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Characterization of NK_3 receptors in rabbit isolated iris sphincter muscle

[1]Andrew D. Medhurst, Andrew A. Parsons, *Jennifer C. Roberts & †Douglas W.P. Hay

Department of Neurology Research and *Department of Biophysical Sciences SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW and †Department of Pulmonary Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, U.S.A.

1 Tachykinin NK_3 receptors were characterized in the rabbit isolated iris sphincter muscle by use of autoradiography and *in vitro* functional studies.

2 [^{125}I]-[MePhe^7]-neurokinin B (NKB) (1 nM), a selective NK_3 receptor agonist, specifically labelled a population of NK_3 receptors that were uniformly distributed throughout the rabbit iris sphincter muscle. This labelling was inhibited by unlabelled [MePhe^7]-NKB (1 μM) but not by the NK_1 receptor antagonist CP 99994 (1 μM).

3 In the presence of CP 99994 (1 μM), the selective NK_3 receptor agonists senktide ($n=14$) and [Pro^7]-NKB ($n=4$), and the natural preferred ligand for the NK_3 receptor, NKB ($n=8$), were potent contractile agents in the rabbit iris sphincter muscle. They all produced monophasic concentration-effect curves with pD_2 values of 9.53 ± 0.08, 8.56 ± 0.09 and 9.75 ± 0.09, and n_H values of 0.93 ± 0.03, 1.53 ± 0.17 and 0.76 ± 0.06, respectively. [MePhe^7]-NKB ($n=12$) was also a potent agonist, but produced shallow concentration-effect curves which appeared biphasic ($n_H=0.45\pm0.04$).

4 Contractile responses to senktide were surmountably antagonized in a concentration-dependent manner by the selective non-peptide NK_3 receptor antagonist, SR 142801 (3–30 nM; $pA_2=8.9$; slope = 0.99) and the non-peptide NK_2/NK_3 receptor antagonist, SR 48968 (3–30 μM; $pA_2=6.1$; slope = 1.5). These pA_2 values were consistent with functional rabbit NK_3 receptors more closely resembling guinea-pig and human NK_3 receptors, than rat NK_3 receptors. SR 142801 (10–100 nM) and SR 48968 (3 and 30 μM) inhibited responses to low ($\leqslant$1 nM) but not higher (>1 nM) concentrations of [MePhe^7]-NKB, and concentration-effect curves to [MePhe^7]-NKB became steeper and monophasic in the presence of either antagonist.

5 SR 142801 (3–30 nM) and SR 48968 (3–30 μM) also surmountably antagonized concentration-effect curves to [Pro^7]-NKB and NKB, although results were more difficult to interpret, since the relationship between log concentration-ratios and the concentration of antagonist used did not adhere to the Schild equation. However, analysis of data with the lowest concentration of SR 142801 (3 nM) tested against NKB, and SR 48968 (3 μM) tested against [Pro^7]-NKB and NKB, yielded apparent pA_2 estimates of 9.3, 6.8 and 6.4, respectively, consistent with blockade of NK_3 receptors.

6 SR 142801 (100 nM) had no effect on contractions induced by transmural nerve stimulation (2 Hz, 0.3 ms, 20 V for 30 s), whereas CP 99994 (1 μM) abolished these responses.

7 Phenoxybenzamine pretreatment (20 μM, 10 min) markedly reduced maximum responses to [MePhe^7]-NKB (from $101\pm6.2\%$ to $38\pm9.5\%$ reference contraction, $n=4$) and induced a marked (10 fold) rightward shift in the concentration-effect curve. The residual responses to [MePhe^7]-NKB after phenoxybenzamine pretreatment were unaffected by 1 μM CP 99994 (maximum response $=41\pm9.4\%$, $n=4$).

8 These results demonstrate autoradiographically and functionally, the presence of NK_3 receptors in rabbit iris sphincter muscle that mediate contractile responses to NK_3 receptor agonists, but not to sensory trigeminal nerve stimulation. The present data with senktide and selective NK_3 receptor antagonists suggest that functional rabbit NK_3 receptors more closely resemble human and guinea-pig NK_3 receptors than rat NK_3 receptors. However, the pharmacological profiles of [MePhe^7]-NKB, SR 142801 and SR 48968 suggest the presence of an 'atypical' NK_3 receptor or a heterogeneous population of NK_3 receptors in this tissue.

Keywords: NK_3 receptors; rabbit iris sphincter muscle; SR 142801; SR 48968; senktide; [MePhe^7]-neurokinin B; neurokinin B; [Pro^7]-neurokinin B

Introduction

Tachykinin receptors have been classified into three subtypes, namely NK_1, NK_2 and NK_3 (Guard & Watson, 1991) and all three human tachykinin receptor genes have been cloned (Nakanishi, 1991). In general, more is known about the pharmacology and pathophysiological roles of NK_1 and NK_2 receptors, than of NK_3 receptors. However, NK_3 receptors have been relatively well characterized in several tissues including guinea-pig ileum (Nguyen *et al*., 1994; Patacchini *et al*., 1995; Emonds-Alt *et al*., 1995; Croci *et al*., 1995), guinea-pig colon (Giuliani & Maggi, 1995), rat portal vein (Nguyen *et al*., 1994; Patacchini *et al*., 1995; Emonds-Alt *et al*., 1995), guinea-pig and rat cerebral cortex (Suman-Chauhan *et al*., 1994; Emonds-Alt *et al*., 1995), and Chinese hamster ovary (CHO) cells stably expressing cloned human NK_3 receptors (Oury-Donat *et al*., 1995; Emonds-Alt *et al*., 1995; Chung *et al*., 1995). There is also evidence for NK_3 receptors in the rabbit isolated iris sphincter muscle (Hall *et al*., 1991; 1993; Wang & Hakanson, 1993), although full characterization of these receptors with selective NK_3 receptor antagonists has not been obtained.

[1] Author for correspondence.

A number of pharmacological tools are available for the study of tachykinin receptors. Senktide (succinyl-[Asp^9,MePhe^8]-substance P (6-11)) and [MePhe^7]-neurokinin B (NKB) are highly selective agonists for NK_3 receptors compared to NK_1 and NK_2 receptors, and tend to be the agonists of choice for the functional characterization of NK_3 receptors (Wormser *et al*., 1986; Drapeau *et al*., 1987; Oury-Donat *et al*., 1995). [Pro^7]-NKB and the natural preferred ligand for the NK_3 receptor, NKB, are also potent NK_3 receptor agonists, although they exhibit less selectivity for the NK_3 receptor versus NK_1 and NK_2 receptors than senktide and [MePhe^7]-NKB (Lavielle *et al*., 1988; 1990; Petitet *et al*., 1993). NK_3 receptor characterization, and assessment of the functional and pathophysiological role of activation of this receptor, has been hindered until recently by the lack of high affinity and selective receptor antagonists. SR 48968 ((**S**)-N-methyl-N[4-acetlyamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide), a potent non-peptide NK_2 receptor antagonist (Advenier *et al*., 1992) with moderate affinity for NK_3 receptors, and the peptide [Trp^7,βA1a^8]-NKA(4–10) have been used, although these possess limited potency and selectivity (Drapeau *et al*., 1990; Petitet *et al*., 1993; Nguyen *et al*., 1994). Importantly, the first potent and selective non-peptide NK_3 receptor antagonist, SR 142801 ((**S**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin - 3 - yl)propyl) - 4 - phenylepiperidin -4-yl)-N-methylacetamide), was recently described (Emonds-Alt *et al*., 1995), and has facilitated more complete characterization of NK_3 receptors (Oury-Donat *et al*., 1995; Patacchini *et al*., 1995; Emonds-Alt *et al*., 1995; Croci *et al*., 1995, Giulianai & Maggi, 1995; Medhurst *et al*., 1996).

The existence of NK_3 receptor subtypes was recently proposed, based on the differing pharmacology of [Trp^7,βAla^8]-NKA(4–10) and SR 48968 in guinea-pig ileum and rat portal vein (Nguyen *et al*., 1994). In addition, SR 142801 has been shown to be 100 fold more potent at inhibiting responses to senktide and [MePhe^7]-NKB in guinea-pig ileum than in rat portal vein (Emonds-Alt *et al*., 1995; Patacchini *et al*., 1995), whilst SR 48968 was over 30 fold more potent at inhibiting [^{125}I]-[MePhe^7]-NKB binding to guinea-pig brain and cloned human NK_3 receptors expressed in CHO cells, than to rat brain NK_3 receptors (Suman-Chauhan *et al*., 1994). However, these results may reflect tissue and species differences in NK_3 receptor characteristics and at present there is no evidence for NK_3 receptor subtypes within the same tissue and species.

Functional studies indicate that the rabbit isolated iris sphincter muscle preparation possesses NK_1 and NK_3 receptors, but not NK_2 receptors (Hall *et al*., 1991; 1993; Wang & Hakanson, 1993). The present study was designed to characterize functionally, by use of NK_3 receptor agonists and antagonists, as well as autoradiography, the NK_3 receptors present in rabbit iris sphincter muscle. Preliminary accounts of this work have been communicated to the British Pharmacological Society (Medhurst *et al*., 1996) and to the Tachykinins '95: from Basic Science to Clinical Applications meeting, Florence, Italy (1995).

Methods

Rabbit isolated iris sphincter muscle preparation

Male New Zealand White rabbits (2–3 kg, Charles River) were killed by i.v. pentobarbitone (Euthatal) followed by exsanguination. The eyes were immediately removed and placed in ice-cold Krebs-Henseleit solution consisting of (mM): NaCl 117.6, KCl 5.4, $NaH_2PO_4.2H_2O$ 1.0, $MgSO_4.7H_2O$ 0.7, glucose 11.1, $NaHCO_3$ 25 and $CaCl_2$ 2.5. Iris sphincter muscles were dissected from the cornea, surrounding connective tissue and dilator muscle, and cut into strips (one per eye). Tissues were either stored at −80°C for autoradiography or used immediately for functional studies.

Autoradiography

Longitudinal sections of iris sphincter muscle strips (20 μm) were cut on a cryostat at −20°C and thaw-mounted onto gelatin-coated slides. Sections were pre-incubated for 30 min in 50 mM Tris-HCl (pH 7.4) containing bovine serum albumin (BSA; 0.02%) at room temperature (22°C). Following pre-incubation, sections were incubated in assay buffer consisting of 50 mM Tris-HCl, BSA (0.02%), $MnCl_2$ (3 mM), chymostatin (2 μg ml^{-1}), bacitracin (40 μg ml^{-1}) and leupeptin (4 μg ml^{-1}). Total binding was defined by incubating the sections in assay buffer containing 1 nM [^{125}I]-[MePhe^7]-NKB for 90 min at room temperature. Non-specific binding and the effect of NK_1 receptor antagonism on specific binding were determined by incubating anatomically adjacent sections in assay buffer containing 1 nM [^{125}I]-[MePhe^7]-NKB in the presence of 1 μM unlabelled [MePhe^7]-NKB and 1 μM CP 99994, respectively, for 90 min at room temperature.

Following incubation, all sections were rinsed three times for 5 min in assay buffer alone at 4°C and then quickly dipped in distilled water, also at 4°C. The sections were then dried in a stream of cool air and exposed to X-ray film (Hyperfilm, Amersham) for 5 days at 4°C. Adjacent sections were stained with 1% cresyl violet for histological identification. Autoradiograms were photographed on PanF film (Ilford).

Functional studies

Iris sphincter muscle strips, isolated as outlined above, were placed in 50 ml organ baths containing Krebs-Henseleit solution maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Each iris strip was attached to a Swema SG4-45 strain guage transducer by means of a stainless steel wire and tension was recorded isometrically on a Watanabe polygraph (Graphtech, Japan).

Tissues were stretched every 10 min to a tension equivalent to 400 mg, over a period of 30 min, and then left to equilibrate for a further 45 min. At the beginning of each experiment, a reference contractile response to carbachol (10 μM) was obtained in each tissue. Preliminary experiments showed this concentration of carbachol to induce maximum responses. After washout, experiments were conducted in the presence of atropine (1 μM; to block indirect cholinergic influences; Ueda *et al*., 1982) and CP 99994 ((+)-(2**S**,3**S**)-3-(2-methoxybenzylamino)-2-phenylpiperidine; 1 μM) to eliminate the involvement of NK_1 receptors (McLean *et al*., 1993).

In some experiments (in the presence or absence of 1 μM CP 99994), tissues were exposed to phenoxybenzamine (20 μM 10 min) or vehicle (ethanol) followed by washout every 5 min for 20 min (as described by Hall *et al*., 1994). A further 30 min later, concentration-effect curves to [MePhe^7]-NKB or senktide were constructed.

To determine the effects of antagonists, tissues were incubated for 120 min with either SR 142801, SR 48968 or [Trp^7,βAla^8]-NKA(4–10), before cumulative concentration-effect curves to NK_3 receptor agonists were determined by sequentially increasing the total concentration by half log unit increments. Concentration-effect curves to NK_3 receptor agonists in time-matched control tissues incubated with vehicle (DMSO) were determined in parallel with antagonist-treated preparations. In some tissues not exposed to NK_3 receptor antagonists, the rates of offset of contractile responses to NK_3 receptor agonists were determined by washing every 5 min until tension returned to pre-dose level.

In a separate series of experiments to compare the effects of NK_3 and NK_1 receptor antagonists on neurogenic contraction, rabbit iris sphincter muscle strips were electrically stimulated (2 Hz, 0.3 ms, 20 V for 30 s) in the presence of atropine (1 μM), before (S1) and after (S2) addition of SR 142801 (100 nM), or CP 99994 (1 μM) for 120 min.

Data analysis

Contractile responses to NK_3 receptor agonists were calculated as a percentage of the carbachol-induced contraction. Agonist concentration-effect curves were fitted by use of Microsoft Excel to the Hill equation:

$$\frac{E}{E_{max}} = \frac{[A]^{n_H}}{[A]_{50^{n_H}} + [A]^{n_H}}$$

where E_{max} is the maximal action of A, n_H is the Hill coefficient and $[A]_{50}$ is the concentration that produces an effect that is 50% of E_{max} (Jenkinson *et al*., 1995). Agonist potency was expressed in terms of absolute potency as pD_2 which represents the $-\log_{10}$ concentration of agonist producing 50% of the maximum response. All pD_2 and n_H values are expressed as mean $\pm$ s.e. mean.

Affinity estimates for antagonists were expressed as pA_2 values calculated according to the method of Arunlakshana & Schild (1959) or by single concentration analysis from the following equation:

$$pA_2 = -\log[B] + \log[CR - 1]$$

where [B] represents the concentration of antagonist and CR represents the ratio of EC_{50} location parameters for agonist concentration-effect curves in the presence and absence of antagonist, respectively.

The percentage inhibition of neurogenic contraction by antagonists was calculated from contraction induced by (S1 − S2)/S1.

Chemicals

[^{125}I]-[MePhe^7]-NKB (specific activity 2200 Ci mmol^{-1}) was obtained from New England Nuclear (Dreieich, Germany). Carbachol, chymostatin, bacitracin and leupeptin were obtained from Sigma (Poole), atropine from BDH Chemicals Limited (Poole) and phenoxybenzamine from Research Biochemicals Incorporated (Natick, U.S.A.). Senktide (succinyl-[Asp^9,MePhe^8]-SP(6 – 11)), [MePhe^7]-NKB, NKB, [Pro^7]-NKB and [Trp^7,βAla^8]-NKA(4 – 10) were purchased from Peninsula Laboratories Inc. (St. Helens). SR 142801 ((**S**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylepiperidin-4-yl)-N-methylacetamide), SR 48968 ((**S**)-N-methyl - N[4 - acetlyamino - 4 - phenylpiperidino) - 2-(3,4-dichlorophenyl)butyl]benzamide) and CP 99994 ((+)-(2**S**, 3**S**)-3-(2-methoxybenzylamino)-2-phenylpiperidine) were synthesised by colleagues in the Department of Medicinal Chemistry at SmithKline Beecham Pharmaceuticals (Milan, Italy). All stock solutions were prepared in distilled water except SR 142801, SR 48968, NKB, and [Trp^7,βAla^8]-NKA(4 – 10) which were dissolved in 100% dimethylsulphoxide (DMSO; final bath concentration $< 0.1\%$), and phenoxybenzamine, which was dissolved in 100% ethanol (final bath concentration $< 0.1\%$) All neuropeptide stock solutions (1 mM) were stored as 50 – 200 μl aliquots at -20°C and used once before being discarded.

Results

Autoradiographical studies

Histological analysis of longitudinal sections of rabbit isolated iris sphincter muscle revealed the undulating epithelium on the pupillary surface, and the dilator muscle margin of the opposing side (Figure 1a). [^{125}I]-[MePhe^7]-NKB (1 nM) labelled a population of NK_3 receptors that appeared uniformly distributed throughout the iris sphincter muscle in an anatomically adjacent section (Figure 1b). This specific binding was inhibited by 1 μM unlabelled [MePhe^7]-NKB in anatomically adjacent sections, where non-specific binding was uniformly low (Figure 1c), whilst CP 99994 (1 μM) had no effect on specific binding (not shown).

Functional studies – NK_3 receptor agonists

Senktide, [MePhe^7]-NKB, [Pro^7]-NKB and NKB were all potent contractile agents in rabbit isolated iris sphincter muscle (Figure 2). Senktide (1 pM – 30 nM) produced monophasic concentration-effect curves with a $pD_2 = 9.53 \pm 0.08$ and $n_H = 0.93 \pm 0.03$ ($n = 14$). Concentration-effect curves to NKB (1 pM – 30 nM) were monophasic with a $pD_2 = 9.75 \pm 0.09$ and $n_H = 0.76 \pm 0.06$ ($n = 8$). [Pro^7]-NKB was a less potent agonist than senktide and NKB, with a $pD_2 = 8.56 \pm 0.09$ ($n = 4$). Furthermore, concentration-effect curves to [Pro^7]-NKB (10 pM – 30 nM) were steeper than those of senktide and NKB with $n_H = 1.53 \pm 0.17$. In contrast to the other NK_3 receptor agonists tested, [MePhe^7]-NKB produced shallow concentration-effect curves which appeared to be biphasic ($n_H = 0.45 \pm 0.04$; $n = 12$), preventing the accurate determination of pD_2 values. After washout, responses to [MePhe^7]-NKB were very slow in offset (mean time to complete reversal = 106 ± 6 min, $n = 4$), whilst responses to [Pro^7]-NKB, senktide and NKB were relatively fast in offset (mean time to complete reversal = 23 ± 2, 20 ± 3, and 24 ± 2 min, respectively, $n = 4$).

Functional studies – NK_3 receptor antagonists

SR 142801 (3 – 30 nM) surmountably antagonized the contractile responses to senktide, resulting in concentration-dependent rightward shifts in the agonist curves (Figure 3a). The pA_2 was calculated to be 8.9, with a slope of 0.99 ± 0.08 ($n = 4$), indicative of competitive antagonism. In contrast, SR 142801 (10 – 100 nM) inhibited the responses to low ($\leqslant$1nM) but not higher (>1 nM) concentrations of [MePhe^7]-NKB (Figure 3b). In the presence of SR 142801 (10, 30 and 100 nM), concentration-effect curves to [MePhe^7]-NKB became steeper and monophasic with n_H values = 0.64 ± 0.03, 0.74 ± 0.04 and 0.94 ± 0.1, respectively. SR 142801 (3 – 30 nM) also surmountably antagonized responses to [Pro^7]-NKB (Figure 3c) and NKB (Figure 3d). For antagonism of [Pro^7]-NKB-induced responses by SR 142801 (3, 10 and 30 nM), log concentration-ratios were 0.18, 0.90 and 1.65, respectively, and the Schild slope was significantly (Student's t test) greater than unity (1.8). Log concentration-ratios for antagonism of NKB-induced responses by SR 142801 (3, 10 and 30 nM) were 0.83, 0.90 and 1.43, respectively. Thus, 3 and 10 nM SR 142801 produced very similar rightward shifts, whilst 30 nM SR 142801 produced a large further rightward shift in the concentration-effect curve to NKB. Analysis of data with the lowest concentration of SR 142801 tested (ie 3 nM) yielded a pA_2 estimate of 9.3. SR 142801 had no contractile activity in any experiments and did not induce relaxation in tissues precontracted with 1 μM carbachol ($n = 3$).

SR 48968 (3 – 30 μM) produced similar effects to SR 142801 against senktide-induced contractile responses, although it was about 630 fold less potent ($pA_2 = 6.1$, slope = 1.5 ± 0.03, $n = 4$; Figure 4a). SR 48968 (3 and 30 μM) also inhibited responses to low ($\leqslant$1 nM) but not higher (>1 nM) concentrations of [MePhe^7]-NKB, but again was less potent than SR 142801 (at least 100 fold; Figure 4b). In the presence of SR 48968, the concentration-effect curves to [MePhe^7]-NKB became steeper and monophasic ($n_H = 0.73 \pm 0.08$ and 1.32 ± 0.08 for 3 and 30 μM, respectively). Contractile responses to [Pro^7]-NKB (Figure 4c) and NKB (Figure 4d) were surmountably antagonized by SR 48968 (3 – 30 μM). However, the antagonism of responses to both agonists was not consistent with the blockade of one receptor population. For antagonism of [Pro^7]-NKB-induced contraction by SR 48968 (3, 10 and 30 μM), log concentration-ratios were 1.38, 1.68 and 2.2, respectively. Although only a small further rightward shift in [Pro^7]-NKB concentration-effect curves was obtained with 10 and 30 μM SR 48968, analysis of data obtained with 3 μM SR 48968 yiel-

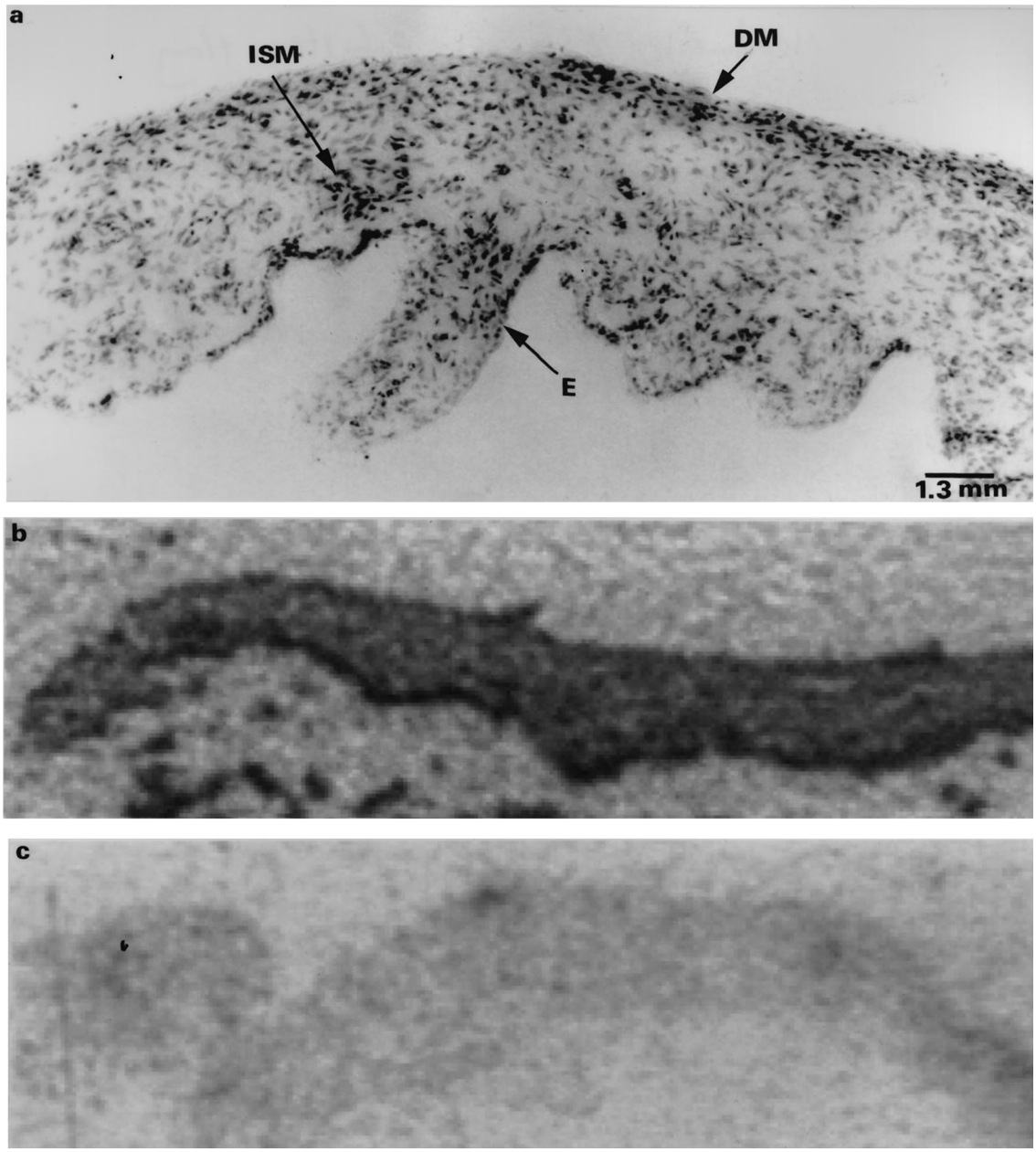

Figure 1 (a) Histological section of rabbit isolated iris sphincter muscle (ISM) showing the undulating epithelium (E) of the pupillary surface and the dilator muscle (DM) margin; (b) autoradiogram of [^{125}I]-[MePhe^7]-neurokinin B (NKB) binding in adjacent section of rabbit isolated iris sphincter muscle, and (c) autoradiogram of [^{125}I]-[MePhe^7]-NKB binding in another adjacent section of rabbit isolated iris sphincter muscle in the presence of 1 μM unlabelled [MePhe^7]-NKB.

ded a pA_2 estimate of 6.8. For inhibition of NKB-induced contraction by SR 48968 (3, 10 and 30 μM), log concentration-ratios were 1.01, 1.10 and 2.11, respectively. Thus, 3 and 10 μM SR 48968 produced almost similar rightward shifts, whilst 30 μM SR 48968 produced a large further rightward shift in the concentration-effect curve to NKB. Analysis of data with the lowest concentration of SR 48968 tested (ie 3 μM) yielded a pA_2 estimate of 6.4. Like SR 142801, SR 48968 had no contractile activity in any experiments and at concentrations up to 100 μM did not relax tissues precontracted with 1 μM carbachol ($n=3$).

[Trp^7,βAla^8]-NKA(4-10) (0.1 μM) had no effect on concentration-effect curves to senktide (Figure 5a) but produced a small antagonism of [MePhe^7]-NKB-induced responses (Figure 5b) which was only statistically significant ($P<0.05$; Student's unpaired t test) at 0.3 nM [MePhe^7]-NKB. Concentrations of [Trp^7,βAla^8]-NKA(4–10) higher than 0.1 μM induced non-specific contractile responses.

Functional studies – neurogenic contraction

Electrical stimulation (2 Hz, 0.3 ms, 20 V for 30 s) induced reproducible contractile responses in the rabbit isolated iris sphincter muscle which were unaffected by 100 nM SR 142801, but abolished by 1 μM CP 99994 (Figure 6).

Functional studies – phenoxybenzamine pretreatment

Concentration-effect curves to [MePhe^7]-NKB were markedly affected by phenoxybenzamine pretreatment (Figure 7). Maximum responses (% carbachol contraction) to [MePhe^7]-NKB were significantly ($P<0.05$, Student's unpaired t test) reduced from control values of $101\pm6.2\%$ to $38\pm9.5\%$ by phenoxybenzamine ($n=4$), and the concentration-effect curve to [MePhe^7]-NKB was markedly (10 fold) shifted to the right. Residual responses to [MePhe^7]-NKB after phenoxybenzamine pretreatment were unaffected by 1 μM CP 99994 (maximum

response = 41 ± 9.4%, $n = 4$). Phenoxybenzamine had a similar effect on senktide-induced contractions, significantly ($P < 0.05$, Student's unpaired t test) reducing the maximum responses from 116 ± 5 to 25 ± 1 ($n = 4$) and markedly shifting the concentration-effect curve of senktide to the right.

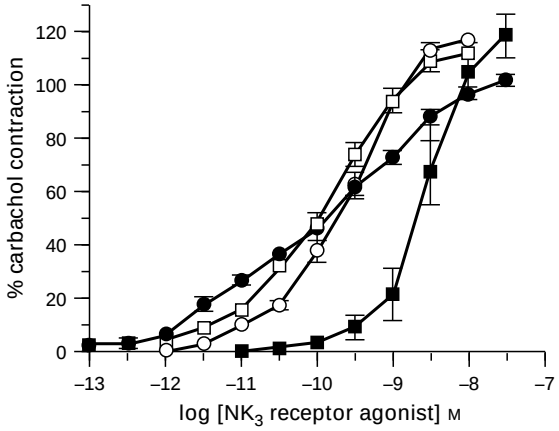

Figure 2 Concentration-effect curves to senktide (○, $n = 14$), [MePhe^7]-neurokinin B (NKB) (●, $n = 12$), NKB (□, $n = 8$) and [Pro^7]-NKB (■, $n = 4$) in rabbit isolated iris sphincter muscle. Responses are expressed as a percentage of the contraction induced by 10 μM carbachol and data are mean with vertical lines showing s.e. mean.

Discussion

The results of the present study with the selective NK_3 receptor antagonist SR 142801 (Emonds-Alt *et al*., 1995), and the NK_2/NK_3 receptor antagonist SR 48968 (Petitet *et al*., 1993), support the role of NK_3 receptor activation mediating contractile responses induced by senktide and [MePhe^7]-NKB in the rabbit iris sphincter muscle (Hall *et al*., 1991; 1993; Wang & Hakanson, 1993). In addition, the pharmacological profile of [MePhe^7]-NKB, SR 142801 and SR 48968 is suggestive of NK_3 receptor heterogeneity in the rabbit iris sphincter muscle. Autoradiographically we have demonstrated high affinity binding sites for the highly selective NK_3 receptor agonist [^{125}I]-[MePhe^7]-NKB, confirming the presence of NK_3 receptors in addition to the substance P binding sites demonstrated previously in rabbit iris sphincter muscle, also by use of autoradiography (Denis *et al*., 1991). The demonstration that SR 142801 failed to inhibit neurogenic contractile responses in the presence of atropine, whilst the NK_1 receptor antagonist, CP 99994 (McLean *et al*., 1993), abolished them, confirms that responses to sensory trigeminal nerve stimulation in the rabbit iris sphincter muscle are the result of NK_1 receptor rather than NK_3 receptor activation (Wang & Hakanson, 1992; Hall *et al*., 1993).

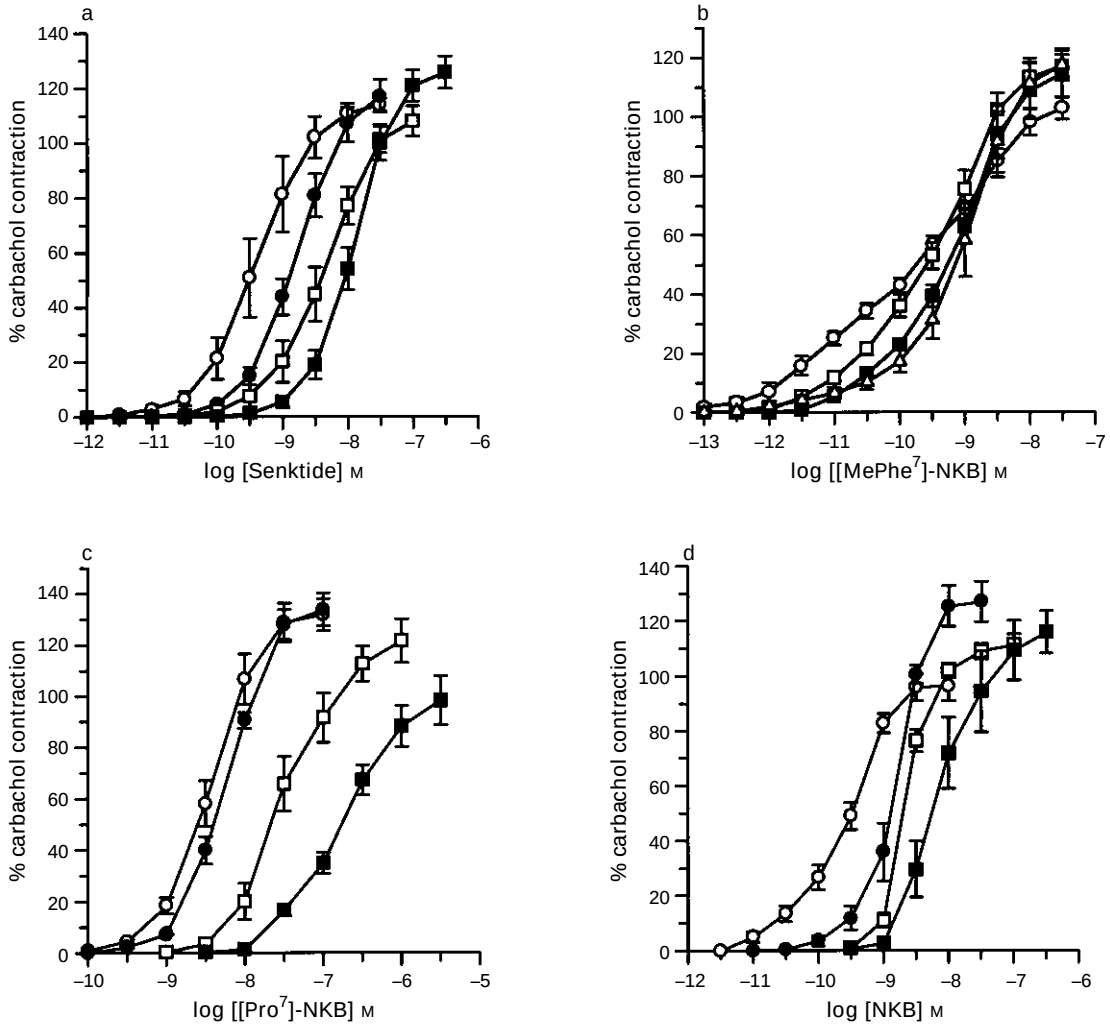

Figure 3 Concentration-effect curves to (a) senktide, (b) [MePhe^7]-neurokinin B (NKB) (c) [Pro^7]-NKB and (d) NKB in the absence (○, $n = 4$) and presence of 3 (●, $n = 4$), 10 (□, $n = 4$), 30 (■, $n = 4$) and 100 nM (△, $n = 4$) SR 142801 in rabbit isolated iris sphincter muscle. Tissues were incubated with SR 142801 for 120 min before agonist concentration-effect curves were constructed. Responses are expressed as a percentage of the contraction induced by 10 μM carbachol and data are mean with vertical lines showing s.e. mean.

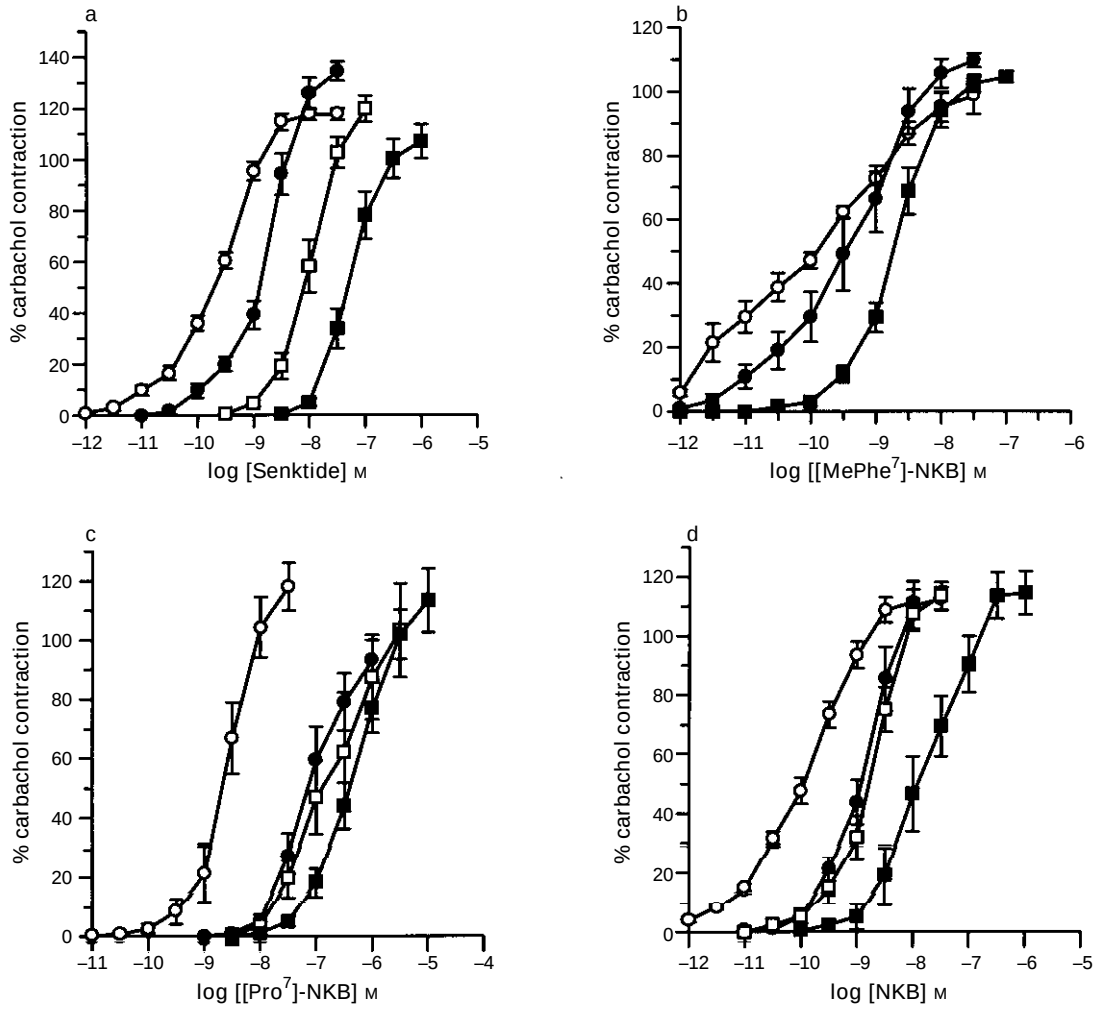

Figure 4 Concentration-effect curves to (a) senktide, (b) [MePhe^7]-neurokinin B (NKB), (c) [Pro^7]-NKB and (d) NKB in the absence (○, $n=4$) and presence of 3 (●, $n=4$), 10 (□, $n=4$), and 30 μM (■, $n=4$) SR 48968 in rabbit isolated iris sphincter muscle. Tissues were incubated with SR 48968 for 120 min before agonist concentration-effect curves were constructed. Responses are expressed as a percentage of the contraction induced by 10 μM carbachol and data are the mean with vertical lines showing s.e. mean.

In the present study, senktide, [MePhe^7]-NKB and NKB potently contracted the rabbit iris sphincter muscle, consistent with previous results obtained in other laboratories (Muramatsu *et al*., 1987; Too *et al*., 1988; Beding-Barnekow *et al*., 1988; Hall *et al*., 1991; 1993; Wang & Hakanson, 1993). Additionally, we have shown that [Pro^7]-NKB is a potent NK_3 receptor agonist in the rabbit iris sphincter muscle, although it was the least potent of the NK_3 receptor agonists tested. The potency estimate for [Pro^7]-NKB in the present study ($pD_2=8.56$) is similar to that previously obtained in guinea-pig ileum ($pD_2=8.51$; Lavielle *et al*., 1988; 1990).

Binding and functional studies have shown that SR 142801 is a potent selective non-peptide NK_3 receptor antagonist (Emonds-Alt *et al*., 1995; Oury-Donat *et al*., 1995; Patacchini *et al*., 1995; Croci *et al*., 1995). In the present study, the surmountable and competitive antagonism by SR 142801 of senktide-induced responses in rabbit iris sphincter muscle was consistent with the blockade of NK_3 receptors. The affinity estimate calculated for SR 142801 against senktide ($pA_2=8.9$) was comparable with that measured in the guinea-pig ileum (apparent $pK_B=8.98-9.27$; Emonds-Alt *et al*., 1995; Patacchini *et al*., 1995; Croci *et al*., 1995) and human NK_3 receptors expressed in CHO cells ($pK_i=9.4-9.68$; Oury-Donat *et al*., 1995; Chung *et al*., 1995), but not in rat portal vein (apparent $pK_B=7.49$; Patacchini *et al*., 1995), suggesting for the first time that rabbit NK_3 receptors more closely resemble guinea-pig and human, rather than rat NK_3 receptors. Further support for the proposal that rabbit NK_3 receptors, more closely resemble human and guinea-pig, rather than rat NK_3 receptors, was provided by the observation that SR 48968 has a similar potency for antagonism of senktide-induced responses in rabbit iris sphincter muscle ($pA_2=6.1$) to that obtained in guinea-pig ileum ($pA_2=6.05$; Nguyen *et al*., 1994) and human, cloned NK_3 receptors ($pK_i=6.46-6.54$; Suman-Chauhan *et al*., 1994; Chung *et al*., 1995) but not rat portal vein ($pA_2=4.8$; Nguyen *et al*., 1994).

SR 48968 and SR 142801 also antagonized responses to NKB and [Pro^7]-NKB in the rabbit iris sphincter muscle, although the results were more difficult to interpret. Apparent pA_2 values of 6.8, 6.4 and 9.3 were estimated from the lowest concentration of antagonist tested for SR 48968 versus [Pro^7]-NKB, SR 48968 versus NKB, and SR 142801 versus NKB respectively, consistent with the affinity of these antagonists for NK_3 receptors demonstrated previously (Nguyen *et al*., 1994; Emonds-Alt *et al*., 1995; Chung *et al*., 1995 Croci *et al*., 1995; Patacchini *et al*., 1995; Oury-Donat *et al*., 1995). Further increases in antagonist concentration disproportionally shifted the concentration-effect curves to [Pro^7]-NKB and NKB compared to data obtained with the lowest concentrations of antagonists tested (i.e. data did not conform to the Schild equation; Jenkinson *et al*., 1995), suggesting that SR 48968 and SR 142801 may block additional non-NK_3 receptor-mediated

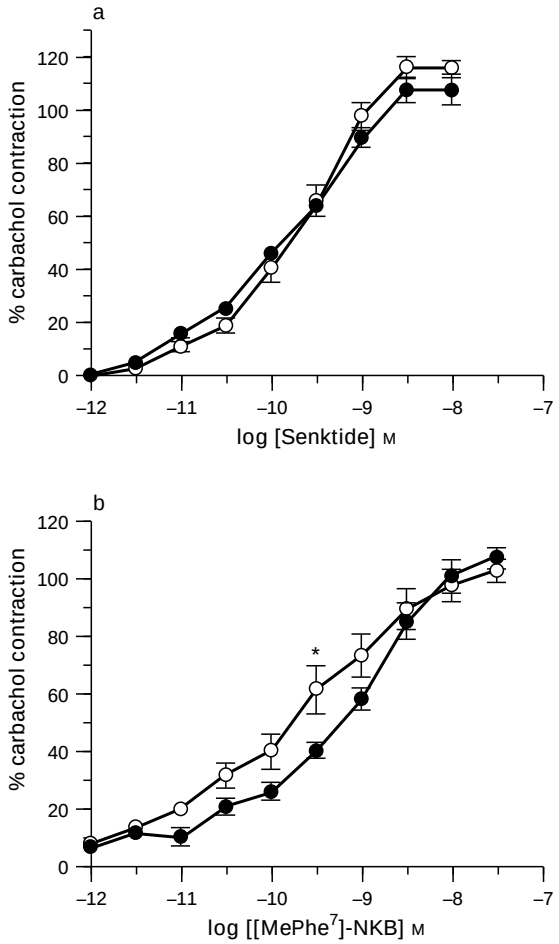

Figure 5 Concentration-effect curves to (a) senktide, and (b) [MePhe^7]-neurokinin B (NKB) in the absence (○, $n=4$) and presence of 0.1 μM (●, $n=4$) [Trp^7,βAla^8]-NKA(4–10) in rabbit isolated iris sphincter muscle. Tissues were incubated with [Trp^7,βAla^8]-NKA(4–10) for 120 min before agonist concentration-effect curves were constructed. Responses are expressed as a percentage of the contraction induced by 10 μM carbachol and the data are mean with vertical lines showing s.e.mean (*$P<$ 0.05, Student's unpaired t test).

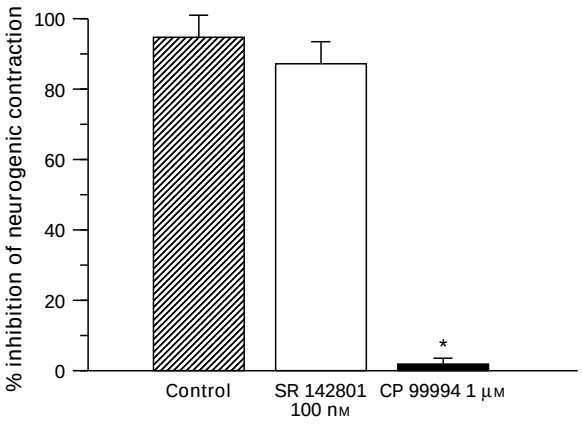

Figure 6 Effect of SR 142801 and CP 99994 on neurogenic contractile responses in rabbit iris sphincter muscle ($n=4$). Tissues were electrically stimulated in the presence of atropine (1 μM) before (S1) and after (S2) the addition of antagonists for 120 min. Data are expressed as % inhibition calculated from contraction elicited by (S1−S2)S1 and are shown as the mean ± s.e. mean. *Significant inhibition ($P<0.05$; Student's unpaired t test).

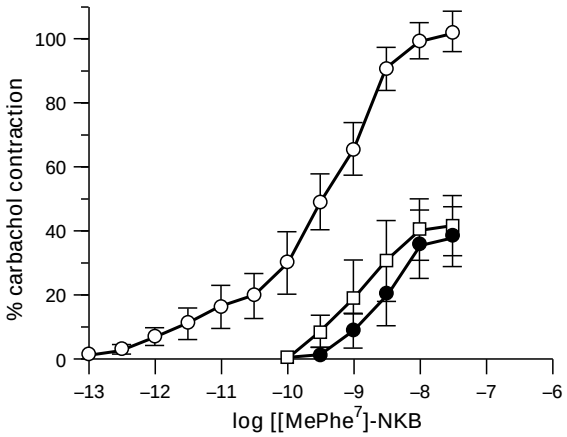

Figure 7 Concentration-effect curves to [MePhe^7]-neurokinin B (NKB) (○), and [MePhe^7]-NKB after phenoxybenzamine pretreatment in the absence (●) and presence (□) of 1 μM CP 99994, in rabbit isolated iris sphincter muscle ($n=4$). Tissues were pretreated with phenoxybenzamine (20 μM for 10 min) or vehicle (ethanol) followed by washout every 5 min for 20 min. After a further 30 min, concentration-effect curves to [MePhe^7]-NKB were constructed. Responses are expressed as a percentage of the contraction induced by 10 μM carbachol and the data are mean with vertical lines showing s.e.mean.

responses. Interestingly SR 142801, which is structurally very similar to SR 48968, has an IC_{50} of 0.1 – 1 μM in binding assays for calcium (verapamil site) and sodium (site 2) channels (Emonds-Alt *et al*., 1994). Despite this, we detected no relaxation of the rabbit iris sphincter muscle precontracted with 1 μM carbachol by 100 μM SR 48968 or 1 μM SR 142801. Alternatively, these results may reflect the poorer selectivity of [Pro^7]-NKB and NKB compared to senktide and [MePhe^7]-NKB for NK_3 receptors over other tachykinin receptors. The complex and variable pharmacological profiles suggest that in the rabbit iris sphincter muscle, senktide may be the agonist of choice for the characterization of 'typical' NK_3 receptors (eg secondary screening for NK_3 receptor antagonists) rather than [MePhe^7]-NKB, NKB or [Pro^7]-NKB.

[Trp^7,βAla^8]-NKA (4 – 10), a potent peptide NK_3 receptor antagonist in rat portal vein but inactive in guinea-pig ileum (Nguyen *et al*., 1994), had very little effect on senktide- and [MePhe^7]-NKB-induced contraction in the rabbit iris sphincter muscle. It also induced contraction at higher concentrations, as previously shown in rat portal vein (Drapeau *et al*., 1990), making it unsuitable for further study.

Putative NK_3 receptor subtypes have been proposed but the differences in NK_3 receptor pharmacology could often be attributed to species- and tissue-specific effects (Nguyen *et al*., 1994; Petitet *et al*., 1993). Intraspecies heterogeneity of NK_3 receptors has been implicated in the guinea-pig enteric nervous system, since SR 142801 had a different affinity for antagonizing nitric oxide-dependent and independent responses to senktide (Giuliani & Maggi, 1995). Some of the present data are suggestive of the presence of NK_3 receptor subtypes in rabbit iris sphincter muscle. For example, concentration-effect curves to [MePhe^7]-NKB appeared shallow and biphasic, as reflected by the low n_H values, implying the possible activation of more than one receptor subtype. Indeed, careful observation of data presented previously by Hall *et al*. (1993), reveals multiphasic concentration-effect curves to [MePhe^7]-NKB with a shallow mean slope of 0.43. Several lines of evidence indicate that contractile responses induced by [MePhe^7]-NKB up to 30 nM (i.e. the highest concentration tested), are likely to be the result of NK_3 receptor activation, rather than other mechanisms such as stimulation of NK_1 or NK_2 receptors. Firstly, [MePhe^7]-NKB is a highly selective agonist for NK_3 receptors with low affinity for both NK_1 and NK_2 receptors (Drapeau *et al*., 1987). In binding studies, [MePhe^7]-NKB was over 80,000 fold selective for cloned human NK_3 receptors

over cloned human NK_1 and NK_2 receptors expressed in CHO cells (H. Sarau, personal communication). Secondly, the NK_1 receptor antagonist CP 99994 (McLean *et al*., 1993) was present throughout our experiments at a concentration (1 μM) that would saturate NK_1 receptors. Thirdly, few, if any, functional NK_2 receptors appear to be present in rabbit iris sphincter muscle (Wang & Hakanson, 1992; 1993; Hall *et al*., 1993), and in the present study the potent NK_2 receptor antagonist SR 48968, did not inhibit responses to high concentrations of [MePhe^7]-NKB. An effect of [MePhe^7]-NKB at other non-tachykinin receptors cannot be discounted, although the combined results of our studies and those of Hall *et al*. (1993) show that the multiphase response profile of [MePhe^7]-NKB occurs in the presence of blockers for muscarinic receptors (atropine), histamine receptors (mepyramine and cimetidine), sympathetic and ganglionic neurotransmission (guanethidine and hexamethonium) and prostanoid formation (ibuprofen).

Additional evidence for the possible existence of NK_3 receptor subtypes in rabbit iris sphincter muscle was obtained from antagonist studies. Both SR 142801 and SSR 48968 blocked responses to low, but not higher, concentrations of [MePhe^7]-NKB, implying the existence of SR 142801- and SR 48968-insensitive and sensitive responses to this highly selective NK_3 receptor agonist. The observation that concentrations of SR 142801 and SR 48968 shown to block senktide-induced responses, also inhibited responses to low concentrations of [MePhe^7]-NKB, suggests that low concentrations of [MePhe^7]-NKB activate 'typical' NK_3 receptors, whilst higher concentrations of [MePhe^7]-NKB may activate 'atypical' NK_3 receptors.

The presence of 'atypical' NK_1 receptors has been proposed in the rabbit isolated iris sphincter muscle which are activated by certain NK_1 receptor agonists including substance P, resulting in contractile responses that are not blocked by NK_1 receptor antagonists (Hall *et al*., 1994). Interestingly, following phenoxybenzamine pretreatment, NK_1 receptor antagonists block substance P-induced responses in the rabbit iris sphincter (Hall *et al*., 1994). Our results with phenoxybenzamine and [MePhe^7]-NKB were quite different from those observed in the 'atypical' NK_1 receptor studies of Hall *et al*. (1994) with identical phenoxybenzamine pretreatment protocols (20 μM for 10 min). Responses to [MePhe^7]-NKB and senktide were extremely susceptible to alkylation by phenoxybenzamine as demonstrated by the large decrease in maximum response and large rightward shift in the concentration-response curve. Indeed, this effect of phenoxybenzamine can be prevented by incubation of tissues with NK_3 receptor antagonists before the administration of phenoxybenzamine (unpublished observations). In addition, the residual contractile responses to [MePhe^7]-NKB remaining after phenoxybenzamine pretreatment were not susceptible to NK_1 receptor blockade by CP 99994 (1 μM), unlike the residual responses to substance P after the same phenoxybenzamine pretreatment which are sensitive to blockade by NK_1 receptor antagonists (Hall *et al*., 1994). However, interpretation of these data is difficult given that phenoxybenzamine could alkylate 'atypical' and 'typical' NK_1 receptors, and 'atypical' and 'typical' NK_3 receptors, all to varying degrees.

The present data could alternatively be explained by preferential interaction with different binding domains on a common NK_3 receptor protein. Thus, [MePhe^7]-NKB may bind to additional domains not accessible to other NK_3 receptor agonists, as well as the antagonists SR 142801 and SR 48968. This may induce conformational or allosteric changes which could prevent antagonist binding or the ability of the antagonists to interact with agonist binding. Our data could theoretically be explained by metabolic instability of peptides, but this is unlikely since it has been shown previously that in rabbit iris sphincter muscle, inhibitors of several peptidases, even in combination, have little effect on activities of a number of tachykinin agonists (Hall *et al*., 1991).

In conclusion, we have confirmed, by use of autoradiography and functional analysis, the presence of NK_3 receptors in rabbit iris sphincter muscle which mediate contractile responses to NK_3 receptor agonists, but not to sensory trigeminal nerve stimulation. Our results with selective NK_3 receptor antagonists suggest that functional rabbit NK_3 receptors more closely resemble human and guinea-pig NK_3 receptors rather than rat NK_3 receptors. The possible existence of intraspecies NK_3 receptor heterogeneity is suggested by the pharmacological profile of [MePhe^7]-NKB, SR 142801 and SR 48968 in the rabbit iris sphincter muscle, but this requires further investigation with subtype-selective antagonists, autoradiography, and/or molecular biology techniques.

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