carbachol), $y_{\max}$ is the estimated maximum response to that tachykinin, A is the tachykinin concentration, A_{50} is the concentration producing 50% of that agonist's maximum response (the EC_{50}), and p is the slope factor (equivalent to the Hill slope). These curve-fitting procedures were carried out using ORIGIN version 2.8 computer software (MicroCal Software Inc., MA, U.S.A.). Tests for significant differences were made using Student's t test for single- or two-samples, as appropriate. The pK_B estimates were determined from individual dose-ratios using the Gaddum-Schild equation: $pK_B = -\log K_B = \log (x-1) - \log [A]$, where x is the dose-ratio and $[A]$ the applied antagonist concentration. Affinities are described as pK_B values when Schild plot analysis showed no significant departure from unity slope, or as apparent pK_B values when such analysis could not be carried out because a limited antagonist concentration range was used (see Results). In experiments measuring the effect of antagonists against responses to substance P, [Pro⁹]-SP, physalaemin or ranamargarin under normal control conditions, in view of the prolonged washout of responses, dose-ratios were estimated between control log concentration-response curves obtained in concurrent time-control preparations (no antagonist) and log concentration-response curves obtained in test preparations (with antagonist), studied in parallel in paired preparations taken from the same animal. Estimates of pK_B obtained with this protocol, and calculated in this manner, with Group 1 agonists did not differ significantly ($P > 0.05$) from those obtained using within-preparation controls. In all cases n refers to the number of estimates (pK_B) or preparations (all other estimates), and represents data obtained from 4–8 animals (except as indicated in Table 1).

Results

Kinetics

The NK₁-receptor-preferring agonists differed in their relative potencies, but not in any consistent way in their slopes or maxima (Table 1; see also Hall *et al.*, 1991; 1993b). The rates of offset of contractile responses to tachykinins and analogues after washout, differed markedly. The responses to Group 1 agonists (GR73632, substance P methyl ester, septide, [Apa⁹⁻¹⁰]-SP, [Glp⁶]-SP(6-11)) had relatively fast offset kinetics (12.0 ± 1.8 , 15.6 ± 3.6 , 13.0 ± 2.0 , 11.2 ± 1.7 , 18.2 ± 3.8 min respectively; $n = 5-8$), and were similar to the mus-

carinic receptor agonist, carbachol (10.5 ± 2.3 min; $n = 6$). Responses to Group 2 agonists (substance P, physalaemin, ranamargarin and [Pro⁹]-SP) were very slow indeed in offset (70.4 ± 8.4 , 54.0 ± 6.7 , 51.4 ± 3.8 , 58.0 ± 3.9 min respectively; $n = 3-4$). Response washout kinetics of substance P methyl ester and substance P are represented in Figure 1. Co-application of EC_{50} concentrations of the Group 1 agonist, septide together with the NK₃-receptor-selective agonist, senktide, did not result in prolonged response offset kinetics (e.g. $t_{1/2}$ of the mixture, 3.4 ± 0.9 min, $n = 4$; cf. substance P, $t_{1/2}$ 35.3 ± 5.4 min; Hall *et al.*, 1991). Tissue pretreatment with phenoxybenzamine greatly accelerated the previously very slow washout-time of Group 2 agonists (e.g. substance P and [Pro⁹]-SP; 24.2 ± 2.4 and 18.3 ± 2.4 min respectively ($P < 0.01$, $n = 7$; $P < 0.01$, $n = 11$; respectively)). There was less acceleration of the time of offset of Group 1 agonists (e.g. substance P methyl ester, septide; 8.5 ± 2.6 and 6.7 ± 0.8 min ($P > 0.05$, $n = 5$; $P < 0.05$, $n = 7$; respectively)), or of carbachol (5.3 ± 1.1 min ($P > 0.05$, $n = 6$)) (see Figure 1).

Antagonist studies

GR82334, ($\pm$)-CP-96,345 and RP67580 antagonized responses to Group 1 agonists (GR73632, substance P methyl ester, septide, [Apa⁹⁻¹⁰]-SP, [Glp⁶]-SP(6-11)) (Table 2). Schild analysis indicated that the antagonism by GR82334 of responses to substance P methyl ester was competitive (Figure 2). However, even at high concentrations the NK₁ antagonists did not inhibit, or only weakly attenuated, the responses to Group 2 agonists (substance P, physalaemin, ranamargarin and [Pro⁹]-SP) (Table 2). Indeed, in some preparations, there was slight potentiation of agonist responses. The effect of GR82334 ($10 \mu\text{M}$) on the averaged log concentration-response curve to substance P is shown in Figure 2. Despite increasing the incubation time for GR82334 ($10 \mu\text{M}$) to 30 min, the antagonist was still without effect on the log concentration-response curve to substance P ($n = 4$; data not shown).

Phenoxybenzamine studies

Concentration-response parameters for two representative agonists from Group 1 and from Group 2 analogues, in control and phenoxybenzamine pretreated (test) preparations, are compared in Table 1. Though there were some significant changes ($P < 0.05$) in the slope or maxima, there seemed little

Table 1 Activities of tachykinin-receptor agonists in contracting the rabbit iris sphincter preparation: effect of phenoxybenzamine pretreatment

Agonist groups	Control s.e.mean	Slope Test s.e.mean	Δ (%) P	Control s.e.mean	Maximum Test s.e.mean	Δ (%) P	Control s.e.mean	pD_2 Test s.e.mean	Δ (log) P
Group 1									
Substance P methyl ester	0.62 0.04; 17	1.07 0.19; 5	172.6 <0.01	100.4 4.8; 17	116.0 9.7; 5	115.5 >0.05	9.16 0.10; 17	7.86 0.23; 5	1.30 <0.01
Septide	0.77 0.05; 13	0.75 0.11; 5	97.5 >0.05	95.3 2.8; 13	95.8 5.7; 5	100.5 >0.05	9.47 0.12; 13	8.84 0.31; 5	0.63 <0.05
Group 2									
Substance P	0.73 0.10; 13	0.91 0.14; 9	124.6 >0.05	92.1 4.3; 13	107.9 4.6; 9	117.1 <0.05	8.45 0.26; 13	9.58 0.24; 9	-1.13 <0.01
[Pro ⁹]-SP	0.74 0.12; 10	0.64 0.08; 5	85.5 >0.05	80.8 3.1; 10	109.8 11.4; 5	136.0 <0.05	9.15 0.16; 10	8.86 0.14; 5	0.29 >0.05

Contractile activities of two groups of tachykinin agonists (Group 1 and Group 2) with differing receptor selectivities (see text), described in terms of logistic parameters (see Methods). Maxima are expressed in terms of % carbachol maximum (where appropriate, prior to phenoxybenzamine treatment), and slope in terms of a slope factor (equivalent to the Hill slope). Differences between the control and the (test) phenoxybenzamine-pretreated ($20 \mu\text{M}$, 10 min) preparations, are expressed as % of control ($\Delta\%$, together with significance of difference, P) on an arithmetic scale for slope and maximum (i.e. 100% indicates no change). Differences in pD_2 values following phenoxybenzamine treatment are shown as logarithms (i.e. 1.0 would indicate a ten fold rightward shift, and -1.0 a ten fold leftward shift).

Figure 1 Kinetics of offset of contractions in the rabbit iris sphincter after washout of substance P methyl ester or substance P; and effect of phenoxybenzamine pretreatment ($20 \mu\text{M}$, 10 min). The upper and middle panels show representative trace of cumulative concentration-responses curves over the concentration-ranges indicated, for substance P (a) and substance P methyl ester (c), respectively; untreated control preparations (a and c), or phenoxybenzamine pretreated preparations (b and d). Note the changed time scale in the latter part of (c). The lower panel shows averaged response *versus* time washout-curves for substance P methyl ester (e) or substance P (f); untreated control preparations ($\bullet$), or phenoxybenzamine pretreated preparations (Δ). Points shown are means and s.e.means of times to reach set response levels expressed as % of pre-washout control level (in 4–11 preparations).

consistent difference between the groups. There was a marked rightward-shift of the concentration-response curves for the Group 1 agonists substance P methyl ester (twenty fold), though this was not so marked with septide (four fold) (Table 1). In contrast, there was a marked leftward-shift of the curve to the Group 2 agonist substance P (thirteen fold), though with no significant change for $[\text{Pro}^9]\text{-SP}$ (Table 1). In contrast to the findings with controls, in phenoxybenzamine-pretreated preparations, GR82334 now antagonized the responses to substance P, physalaemin, ranamargarin and $[\text{Pro}^9]\text{-SP}$ (Table 2). The overall results for substance P are shown in Figure 2, where antagonism was found to be compatible with simple equilibrium competitive interaction at a single site (see Table 2). Following phenoxybenzamine pretreatment, the pK_B estimates obtained for GR82334 against the Group 1 agonists substance P methyl ester and septide showed no difference ($P < 0.05$) as compared to controls (Table 2): further, Schild analysis (Figure 2) showed that the antagonism by GR82334 of responses to substance P methyl ester was apparently competitive under both conditions. It may be noted that GR82334 always produced an initial fast contractile response in phenoxybenzamine-treated preparations, and occasionally in untreated preparations (data not shown).

Discussion

Within the tachykinin receptor analogues that are considered to be NK_1 -receptor-preferring, two groups could be distinguished in terms of their common profile of pharmacological properties in the rabbit iris sphincter preparation. Group 1 consisted of septide, $[\text{Glp}^6]\text{-SP}(6-11)$, substance P methyl ester, GR73632 and $[\text{Apa}^{9-10}]\text{-SP}$; whereas Group 2 included substance P, physalaemin, ranamargarin and $[\text{Pro}^9]\text{-SP}$. The responses to Group 1 agonists reversed rapidly on washout and were inhibited with high affinity by various NK_1 -receptor-selective antagonists. In contrast, responses to Group 2 agonists reversed only very slowly on washout, and were not inhibited by NK_1 receptor antagonists under normal conditions: only after phenoxybenzamine pretreatment were such antagonists effective. These contrasting characteristics of the two groups seem not to result from differences in

Table 2 Affinity estimates in the rabbit iris preparation for three tachykinin receptor antagonists tested against two groups of tachykinin receptor agonists

Agonist groups	$(\pm) \text{CP-96,345}$		GR82334	
	– PBZ	+ PBZ	– PBZ	+ PBZ
Group 1				
Substance P methyl ester	8.9 (0.3; 6)	7.0 (0.1; 6)	7.5 (0.1; 18)	7.2 (0.2; 15)
Septide	9.3 (0.3; 6)	7.4 (0.2; 6)	7.7 (0.2; 9)	7.9 (0.2; 7)
$[\text{Apa}^{9-10}]\text{-SP}$	9.5 (0.5; 6)	n.d.	7.1 (0.1; 7)	7.1 (2)
$[\text{Glp}^6]\text{-SP}(6-11)$	n.d.	n.d.	8.2 (0.3; 7)	7.7 (2)
GR73632	n.d.	n.d.	7.5 (0.2; 15)	n.d.
Group 2				
Substance P	<7.0 (3)	<6.0 (3)	<5.5 (6)	6.4 (0.1, 15)
$[\text{Pro}^9]\text{-SP}$	<7.0 (3)	<6.0 (3)	<6.0 (5)	6.8 (0.3, 7)
Physalaemin	n.d.	n.d.	<6.0 (4)	7.1 (2)
Ranamargarin	n.d.	n.d.	<6.0 (8)	6.8 (2)

Antagonist affinity estimates are given as apparent pK_B values ($\pm$ s.e.mean; n), when tested against two groups of agonists (Group 1 and Group 2) with differing receptor selectivities. The antagonist GR82334 was tested both with and without pretreatment of preparations with phenoxybenzamine ($20 \mu\text{M}$, 10 min; see Methods); n.d. denotes not determined.

Figure 2 The effect of NK₁-receptor selective antagonists on responses to substance P, or substance P methyl ester, in control and phenoxybenzamine pretreated (20 μ M, 10 min) preparations of rabbit iris sphincter. The top panels show averaged log concentration-response curves to substance P methyl ester (a) or substance P (b) determined in control tissue or, middle panels, in phenoxybenzamine pretreated tissues (c and d). Each panel shows control curves in the absence ($\bullet$), or test, after a 5 min incubation with the NK₁-receptor-selective antagonist GR82334 at 1 μ M ($\blacktriangle$), 3.2 μ M ($\blacktriangledown$), or 10 μ M ($\blacklozenge$). The lower panels show Schild regressions for antagonism by GR82334 of responses to substance P methyl ester in control preparations ($\bullet$), and in preparations pretreated with phenoxybenzamine ($\blacktriangle$) (e); and of responses to substance P in phenoxybenzamine-treated preparations only (f). The calculated slopes are ($b \pm$ s.e.mean; n) 0.87 ± 0.36 ; 18; 1.12 ± 0.42 ; 15; and 1.30 ± 0.18 ; 15, respectively: in no case do these Schild regressions differ significantly from unity ($P < 0.05$), so unity slopes are imposed. For pK_B estimates, see Table 2. Experiments were carried out in the presence of hexamethonium (100 μ M), mepyramine, cimetidine, guanethidine and ibuprofen (all 1 μ M). Atropine (1 μ M) was included only in experiments without phenoxybenzamine.

metabolic stability of the peptides, since we have shown elsewhere (Hall *et al.*, 1991), that in the rabbit iris, even in combination, inhibitors of several different peptidases have little effect on activities of a number of such tachykinin receptor agonists; though we have found in certain other preparations that these inhibitors can modestly prolong tachykinin action (Hall *et al.*, 1990).

Interactions of Group 1 agonists with 'typical' NK₁ receptors

The Group 1 agonists (GR73632, substance P methyl ester, septide, [Apa⁹⁻¹⁰]-SP, [Glp⁶]-SP(6-11)) certainly can contract the rabbit iris sphincter via an interaction with 'typical' NK₁ receptors, since conventional NK₁ receptor antagonists (GR82334, RP67580, ($\pm$)-CP-96,345) competitively inhibited

their responses with affinities consistent with published data for NK₁ receptors in other rabbit preparations (Hall *et al.*, 1993a; see Maggi *et al.*, 1993). For example, ($\pm$)-CP-96,345 had a similar affinity in the iris (pK_B 8.9–9.5) to that reported in the rabbit jugular vein (pK_B 8.33; Patacchini *et al.*, 1992), jugular vein and vena cava (pK_B 8.9 and 9.5, respectively; Rouissi *et al.*, 1991), and rabbit aorta (pK_B 8.81; Beresford *et al.*, 1991). It should be noted that for the present experiments in the rabbit iris sphincter, antagonism of responses to substance P methyl ester by GR82334 (both under control and phenoxybenzamine-pretreated conditions) was compatible with fully competitive equilibrium interaction at a single site, since the Schild regression appeared linear with a slope close to unity. However, there was some variation in the apparent affinity of a given antagonist depending on the agonist under study, and it may well be that some of the particular agonists which we designed as Group 1 for the purposes of discussion, do not have a total selectivity for this site. In preparations treated with the protein alkylator phenoxybenzamine, the log concentration-response curves to Group 1 agonists were shifted markedly to the right (twenty fold for substance P methyl ester or four fold for septide) (Table 1). However, most interestingly, there was no significant change ($P > 0.05$) in the affinity of GR82334 tested against Group 1 agonists (Table 2; e.g. pK_B against substance P methyl ester, 7.5 and 7.2; and against septide 7.7 and 7.9, respectively). These various observations, taken together, suggest that phenoxybenzamine pretreatment reduces the receptor number at the sites where Group 1 agonists act (as might be expected by analogy with other receptor types) but does not change the receptor recognition properties.

'Atypical' characteristics of Group 2 agonists

The responses to the Group 2 agonists (substance P, physalaemin, ranamargarin, [Pro⁹]-SP) had very prolonged response offset kinetics and were little inhibited, or in some instances potentiated, by the NK₁ receptor antagonists. Thus, Group 2 agonists must mediate contraction of iris smooth muscle via another route as compared to that of Group 1 agonists. An interaction with NK₂ receptors can be ruled out, since we have previously demonstrated insignificant activity of various NK₂-receptor-selective ligands in the iris preparation (Hall *et al.*, 1991; 1993b). Further, it seems unlikely that simultaneous synergistic activation of NK₁ and NK₃ receptors account for the unusual pharmacology of Group 2 ligands, since the co-administration of Group 1 NK₁- and NK₃-receptor-selective, ligands (septide and senktide, respectively) does not result in a contractile response similar in profile to that of the Group 2 agonists. Also, it may be noted that the Group 2 agonist, [Pro⁹]-SP, is well established in other systems as a very selective NK₁ receptor agonist, being virtually inactive at NK₃ receptors (Lavielle *et al.*, 1986; Beaujouan *et al.*, 1991; Petit *et al.*, 1992). Moreover, tachykinins such as neurokinin A which are known to activate both NK₁ and NK₃ receptors, do not exhibit prolonged offset kinetics (Too *et al.*, 1988; Hall *et al.*, 1993b); and, as might be expected, the responses to such non-selective agonists are indeed partially inhibited by NK₁-receptor antagonists (Hall *et al.*, 1993b). Another mechanism that needs to be considered in attempting to explain the unusual characteristics of Group 2 agonists, is whether they act indirectly via the release of further mediators which, in principle, could influence both offset kinetics and sensitivity of responses to antagonism by NK₁ antagonists. Of the possible mediators that we have tested (endothelin, [Arg⁸]-vasopressin, bombesin, angiotensin II, bradykinin), only endothelin had prolonged response offset kinetics in the rabbit iris sphincter, but was found to be insensitive to phenoxybenzamine pretreatment (unpublished observations).

It must be considered whether Group 2 agonists do, in fact, interact with 'typical' NK₁ receptors, but perhaps their

prolonged action and insensitivity to NK₁ receptor antagonists results from, for example, some prolonged non-equilibrium state in the biophase. There have been a number of reports of peptide antagonists that appear to become inserted in the membrane by some lipophilic binding (e.g. Hoe 140; Hall, 1992), and a similar phenomenon might well occur for agonists (as established for the nonpeptide β_2 -adrenoceptor agonist salmeterol (Ball *et al.*, 1991)). It is difficult to test for such phenomena, but it may be noted that the prolonged action of Group 2 agonists is not prevented even by 30 min prior incubation with NK₁ antagonists. That phenoxybenzamine-pretreatment prevents the agonists' prolonged effect, could be interpreted in a number of ways, given this reagent's wide-ranging alkylating actions. Possible explanations include: (1) reduction of the receptor density reducing the diffusional drag limiting washout of agonist molecules from high-affinity binding in the biophase (see Bailey, 1985); and here it is noteworthy that several NK₁ agonists have unusually high potency in this preparation (Hall *et al.*, 1991) suggesting a large receptor concentration and/or good coupling-efficiency (this supposition of a large receptor reserve being supported by the failure of phenoxybenzamine alkylation to reduce agonists' maximum responses or slope, even when there was a substantial shift of the concentration-response curve to the right); (2) reduction of high-affinity binding at sites not actually involved in the response ('silent receptors') but which exert a diffusional drag; or (3) modification of the receptor-effector coupling-mechanisms downstream of the receptor site (for instance, by blocking Ca²⁺-channels). However, no single explanation seems to account for all the atypical characteristics of Group 2 agonists. It would be helpful if the differences in pharmacology of Group 1 and Group 2 agonists could be attributed to some specific chemical feature; but though all the Group 2 agonists are full-length peptides, whereas several Group 1 agonists are made up of the truncated SP(6-11) sequence, there are the notable exceptions within Group 1 of the full-sequence agonists substance P methyl ester and [Apa⁹⁻¹⁰]-SP(1-11).

Discounting the potential complications discussed above, the simplest remaining explanation for our findings is that the rabbit iris sphincter contains two differing 'NK₁-like' receptor populations. Here, there is the possibility that these represent two genetically-distinct receptor subtypes: alternatively they may reflect receptor isoforms, resulting, for example, from alternative pre-mRNA splicing (as has been shown with the cloned human NK₁ receptor; Fong *et al.*, 1992). One receptor subtype in the iris, which Group 1 agonists preferentially activate, appears to have characteristics similar to those previously described for 'typical' rabbit NK₁ receptors. Thus, ($\pm$)-CP-96,345, GR82334 and RP67580 have their characteristic affinities (see Maggi *et al.*, 1993), and responses following receptor activation reverse fairly rapidly on agonist washout. In contrast, Group 2 agonists seem preferentially to activate a very different (in terms of recognition properties) 'atypical' receptor-subtype or receptor-isoform, which is insensitive to blockade by ($\pm$)-CP-96,345, GR82334 or RP67580, and where receptor activation results in prolonged washout offset-kinetics. It might be supposed that phenoxybenzamine preferentially alkylates this form of receptor, unmasking the 'typical' NK₁ receptor at which Group 2 agonists have some agonist activity, so that their actions are now rapid in offset and are inhibited by NK₁ receptor antagonists. However, since Group 1 agonist activities are attenuated by phenoxybenzamine, it would seem that 'typical' NK₁ sites are also susceptible to alkylation.

In practice, it is difficult to distinguish between the above proposal of the presence of two distinct receptor proteins, from a plausible and similar model where Group 1 and Group 2 agonists bind to different domains of the *same* (NK₁) receptor protein. For instance, Group 2 agonists may bind to additional domains not accessible to NK₁ agonists,

and this could induce conformational or allosteric changes resulting in prolonged responses, and prevention of antagonist binding or the ability of the antagonists actually to interfere with agonist binding. Binding of agonist and antagonist ligands each to different domains of NK₁ receptors, has been described as the cloned NK₁ receptors of rat and man (see Hall *et al.*, 1993a). It could be envisaged that the additional binding domain occupied by Group 2 agonists is susceptible to alkylation by phenoxybenzamine; and thus following alkylation of this site, Group 2 agonists interact with the same receptor domains as those recognizing Group 1 agonists.

It is of interest that Petitet *et al.* (1992) have recently reported pharmacological and radioligand binding data which they interpret as indicating the existence of two NK₁-like tachykinin receptor subtypes in the guinea-pig ileum. In their studies, two groups of agonists could also be distinguished. Substance P, [Pro⁹]-SP and [Sar⁹, Met(O₂)¹¹]-SP were proposed to belong to one group which had high affinity for [³H]-[Pro⁹]-SP binding sites; and further, contractile responses to a representative agonist from this group ([Pro⁹]-SP) were only weakly inhibited by the NK₁ receptor antagonist GR71251 (pK_B 6.5). In contrast, [Glp⁶, L-Pro⁹]-SP(6-11) (septide), substance P methyl ester, [Apa⁹⁻¹⁰]-SP and [Pro^{9,10}]-SP were all considered to belong to a second group having low affinity for [³H]-[Pro⁹]-SP binding sites, and responses to agonists in this group (septide and [Apa⁹⁻¹⁰]-SP) were inhibited by the NK₁ antagonist, GR71251 with relatively high affinity (pK_B 7.9 and 8.1, respectively). Further evidence for the existence of subtypes of the NK₁ receptor in the guinea-pig ileum was provided by the fact that [D-Pro⁹, MeLeu¹⁰, Trp¹¹]-SP was equipotent as an antagonist against either [Pro⁹]-SP or septide evoked contractions (pA₂ 6.6) (Chassaing *et al.*, 1992). Unfortunately, in the rabbit iris sphincter, we found this analogue to be a partial agonist (unpublished observations).

The grouping of agonists by Petitet *et al.* (1992) in the guinea-pig ileum is largely in accord with our own grouping of agonists in the rabbit iris sphincter. An obvious interpretation of this correspondence, is that there are two within-preparations subtypes of the NK₁ receptor; which in turn have some common between-preparations properties. Indeed, others have claimed some inter-species similarities in NK₁ receptors of the guinea-pig and rabbit (see Hall *et al.*, 1993a). However, it would be premature to propose intra-species variants of the NK₁ receptor on the present evidence, especially given some of our reservations over interpretation of the experimental evidence. Final conclusions would be aided by the development of selective antagonists for the NK₁ receptor antagonist-insensitive 'atypical' site; but more important would be the demonstration, by the techniques of molecular biology, of the existence of genetically-distinct NK₁ receptor-subtypes, or of receptor-isoforms, within the single species; or alternatively, the identification of different binding domains, for each of the two agonist groups, on a single NK₁-receptor-protein. However, irrespective of such conclusions, the contrasting operational pharmacological characteristics of the two groups of ligands is striking, and the physiological importance of this, at the level of receptor function, may well be considerable.

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