Novel selective agonists and antagonists confirm neurokinin NK₁ receptors in guinea-pig vas deferens

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1 This study investigated the recognition characteristics of neurokinin receptors mediating potentiation of the contractile response to field stimulation in the guinea-pig vas deferens.

2 A predominant NK₁ receptor population is strongly suggested by the relative activities of the common naturally-occurring tachykinin agonists, which fall within less than one order of magnitude. This conclusion is supported by the relative activities of the synthetic NK₁ selective agonists substance P methyl ester, [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ and δ -aminovaleryl-[L-Pro⁹,N-MeLeu¹⁰]-SP₍₇₋₁₁₎ (GR73632) which were 0.78, 9.3 and 120 as active as substance P, respectively. Furthermore, the NK₂ selective agonist [Lys³, Gly⁸,R- γ -lactam-Leu⁹]-NKA₍₃₋₁₀₎ (GR64349) was active only at the highest concentrations tested (> 10 μ M), and the NK₃ selective agonist, succ-[Asp⁶,N-MePhe⁸]-SP₍₆₋₁₁₎ (senktide) was essentially inactive (10 nM–32 μ M).

3 [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ antagonized responses to neurokinin A, neurokinin B, physalaemin, elodeisin, [Glp⁶,D-Pro⁹]-SP₍₆₋₁₁₎, GR73632 and GR64349 (apparent pK_Bs 5.6–6.2), but was less potent in antagonizing responses to substance P, substance P methyl ester and [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ (apparent pK_Bs $\leq$ 5.0–5.0).

4 In contrast, the recently developed NK₁-selective receptor antagonist [D-Pro⁹[Spiro- γ -lactam]Leu¹⁰, Trp¹¹]-SP₍₁₋₁₁₎ (GR71251) did not produce agonist-dependent pK_B estimates. Schild plot analysis indicated a competitive interaction with a single receptor population where the antagonist had an estimated overall pK_B of 7.58 $\pm$ 0.13 for the four agonists of differing subtype selectivity tested (GR73632, GR64349, substance P methyl ester and neurokinin B). This estimate is similar to that we obtained for NK₁-mediated (substance P methyl ester) contraction in the guinea-pig ileum preparation (pK_B = 7.86 $\pm$ 0.05).

5 Tachykinin action appears not to depend on release of a number of intermediary mediators including acetylcholine, histamine or cyclo-oxygenase products, nor to involve interaction with neuronal mechanisms including α_2 -adrenoceptor feedback, noradrenergic Uptake-1 or opioid-release, since antagonism or inhibition of these mechanisms did not modify responses to tachykinins.

6 We conclude that tachykinin action in the field-stimulated guinea-pig vas deferens preparation is mediated through interaction with a predominant neurokinin NK₁ receptor population and this preparation can therefore be used to study NK₁ modulation of sympathetic neurotransmission.

Introduction

There is now considerable support for the proposed existence of three functional neurokinin receptor subtypes, NK₁, NK₂ and NK₃ (see, Henry, 1987). The evidence for this proposal has come, until comparatively recently, almost exclusively from the determination of activities of naturally-occurring tachykinin agonists including the mammalian neurokinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) in functional studies in the periphery and radio-ligand binding studies both in the periphery and in the CNS (for reviews see, Watson, 1984; Lee *et al.*, 1986; Regoli *et al.*, 1987; Quirion & Dam, 1988).

The existence of three neurokinin receptor subtypes has been further substantiated through use of synthetic subtype selective neurokinin receptor agonists. Such agonists that have proven valuable for classificational purposes include; the NK₁ selective agonists substance P methyl ester (SPOMe, Cascieri *et al.*, 1981; Watson *et al.*, 1983), [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ (septide, Piercey *et al.*, 1985; Laufer *et al.*, 1986a), and [Sar⁹, Met(O₂)¹¹]-SP (Drapeau *et al.*, 1987), the NK₂ selective agonist [β -Ala⁸]-NKA₍₄₋₁₀₎ (Rovero *et al.*, 1989), and the NK₃ selective agonists succ-[Asp⁶,N-MePhe⁸]-SP₍₆₋₁₁₎ (senktide, Wormser *et al.*, 1986) and [N-MePhe⁷]-NKB (Drapeau *et al.*, 1987).

Selective and potent competitive neurokinin receptor antagonists are particularly necessary in neurokinin receptor studies in order to provide confirmatory data to support the

current putative classificational schemes largely based on agonist activities. The earlier developed neurokinin receptor antagonists, however, have been of little value for such purposes. For example, many antagonists were found to be low in potency, showed no clear neurokinin subtype selectivity, and several produced agonist-dependent potency estimates in hitherto presumed mono-receptor systems for reasons that are unclear (for discussion see, Bailey *et al.*, 1986). Furthermore, a number of early antagonists additionally have actions not related to receptor antagonism (Karlsson *et al.*, 1984; Lembeck *et al.*, 1986), and several show antagonism at other receptors such as those for bombesin (Folkers *et al.*, 1984; Jensen *et al.*, 1984; Yachnis *et al.*, 1984; Mizrahi *et al.*, 1985).

Very recently several novel agonists and antagonists have been proposed as having high potency and neurokinin receptor subtype selectivity. Such agonists include the NK₁ selective agonist δ -aminovaleryl-[L-Pro⁹, N-Me-Leu¹⁰]-SP₍₇₋₁₁₎ (GR73632) and the NK₂ selective agonist, [Lys³, Gly⁸,R- γ -lactam-Leu⁹]-NKA₍₃₋₁₀₎ (GR64349) (Hagan *et al.*, 1989). Novel neurokinin receptor antagonists include the NK₁ selective antagonist [D-Pro⁹[Spiro- γ -lactam]Leu¹⁰, Trp¹¹]-SP₍₁₋₁₁₎; GR71251 (Hagan *et al.*, 1990; Ward *et al.*, 1990), and the NK₂ selective antagonist cyclo[Gln,Trp,Phe,Gly,Leu,Met] (L659,877; McKnight *et al.*, 1988; Williams *et al.*, 1988).

The contractile response to sympathetic field-stimulation of the guinea-pig vas deferens is potentiated by substance P (SP) and related tachykinins (von Euler & Hedqvist, 1974a,b). However, since its original classification as an SP-P (= NK₁) (Watson *et al.*, 1983), this preparation has been little studied in terms of neurokinin receptor subtypes or involvement of other

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mediators in responses to tachykinins. Pharmacological investigation of the site of action of tachykinins (Ellis & Burnstock, 1989) and the autoradiographic localisation of neurokinin receptors (Mussop *et al.*, 1989) have, however, recently been reported. The rat vas deferens in contrast has been used extensively to study sympathetic neuromodulation, though here tachykinin action is mediated through interaction with NK₂ receptors.

We describe, in this paper, an extensive re-examination of neurokinin receptors in the field-stimulated guinea-pig vas deferens *in vitro* and we report, for the first time, activities of some novel agonist and antagonist analogues in this preparation.

Preliminary accounts of this work have been communicated to The British Pharmacological Society (Hall *et al.*, 1987) and to X Satellite IUPHAR (Sydney) (Hall *et al.*, 1988).

Methods

Tissue preparation and recording arrangements

Male Dunkin-Hartley guinea-pigs (450–800 g) were killed by stunning and exsanguination. The abdomen was opened by mid-line incision, and the two vasa deferentia were removed, cleared of superficial blood vessels and connective tissue and divided into separated prostatic and epididymal sections by length. Each section was mounted on a stainless-steel tissue holder, with a platinum ring electrode around the base of the preparation and with the upper section of the preparation tied with 0.1 mm fully-softened stainless-steel wire. The preparations were attached to Grass FT03B force-displacement transducers under an initial resting tension of 0.5–1.0 g, the wire being used both to attach the tissue to the strain gauge and to act as the upper electrode. Four preparations were set up in parallel in 1 ml silanised glass organ baths, with contractions recorded on Grass model 79C polygraphs after d.c. amplification. Preparations were stimulated via Grass SD9 stimulators individual to each preparation, collectively gated by a single Grass S44 stimulator at 0.05 Hz generating a 0.25 ms gating pulse. Stimulation parameters were a modification of those used by Watson *et al.* (1983) and were trains of 5 pulses at 20 Hz at a 1 ms pulse-width every 20 s. At sub-maximal voltage (40–60 V) the tissue-electrode-bath assembly drew 20–30 mA. The neurally-mediated 'twitch' response so obtained may in fact be regarded as a short tetanus since some summation occurs.

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.

Relative activities of tachykinin agonists

Tachykinins were assayed for their ability to potentiate responses to nerve stimulation at normally four dose-levels (10 nM–10 μM) in a fully randomised (concentration and agonist) serial block design, with each preparation receiving each concentration of each agonist. A 40–60 s contact time and 7 min dose cycle with SP (1 μM) applied at the start of each block of four doses was used to allow normalisation of responses which are expressed in terms of % increase in twitch height over basal. Similar results were obtained on expression of the data by a number of different methods. Random orders for dosing were determined from computer-generated numbers. In all experiments, the Krebs solution contained atropine (1 μM), mepyramine (1 μM) and hexamethonium (10 μM).

Six of the naturally-occurring tachykinins SP, NKA, NKB, physalaemin (Phy), eledoisin (Ele) and kassinin (Kas) were assayed along with the synthetic NK₁ selective analogue SPOMe.

The D- and L-isomers of [Glp⁶,*Pro⁹]-SP₍₆₋₁₁₎, and the NK₁ and NK₂ selective analogues GR73632 and GR64349 respectively, as well as [pGlu⁵,MePhe⁸,Sar⁹]-SP₍₅₋₁₁₎ (DiMeC7), were assayed in two separate series of experiments, along with SP. The NK₃ selective analogue, senktide, was found to be virtually inactive (10 nM–32 μM) and thus was not included in any formal assay procedure.

Estimation of pK_B values for neurokinin receptor antagonists

[D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ (10 μM) was tested against responses to several tachykinins. After establishing control submaximal responses to 2 or 3 dose-levels of selective tachykinins (SP, NKA, NKB, Phy, Ele, SPOMe, [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎, [Glp⁶,D-Pro⁹]-SP₍₆₋₁₁₎, GR73632 and GR64349), the antagonist was left in contact with the preparation for 7 min and suitable concentrations of agonist applied to enable estimation of dose-ratios after antagonist application. The rapid reversibility of antagonist effects on washout allowed the assay of two agonists in each preparation. Schild plot analysis was not possible with this antagonist because of its low potency coupled with the shallow agonist log concentration-response curve which thus did not permit accurate estimation of small dose-ratios.

For studies with the NK₁-selective antagonist, GR71251, four preparations were set up in parallel and control cumulative concentration-response curves to the NK₁ selective agonists GR73632 and SPOMe, the NK₂ selective agonist GR64349, and the relatively non-selective agonist NKB were obtained in individual preparations. A cumulative dosing protocol was used in these experiments in order to determine antagonist effects on the whole concentration-response curve. Cumulative concentration-response curves were then repeated after a 7 min equilibration with GR71251 (0.32, 1.0 or 3.2 μM). A 20–60 min dose-cycle was used, depending on agonist washout time (see, Results). Washout of antagonist effect was established in each preparation, and the procedure was repeated with higher antagonist concentrations (allowing up to three antagonist concentrations to be tested in each preparation).

In all antagonist experiments, the Krebs solution contained atropine (1 μM), hexamethonium (10 μM), mepyramine and cimetidine (both 1 μM). Mepyramine and cimetidine were included in view of the known histamine-releasing properties of [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ and GR71251 (Folkers *et al.*, 1984; Hagan *et al.*, 1990).

Controls for antagonist experiments

Control cumulative concentration-response curves were determined in the absence of antagonist throughout the experimental periods in order to investigate possible time effects and desensitization on tachykinin concentration-response curves. Confirmation of competitive antagonism with GR71251 against NK₁-mediated contraction of the whole guinea-pig ileum was obtained by Schild plot analysis for the antagonist against SPOMe in the presence of hexamethonium (10 μM), atropine, mepyramine, ibuprofen, naloxone and guanethidine (all 1 μM). Three-point concentration-response curves were determined in the absence, and then after a 7 min incubation with GR71251 at increasing antagonist concentrations (0.1–10 μM). Reversibility of antagonism was established in each individual preparation.

Test for involvement of other mediators or interaction with other mechanisms

Responses to single sub-maximal concentrations of SP, Ele, Phy, NKA and NKB were obtained first in the absence, and then after a 30 min equilibration with atropine (1 μM) or mepyramine (1 μM), with each preparation receiving every tachykinin. Two-point concentration-response curves were

obtained to SP, Ele and Phy and were repeated after a 30 min incubation with cimetidine (1 μ M). Also, ipromidine (10 μ M) was tested for activity in all preparations. The involvement of cyclo-oxygenase products in responses to SP, Ele and Phy was investigated by comparing concentration-response curves (10 nM–10 μ M) obtained before and after a 30 min equilibration with ibuprofen (1 μ M). Responses to sub-maximal concentrations of SP, Ele and Phy were obtained in the absence, and then after a 30 min equilibration with naloxone (1 μ M) or yohimbine (0.32 μ M). Full concentration-response curves were obtained for SP, Phy and [Glp⁶,D-Pro⁹]-SP₍₆₋₁₁₎ and then repeated after a 30 min equilibration with cocaine (2 μ M), a blocker of the noradrenergic Uptake-1 system, in the presence of yohimbine (0.1 μ M) to circumvent α_2 -adrenoceptor pre-junctional autofeedback caused by accumulation of noradrenaline.

Source of materials

Agents were obtained as follows: substance P, eldeoisin, physalaemin, kassinin, neurokinin A, neurokinin B, substance P methyl ester, [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎, [pGlu⁵,MePhe⁸,Sar⁹]-SP₍₅₋₁₁₎ and yohimbine hydrochloride (Peninsula Laboratories Europe). Ibuprofen, atropine sulphate, naloxone and hexamethonium bromide, cocaine hydrochloride (Sigma, U.K.), mepyramide maleate (May & Baker, U.K.), cimetidine and ipromidine (Smith, Kline & French, U.K.). All salts used were of analytical grade and were obtained from B.D.H., U.K. δ -Aminovaleryl-[L-Pro⁹,N-Me-Leu¹⁰]-SP₍₇₋₁₁₎ (GR73632), [Lys³,Gly⁸-R- γ -lactam-Leu⁹]-NKA₍₃₋₁₀₎ (GR64349) and [D-Pro⁹[Spiro- γ -lactam]Leu¹⁰,Trp¹¹]-SP₍₁₋₁₁₎ (GR71251) were kind gifts from Glaxo Group Research, Ware, Hertfordshire, U.K. Succ-[Asp⁶,N-MePhe⁸]-SP₍₆₋₁₁₎ (senktide), [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ and [Glp⁶,D-Pro⁹]-SP₍₆₋₁₁₎ were kind gifts from Dr B. Williams, Merck, Sharp and Dohme, U.K.

Neurokinin B was dissolved in dimethylsulphoxide (DMSO). All other peptides were dissolved in 0.01 M acetic acid. Ibuprofen was dissolved in 5% Na₂CO₃. All other agents were dissolved in distilled water. Peptides were stored deep frozen under N₂ with dilutions discarded at the end of each experiment.

Analysis of results

Responses of the prostatic and epididymal sections of the vas deferens did not differ, so data from both sections were pooled. Tests for significance were made using Student's *t* test for independent or paired samples or by Analysis of Variance as appropriate. pK_B estimates for antagonist experiments were determined from individual dose-ratios using the Gaddum-Schild equation for competitive inhibition at equilibrium, $pK_B = \log_{10}(x - 1) - \log_{10}[A]$, where *x* is the dose-ratio and [A] the applied antagonist concentration. Data described as apparent pK_Bs are where Schild plot analysis was not carried out; otherwise pK_B values are quoted where analysis showed slopes of the Schild plots not to differ significantly from unity. Log₁₀ activity estimates for agonists were calculated for individual preparations at the arbitrary level of 100% potentiation of the twitch response.

Results

General

Tachykinins were without consistent effect on baseline tension of the guinea-pig vas deferens; this contrasts with tachykinin actions in the rat vas deferens where marked contraction of the smooth muscle is seen in response to tachykinins (data not shown). In experiments using both serial and cumulative dosing procedures, potentiation of contractile responses to field stimulation by all tachykinins was rapid in onset with

Figure 1 Typical traces showing potentiation of the contractile response to field stimulation in the guinea-pig vas deferens. Responses to naturally-occurring (a and c) and synthetic (b and d) tachykinin analogues were obtained with a serial (a and b) or cumulative (c and d) dosing protocol. Responses shown are to 5-pulse trains at 20 Hz of 1 ms duration every 20 s. Note the delayed onset and offset of response to the synthetic agonists GR73632 and GR64349 compared with the naturally-occurring agonists substance P, eldeoisin and neurokinin B. W indicates washout. Agonist concentrations are given as log₁₀.

peak responses to each agonist concentration occurring within ca. 40–60 s, though responses to GR73632 and GR64349 were often somewhat slower in onset than the other agonists (see Figure 1). Responses to the novel NK₁ and NK₂ selective agonists GR73632 and GR64349 respectively, however, were consistently slower in offset than responses to the other tachykinins both after serial and cumulative dosing protocols. For example, washout of agonist responses after cumulative concentration-response curves showed the following times to return to pre-agonist twitch level; GR73632 and GR64349, 37 ± 7 min and 22 ± 6 min respectively compared to 12 ± 4 min and 12 ± 3 min for SPOMe and NKB respectively (see also Figure 1). For comparison, from an earlier series of experiments (see, Hall *et al.*, 1990) washout times after cumulative concentration-response curves to SP, NKA, NKB and Phy were 11 ± 2 min, 14 ± 3 min, 9 ± 3 min and 15 ± 4 min respectively.

Inhibitors or antagonists of several mechanisms implicated in tachykinin actions in other peripheral preparations were without significant effect on responses to various tachykinins. Thus, atropine (1 μ M) or mepyramine (1 μ M) were without effect on responses to SP, Ele, Phy, NKA and NKB; cimetidine (1 μ M), ibuprofen (1 μ M), naloxone (1 μ M) or yohimbine (0.32 μ M) were without effect on responses to SP, Ele or Phy and cocaine (2 μ M) was without effect on responses to SP, Phy or [Glp⁶,D-Pro⁹]-SP₍₆₋₁₁₎ (data not shown).

Relative activities of tachykinin agonists

The log concentration-response relationships shown in Figure 2 are for three separate series of experiments where, in each series, several tachykinins were assayed along with SP. The

Table 1 Relative activities of tachykinins in the field-stimulated guinea-pig vas deferens

Agonist	Activity ($-\log_{10}$)	s.e.mean	Relative activity	n
Substance P	6.2	0.07	1.00	52
Neurokinin A	5.9	0.16	0.56	24
Neurokinin B	5.7	0.12	0.36	24
Physalaemin	7.0	0.06	7.20	24
Kassinin	6.1	0.10	0.80	24
Eledoisin	6.3	0.10	1.50	24
Substance P methyl ester	6.0	0.07	0.78	24
[Glp ⁶ ,L-Pro ⁹]-SP ₍₆₋₁₁₎	6.9	0.15	9.30	20
[Glp ⁶ ,D-Pro ⁹]-SP ₍₆₋₁₁₎	5.3	0.11	0.23	20
Senktide	<4.5	—	<0.03	4
GR73632	8.7	0.08	120.00	8
GR64349*	5.6	0.05	0.05	8
[pGlu ⁵ ,Me-Phe ⁷ ,Sar ⁸]-SP ₍₅₋₁₁₎	5.2	0.37	0.37	8

Activities of naturally-occurring and synthetic tachykinin agonists in the field-stimulated guinea-pig vas deferens preparation. The activity estimates shown, were calculated for individual preparations at the arbitrary level of 100% potentiation of the twitch response, and are given as the $-\log_{10}$ of the concentration required (M). Relative activities are expressed as compared to substance P (activity = 1.00) in that series of experiments.

* It should be noted that this analogue was inactive at $\leq 1 \mu\text{M}$, but had marked activity at $> 10 \mu\text{M}$ (see, Results).

Figure 2 Log concentration-response curves to tachykinins from three series of experiments in the field-stimulated guinea-pig vas deferens preparation. (a) Substance P (●), neurokinin A (□), neurokinin B (◆), kassinin (△), eledoisin (◇), physalaemin (○) and substance P methyl ester (▲); (b) substance P (●), [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ (○) and [Glp⁶,D-Pro⁹]-SP₍₆₋₁₁₎ (△), and (c) substance P (●), [pGlu⁵,MePhe⁷,Sar⁹]-SP₍₅₋₁₁₎ (▲), GR73632 (○) and GR64349 (△). Senktide was inactive (0.1 nM–32 μM). Experiments were carried out in Krebs solution at 37°C containing atropine and mepyramine (both 1 μM) and hexamethonium (10 μM). Each point is the mean for 8–24 preparations; s.e.mean shown by vertical bars. Responses are expressed as % increase in twitch height over basal normalised to responses to substance P (1 μM).

log concentration-response curves are rather shallow in all cases, with thresholds ca. 10 nM and maxima greater than 10 μM for active analogues, though true maxima could not be estimated for reasons of cost, solubility and possible solvent effects. It is evident that though tachykinins of roughly equal activity have log concentration-response curves with similar slopes, those of both the more active and least active agents in each series diverge from parallelism. Activity estimates shown in Table 1 are expressed relative to SP in that individual series. The absolute potency of SP did not differ significantly ($P > 0.05$) between the different series.

Estimated pK_B values for neurokinin receptor antagonists

Table 2 shows estimates of apparent pK_B values for the antagonist [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ when tested against responses to several tachykinin agonists. This antagonist gave lower apparent pK_B estimates when tested against SP, SPOMe and [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ than against the other agonists. It should be pointed out that rigorous Schild plot analysis of antagonism by [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ over a reasonable concentration-range was not possible because of the low potency of the antagonist in this system, with the consequence inaccuracies in estimation of small dose-ratios. However, this antagonist at 10 μM did not produce a parallel shift to the right of the concentration-response line to all those agonists against which it was active

Table 2 Estimated apparent pK_B values for [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ against tachykinins in the guinea-pig vas deferens

Agonist	pK_B	s.e.mean	n
Substance P methyl ester	<5.0	—	6
Substance P	5.0	0.2	6
[Glp ⁶ ,L-Pro ⁹]-SP ₍₆₋₁₁₎	5.0	0.2	6
Neurokinin A	5.6	0.1	6
Eledoisin	5.6	0.2	6
GR64349	5.9	0.1	5
Physalaemin	5.8	0.1	5
GR73632	6.0	0.1	5
[Glp ⁶ ,D-Pro ⁹]-SP ₍₆₋₁₁₎	6.1	0.3	4
Neurokinin B	6.2	0.2	6

pK_B [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ values were estimated from calculation of dose-ratios in individual preparations using the Gaddum-Schild equation $pK_B = \log(x - 1) - \log[\text{Ant}]$ where x is the dose-ratio and $[\text{Ant}]$ the applied antagonist concentration. <5.0 denotes inactive at 10 μM .

Figure 3 Schild plots for the NK₁ selective antagonist GR71251 against (a) the NK₁ selective agonist substance P methyl ester (●) in the guinea-pig ileum in the presence of atropine, (b) the NK₁ selective agonist GR73632 (●), and the NK₂ selective agonist GR64349 (○) in the guinea-pig vas deferens. The slope estimates ($b = 1.06, 1.25$ and 1.25 for substance P methyl ester, GR73632 and GR64349 respectively) did not differ significantly from 1.00 ($P > 0.05$), so unity slopes were imposed in the Schild plot shown. Estimated pK_B values are given in Table 3.

which was rapidly reversible on washout. The antagonist had no action *per se* on the twitch response.

The novel NK₁ selective antagonist, GR71251, was much more potent as an antagonist but, in addition showed concentration-related inhibition of the twitch response at high concentrations (above $3.2 \mu\text{M}$). Consequently a relatively lower concentration range (0.32 – $3.2 \mu\text{M}$) was employed in obtaining the Schild plots shown in Figure 3b against the NK₁ selective agonist, GR73632, and the NK₂ selective agonist, GR64349. Table 3 shows pK_B estimates for GR71251 against a range of tachykinin agonists, and Analysis of Variance showed no sig-

Table 3 Estimated pK_B values for GR71251 against tachykinins of differing subtype selectivities in the guinea-pig vas deferens

Agonist	pK_B	s.e.mean	n
Substance P methyl ester	7.61	0.25	10
GR73632	7.65	0.25	16
GR64349	7.43	0.22	15
Neurokinin B	7.57	0.28	7
Overall estimate	7.58	0.13	48

pK_B values for GR71251 were estimated from calculation of dose-ratios in individual preparations using the Gaddum-Schild equation $pK_B = \log(x - 1) - \log[\text{Ant}]$ where x is the dose-ratio and $[\text{Ant}]$ the applied antagonist concentration. Analysis of Variance showed no significant difference ($P > 0.05$) between agonists, so an overall pK_B estimate is shown.

nificant evidence of agonist-dependent pK_B estimates (mean overall $pK_B = 7.58 \pm 0.13$, $n = 48$; $P > 0.05$). The maximum potentiation of responses to field stimulation by the four agonists was unchanged in the presence of this antagonist ($P > 0.05$). For the purpose of comparison, GR71251 was also tested, over a more extensive concentration range (0.1 – $10 \mu\text{M}$), against NK₁ mediated (SPOMe) contraction of the guinea-pig ileum in the presence of atropine, and Schild plot analysis (Figure 3a) indicated a competitive interaction with a pK_B estimate of $7.86 (\pm 0.05, n = 20)$. In the ileum preparation, this antagonist was without effect on basal tension in its own right. In both preparations, the antagonism was fully reversible on washout. The overall pK_B for GR71251 in the vas deferens did not differ significantly from that obtained in the ileum ($P > 0.05$).

Discussion

This study investigated neurokinin receptors mediating potentiation of the twitch response to field-stimulation of the guinea-pig vas deferens *in vitro*.

Inhibitors and antagonists of several intermediary mediators implicated in tachykinin actions in other systems were without effect on responses to tachykinins. This suggests a direct interaction of tachykinins with neurokinin receptors at a pre- and/or post-junctional site. The site of action of tachykinins in the guinea-pig vas deferens is unclear. A post-junctional site on the smooth muscle has been suggested by Ellis & Burnstock (1989) who reported potentiation of the contractile response to exogenously applied $\alpha\beta$ -methylene ATP (though not to noradrenaline) by SP, but failed to measure an increase in release of ATP or [^3H]-noradrenaline overflow. However, although we confirm that there is no increase in [^3H]-noradrenaline overflow in this preparation in response to several tachykinins of differing subtype selectivities, we fail to confirm potentiation of the contractile response to exogenously applied $\alpha\beta$ -methylene ATP by SP or other tachykinins (data not shown). The presence of neurokinin receptors (NK₁) both on sensory nerve endings in the outer longitudinal muscle of the guinea-pig vas deferens, and post-junctional neurokinin (NK₁) receptors on the inner longitudinal smooth muscle has been proposed from autoradiographic studies (Mussop *et al.*, 1989). It therefore seems unresolved whether both pre- (on sensory or sympathetic) and post-junctional receptors are functionally involved in the potentiation of twitch responses by tachykinins. Atropine and hexamethonium were without effect on responses to tachykinins, thus discounting an interaction with parasympathetic nerves (data not shown). It should be noted in relation to later discussion, that autoradiographic studies detected only NK₁ binding sites in this preparation (Mussop *et al.*, 1989).

Neurokinin receptor subtypes; agonist studies

The relative activities of the naturally-occurring tachykinin agonists fall within less than one order of magnitude which suggests the presence of a predominant NK₁ receptor population according to the criteria originally defined by Lee *et al.* as SP-P (Lee *et al.*, 1982). These present data in the guinea-pig vas deferens confirm an early report by Watson *et al.* (1983), who, using the limited number of tachykinin analogues available at the time, also proposed the presence of an SP-P (NK₁) receptor population in this preparation.

This conclusion is further substantiated by results obtained with several subtype selective synthetic agonist analogues. The well established NK₁ receptor agonist SPOMe had an activity of 0.78 relative to SP, a value similar to that reported previously in this preparation (0.52; Watson *et al.*, 1983). [$\text{Glp}^6, \text{L-Pro}^9$]-SP₍₆₋₁₁₎ had an activity of 9.3 and the novel NK₁ receptor agonist, GR73632 an activity of 120. Furthermore, the ratio of activity of the L- and D-isomers of [$\text{Glp}^6, \text{Pro}^9$]-SP₍₆₋₁₁₎ was 41, which also indicates interaction with an NK₁

receptor population (Lee *et al.*, 1986; Bristow *et al.*, 1987; Fletcher *et al.*, 1987). The established NK₃ selective agonist, senktide, was inactive (10 nM–32 µM). This agonist is inactive in several other NK₁ preparations (Laufer *et al.*, 1986a,b; Wormser *et al.*, 1986). The novel NK₂ agonist GR64349 had very little activity over the concentration-range 10 nM–1 µM, but at concentrations greater than 10 µM, potentiation of the twitch response was seen to give a very steep log concentration response curve in this region and approached the magnitude of effect of several other agonists. These latter results with GR64349 could be interpreted to indicate the presence of either a relatively subsidiary NK₂ sub-population in the preparation, or alternatively an interaction of GR64349 with NK₁ receptors as might be expected at such a high concentration of agonist, even if the agent is relatively selective. GR64349 has ca. 0.01 the activity of SP in some other NK₁ preparations (Hagan *et al.*, 1989). The former conclusion is supported by the antagonist data with GR71251 discussed below.

The agonist data discussed seem clearly to indicate the presence of a predominant NK₁ receptor population mediating the action of tachykinins. It should also be noted that we have previously investigated, in the guinea-pig vas deferens, factors related to in the interpretation of agonist studies for receptor classification, particularly those involving possible failure to reach an equilibrium or known biophase concentration due to diffusional barriers or degradation (for discussion, see Kenakin, 1987). We have studied the effect of several peptidase inhibitors on tachykinin activity estimates and kinetics of clearance in the guinea-pig vas deferens, and conclude that agonist breakdown is not a primary determinant of onset of agonist activity or peak response, though slower processes such as offset may well be affected (Hall *et al.*, 1990; see also, Watson, 1983a). This is unlike the situation in other slower responding preparations where peak responses to agonists may indeed be modified by agonist breakdown (eg Stephens-Smith *et al.*, 1988).

In the present series of experiments, it was shown that, in the absence of peptidase inhibitors, the offset times of responses after washout for the synthetic agonists GR73632 and GR64349 were more prolonged as compared to the other agonists. These observations offer support for the claims that these agonists are metabolically stable (Hagan *et al.*, 1989; see, Hall *et al.*, 1990).

Neurokinin receptor subtypes; antagonist studies

The neurokinin receptor antagonist [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ was tested against several tachykinins of dif-

fering subtype selectivities (see also, Watson, 1983b). The antagonist (10 µM), though active against most agonists tested, produced lower apparent pK_B when tested against SP, the NK₁ selective agonist, [Glp⁶,L-Pro⁹]-SP₍₆₋₁₁₎ and was inactive when tested against the NK₁ selective agonist, SPOMe. It should be noted that the antagonist was active when tested against the NK₁ selective agonist, GR73632, with an apparent pK_B of 6.0. That the agonist-dependent apparent pK_B estimates obtained with [D-Arg¹,D-Pro²,D-Trp^{7,9},Leu¹¹]-SP₍₁₋₁₁₎ could indicate the presence of a mixed NK₁ and NK₂ neurokinin receptor population is unlikely in view of this latter observation along with the data obtained with GR71251 discussed below.

The putative NK₁ selective antagonist, GR71251 (Hagan *et al.*, 1990; Ward *et al.*, 1990), was tested against agonists of differing subtype selectivity (SPOMe, GR73632, GR64349 and NKB) in the guinea-pig vas deferens. Schild plot analysis indicated a competitive interaction with a single population of receptors having recognition properties (pK_B = 7.58) virtually identical to those described previously in the guinea-pig ileum in the presence of atropine (pK_B = 7.72; Hagan *et al.*, 1990; Ward *et al.*, 1990), and confirmed in this study (pK_B = 7.86). GR71251 has a reported pK_B of 4.8 at NK₂ receptors (Ward *et al.*, 1990). In view of the similar pK_B estimates being obtained for the four different agonists in the guinea-pig vas deferens, and that the overall estimate is similar to that obtained against NK₁-mediated contraction of the guinea-pig ileum, these data indicate that GR71251 interacts with a homogeneous NK₁ receptor population in this preparation. Further, the pK_B estimate obtained against GR73632 was not significantly different from that obtained against GR64349, showing an interaction of the latter agonist with NK₁ receptors in this preparation.

In conclusion, evidence obtained in this present series of experiments suggests a direct interaction of tachykinins with a homogeneous NK₁ receptor population mediating potentiation of the contractile response to field stimulation in the guinea-pig vas deferens. This preparation has value as a model system where NK₁ modulation of sympathetic neurotransmission is to be studied.

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