British Journal of Pharmacology (1997) 122, 469–476

In vitro and *in vivo* characterization of NK_3 receptors in the rabbit eye by use of selective non-peptide NK_3 receptor antagonists

[1]Andrew D. Medhurst, *Douglas W.P. Hay, Andrew A. Parsons, †Lenox D. Martin & †Don E. Griswold

Department of Neurosciences Research, SmithKline Beecham Pharmaceuticals, Third Avenue, Harlow, Essex CM19 5AW, U.K. and Departments of *Pulmonary Pharmacology and †Immunopharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, U.S.A.

1 Inhibition of NK_3 receptor agonist-induced contraction in the rabbit isolated iris sphincter muscle was used to assess the *in vitro* functional activity of three 2-phenyl-4-quinolinecarboxamides, members of a novel class of potent and selective non-peptide NK_3 receptor antagonists. In addition, an *in vivo* correlate of this *in vitro* response, namely NK_3 receptor agonist-induced miosis in conscious rabbits, was characterized with some of these antagonists.

2 *In vitro* senktide (succinyl-[Asp^9,$MePhe^8$]-substance P (6-11) and [$MePhe^7$]-neurokinin B ([$MePhe^7$]-NKB) were potent contractile agents in the rabbit iris sphincter muscle but exhibited quite different profiles. Senktide produced monophasic log concentration-effect curves with a mean $pD_2 = 9.03 \pm 0.06$ and mean $n_H = 1.2 \pm 0.02$ ($n = 14$). In contrast, [$MePhe^7$]-NKB produced shallow log concentration-effect curves which often appeared biphasic ($n_H = 0.54 \pm 0.04$, $n = 8$), preventing the accurate determination of pD_2 values.

3 The contractile responses to the NK_3 receptor agonist senktide were antagonized in a surmountable and concentration-dependent manner by SB 223412 ((−)-(**S**)-N-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide; 3–30 nM, $pA_2 = 8.4$, slope $= 1.8 \pm 0.3$, $n = 4$), SB 222200 ((−)-(**S**)-N-(α-ethylbenzyl)-3-methyl-2-phenylquinoline-4-carboxamide; 30–300 nM, $pA_2 = 7.9$, slope $= 1.4 \pm 0.06$, $n = 4$) and SB 218795 ((−)-(**R**)-N-(α-methoxycarbonylbenzyl)-2-phenylquinoline-4-carboxamide; 0.3 and 3 μM apparent $pK_B = 7.4 \pm 0.06$, $n = 6$).

4 Contractile responses to the NK_3 receptor agonist [$MePhe^7$]-NKB in the rabbit iris sphincter muscle were unaffected by SB 218795 (0.3 and 3 μM, $n = 8$). In contrast, SB 223412 (30 and 300 μM, $n = 4$) and SB 222200 (0.3 and 3 μM, $n = 4$) inhibited responses to low concentrations ($\leqslant 1$ nM), to a greater extent than higher concentrations (> 1 nM) of [$MePhe^7$]-NKB. Furthermore, log concentration-effect curves to [$MePhe^7$]-NKB became steeper and monophasic in the presence of each antagonist.

5 SB 218795 (3 μM, $n = 4$) had no effect on contractions induced by transmural nerve stimulation (2 Hz) or substance P, exemplifying the selectivity of this class of antagonist for functional NK_3 receptors over NK_1 receptors in the rabbit.

6 *In vivo*, senktide (1, 10 and 25 μg i.v., i.e. 1.2, 11.9 and 29.7 nmol, respectively) induced concentration-dependent bilateral miosis in conscious rabbits (maximum pupillary constriction $= 4.25 \pm 0.25$ mm; basal pupillary diameter 7.75 ± 0.48 mm; $n = 4$). The onset of miosis was within 2–5 min of application of senktide and responses lasted up to 30 min. Responses to two i.v. administrations of 25 μg senktide given 30 min apart revealed no evidence of tachyphylaxis. Topical administration of atropine (1%) to the eye enhanced pupillary responses to 25 μg senktide. This was probably due to the mydriatic effect of atropine since it significantly increased baseline pupillary diameter from 7.0 ± 0.4 mm to 9.0 ± 0.7 mm ($n = 4$), thereby increasing the maximum capacity for miosis. Senktide-induced miosis was inhibited by SB 222200 (1 and 2 mg kg^{-1}, i.v., i.e. 2.63 and 5.26 μmol kg^{-1}; maximum inhibition 100%; $n = 3–4$), SB 223412 (0.5 and 1 mg kg^{-1}, i.v., i.e. 1.31 and 2.61 μmol kg^{-1}; maximum inhibition 100%; $n = 3$), SB 218795 (0.5 and 1 mg kg^{-1}, i.v., i.e. 1.26 and 2.52 μmol kg^{-1}; maximum inhibition 78%; $n = 3$), and the structurally distinct NK_3 receptor antagonist SR 142801 ((**S**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylepipiperidin-4-yl)-N-methylacetamide; 1.5 mg kg^{-1}, i.v., i.e. 2.47 μmol kg^{-1}, maximum inhibition 92%; $n = 3$).

7 Topical administration of senktide (25 μg; 29.7 nmol) to the eye induced unilateral miosis in the treated eye only. At this dose there was no significant difference ($P < 0.05$) between pupillary constriction obtained by topical or i.v. senktide, and topically administered atropine had no significant effect on responses to topical senktide ($n = 4$).

8 [$MePhe^7$]-NKB (125, 250 and 500 μg, i.v., i.e. 98.31, 196.62 and 393.24 nmol, respectively) also induced bilateral miosis in conscious rabbits (maximum pupillary constriction $= 4.13 \pm 0.30$ mm; $n = 4$), but in contrast to *in vitro* studies this agonist was approximately 100 fold less potent than senktide. [$MePhe^7$]-NKB-induced miosis was inhibited by SB 222200 (5 mg kg^{-1}, i.v., i.e. 13.14 μmol kg^{-1}; maximum inhibition 69%; $n = 3$).

9 In summary, SB 223412, SB 222200 and SB 218795 are potent and selective antagonists of NK_3 receptor-mediated contraction in the rabbit isolated iris sphincter muscle. In addition, NK_3 receptor agonist-induced miosis in conscious rabbits is a good *in vivo* correlate of the *in vitro* rabbit iris sphincter muscle preparation and appears to be a useful model for characterizing the pharmacodynamic profile and efficacy of structurally distinct NK_3 receptor antagonists, such as SB 222200, SB 223412, SB 218795 and SR 142801.

Keywords: NK_3 receptors; rabbit iris sphincter muscle; pupillary constriction; senktide; SR 142801; SB 223412; SB 222200; SB 218795; NK_3 receptor antagonists

[1] Author for correspondence.

Introduction

Substance P, neurokinin A (NKA) and neurokinin B (NKB), the preferred endogenous ligands for tachykinin NK$_1$, NK$_2$ and NK$_3$ receptors, respectively, have previously been implicated in the ocular response to injury in the rabbit, including the mediation of miosis (Beding-Barnekow *et al*., 1988). Further evidence for the involvement of tachykinins in ocular pharmacology is suggested by the presence of NK$_1$ and NK$_3$ receptors in the rabbit isolated iris sphincter muscle (Hall *et al*., 1991; 1993; Wang & Håkanson, 1993; Medhurst *et al*., 1997) and the presence of substance P, NKA and NKB in various ocular tissues (Taniguchu *et al*., 1986; Beding-Barnekow *et al*., 1988). NK$_1$ receptors mediate contractile responses resulting from chemical (eg capsaicin) or electrical stimulation of the trigeminal nerve innervating the iris sphincter, as well as contractile responses induced by selective NK$_1$ receptor agonists (Ueda *et al*., 1982; 1984; Hall *et al*., 1991; Wang & Håkanson, 1992).

Further characterization of the effects of NK$_3$ receptor activation in the iris has been hampered by the lack of selective NK$_3$ receptor antagonists. However, we recently showed that the first potent and selective non-peptide NK$_3$ receptor antagonist described in the literature, SR 142801 (Emonds-Alt *et al*., 1995), blocked responses to the NK$_3$ receptor agonist senktide with similar affinity to the binding affinity seen for the human cloned NK$_3$ receptor expressed in Chinese hamster ovary (CHO) cells, but had no effect on contractile responses to electrical nerve stimulation (Medhurst *et al*., 1997). Stimulated by these findings, we used the rabbit isolated iris sphincter muscle to assess the functional activity of three members of a novel class of potent and selective non-peptide NK$_3$ receptor antagonists (the 2-phenyl-4-quinolinecarboxamides; Giardina *et al*., 1996), namely SB 223412 ((−)-(**S**)-N-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide), SB 222200 ((−)-(**S**)-N-(α-ethylbenzyl)-3-methyl-2-phenylquinoline-4-carboxamide) and SB 218795 ((−)-(**R**)-N-(α-methoxycarbonylbenzyl)-2-phenylquinoline-4-carboxamide). In addition, we investigated the possibility of using NK$_3$ receptor agonist-induced miosis in the conscious rabbit as an *in vivo* correlate of NK$_3$ receptor-mediated contraction in the rabbit isolated iris sphincter muscle, to characterize novel NK$_3$ receptor antagonists.

Methods

In vitro*: rabbit isolated iris sphincter muscle preparation*

Methods for *in vitro* studies in the rabbit iris sphincter muscle were as previously described (Medhurst *et al*., 1997). Eyes were removed from male New Zealand White rabbits (2–3 kg, Charles River, Margate, U.K.) killed by i.v. pentobarbitone (Euthatal), and were placed immediately 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). Each strip was then prepared for the isometric measurement of tension in 50 ml organ baths containing Krebs-Henseleit solution maintained at 37°C and gassed with 95% O_2 and 5% CO_2. The tissues were attached to a Swema SG4-45 strain gauge transducer by means of a stainless steel wire and force was recorded on a Watanabe polygraph (Graphtech, Japan).

In vitro*: effects of selective NK$_3$ receptor agonists and antagonists*

Following two washouts five minutes apart, tissues were stretched three times to a tension equivalent to 400 mg over a period of 30 min and left to equilibrate for 45 min. At the beginning of each experiment, a reference contractile response to carbachol (10 μM) was obtained in each tissue. After plateau of the carbachol-induced response, tissues were washed three times over 15 min. Tissues were then exposed to atropine (1 μM; to block indirect cholinergic influences; Ueda *et al*., 1982) and the NK$_1$ receptor antagonist CP 99994 ((+)-(2**S**,3**S**)-3-(2-methoxybenzylamino)-2-phenylpiperidine; 1 μM, McLean *et al*., 1993), for the remainder of the experiments. To investigate the effects of NK$_3$ receptor antagonists, tissues were incubated for 120 min with either SB 223412, SB 222200 or SB 218795, before cumulative concentration-effect curves to senktide or [MePhe^7]-NKB were determined, by sequentially increasing the total concentration by half log increments. The incubation time of 120 min was used to enable comparisons with previous data generated with another NK$_3$ receptor antagonist, SR 142801 (see Medhurst *et al*., 1997). Concentration-effect curves to NK$_3$ receptor agonists in time-matched control tissues incubated with vehicle (DMSO) were determined in parallel.

In a separate series of experiments in the absence of CP 99994, the effects of SB 218795 on electrically-induced and substance P-induced contractions were investigated. We chose to investigate only one antagonist from the 2-phenyl-4-quinolinecarboxamide series as an example, since previous results with SR 142801 (Emonds-Alt *et al*., 1995), an NK$_3$ receptor antagonist of a different structural class, had no effect on neurogenic contraction (Medhurst *et al*., 1997). The effect of the NK$_1$ receptor antagonist CP 99994 on neurogenic contraction was determined as a comparison. 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 SB 218795 (3 μM) for 120 min. After a further 30 min, concentration-effect curves to substance P were determined. Control tissues were again run in parallel.

In vitro*: analysis of data*

Contractile responses to NK$_3$ receptor agonists were calculated as a percentage of the carbachol-induced contraction. Concentration-effect curves were fitted by use of Microsoft Excel to the logistic function:

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

where E_{max} is the maximal action of A, n_H is the Hill coefficient (slope factor) and $[A]_{50}$ is the concentration that produces an effect that is 50% of E_{max}. 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 ± s.e.mean.

Affinity estimates for antagonists were expressed as pA_2 values calculated according to the method of Arunlakshana & Schild (1959). When a limited antagonist concentration range was used, apparent pK_B estimates were calculated by single concentration analysis from the following Gaddum-Schild equation:

$$-\log K_B = pK_B = -\log[B] + \log[CR - 1]$$

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.

In vivo*: induction and measurement of miosis in conscious rabbits*

Male New Zealand White rabbits (2–4 kg, H.A.R.E. Rabbitry, Hewitt, NJ 07421, U.S.A.) were utilized for *in vivo* studies and all protocols were approved by the SmithKline Beecham Pharmaceuticals Animal Care and Use Committee. The left pupil of each rabbit was measured under normal ambient fluorescent lighting with a comparator reticle (Finescale Comparator Reticle scaled to 0.1 mm; Orange, CA, U.S.A.). This value was recorded as the baseline in mm. The rabbit was then given an i.v. bolus of different doses of senktide or [MePhe[7]]-NKB in a volume of 0.2 ml in 50 μl DMSO/950 μl sal-

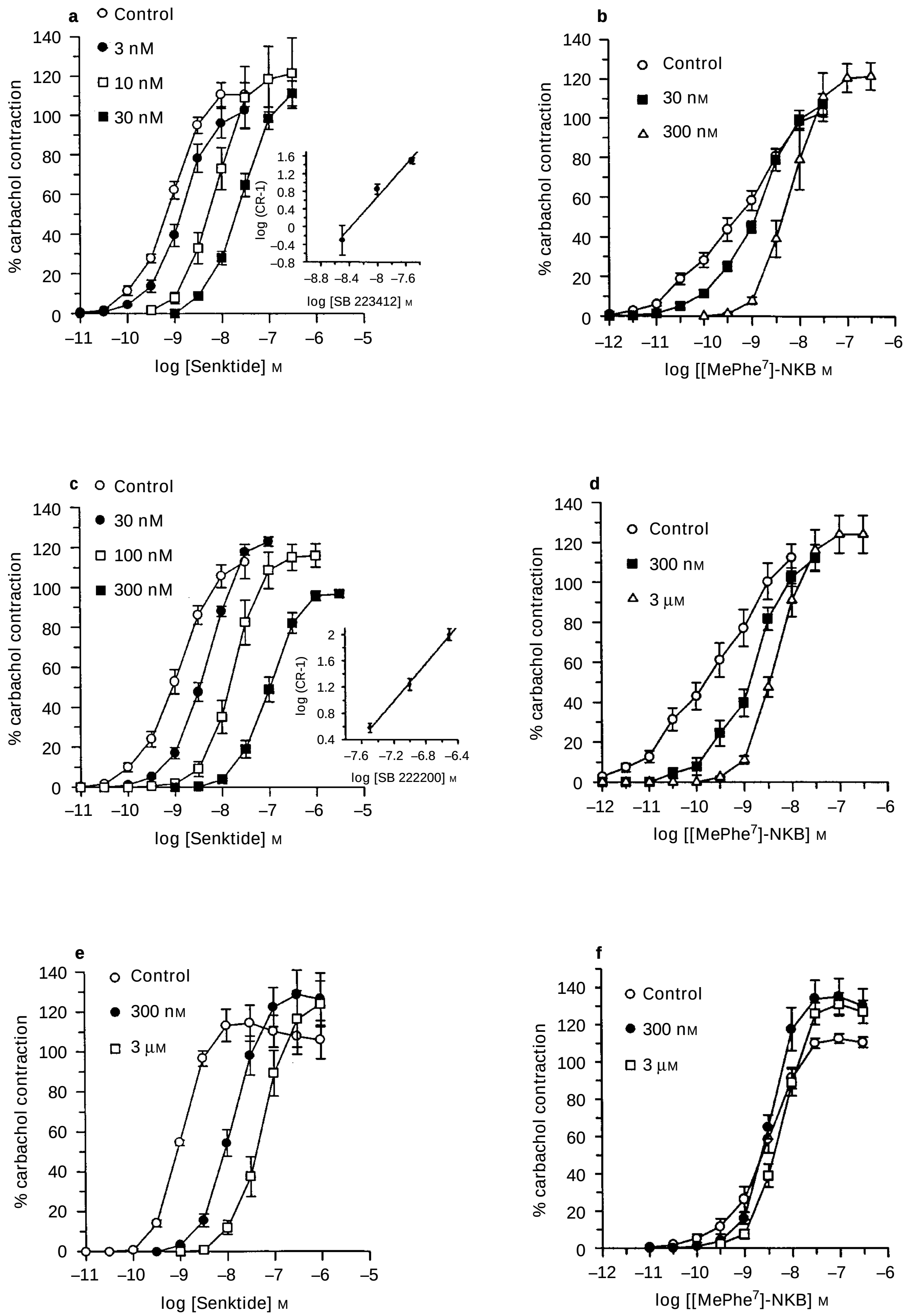

Figure 1 Log concentration-effect curves to senktide (a, c and e) in the absence and presence of (a) SB 223412 ($n=4$), (c) SB 222200 ($n=4$) and (e) SB 218795 ($n=6$), and concentration-effect curves to [MePhe[7]]-NKB (b, d and f) in the absence and presence of (b) SB 223412 ($n=4$), (d) SB 222200 ($n=4$) and (f) SB 218795 ($n=8$), in the rabbit isolated iris sphincter muscle. Results are expressed as a percentage of the response to 10 μM carbachol and data are mean with vertical lines showing s.e.mean. Schild plots for antagonism of senktide-induced responses by SB 223412 (a) and SB 222200 (c) are shown in the insets.

ine. The pupil measurements were then taken at times between 2.5 and 30 min after agonist administration. In the antagonist studies, SR 142801, SR 223412, SB 218795 or SB 222200 were administered as 0.2 ml bolus injection (i.v.) 2.5 min before the addition of senktide or [MePhe[7]]-NKB. For topical application, a volume of 10 μl of test compound was carefully placed on the surface of the eye by a micropipette.

In vivo: *data analysis*

Determination of the extent of miosis was expressed as absolute pupillary constriction (mm), i.e., initial pupil diameter minus smallest pupil diameter recorded. All data are mean ($\pm$s.e.mean) and Student's unpaired *t* test was used to determine statistical significance with $P<0.05$ considered significant.

Materials

Carbachol was obtained from Sigma (U.K.), atropine from BDH Chemicals Limited (Poole, U.K.) and from Sigma Chemical Co. (St. Louis, MO 63178). Senktide and [MePhe[7]]-NKB were purchased from California Peptide Research Inc. (Napa, CA, U.S.A.) and Peninsula Laboratories, Inc. (Europe). SB 218795 ((−)-(**R**)-N-(α-methoxycarbonylbenzyl)-2-phenylquinoline-4-carboxamide), SB 222200 ((−)-(**S**)-N-(α-ethylbenzyl)-3-methyl-2-phenylquinoline-4-carboxamide), SB 223412 ((−)-(**S**)-N-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide), SR 142801 ((**S**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylepipiperidin-4-yl)-N-methylacetamide) and CP 99994 ((+)-(2**S**,3**S**)-3-(2-methoxybenzylamino)-2-phenylpiperidine) were synthesized by colleagues in the Department of Chemistry at SmithKline Beecham S.p.A. (Milan, Italy). All stock solutions were prepared in distilled water, except SB 218795, SB 222200 and SB 223412 which were dissolved in dimethylsulphoxide (DMSO). All neuropeptide stock solutions were stored as 50–200 μl aliquots at -20°C.

Results

In vitro: *rabbit isolated iris sphincter muscle*

As demonstrated previously (Medhurst *et al.*, 1997), senktide and [MePhe[7]]-NKB were potent contractile agents in the rabbit iris sphincter muscle and exhibited quite different profiles. Thus, in the present study, senktide produced monophasic concentration-effect curves with a mean $pD_2=9.03\pm0.06$ and mean $n_H=1.2\pm0.02$ ($n=14$). In contrast, [MePhe[7]]-NKB produced shallow concentration-effect curves which often appeared biphasic ($n_H=0.54+0.04$, $n=8$), preventing the accurate determination of pD_2 values.

SB 223412 (3–30 nM, $n=4$) antagonized contractile responses to senktide in a surmountable manner, resulting in concentration-dependent rightward shifts in the log concentration-effect curves (Figure 1a). Log concentration-ratios were 0.25 ± 0.10, 0.91 ± 0.09 and 1.51 ± 0.06 for 3, 10 and 30 nM SB 223412, respectively, and the Schild slope was significantly ($P<0.05$) greater than unity (1.8 ± 0.3). A pA_2 of 8.4 was calculated for antagonism of senktide-induced contraction. In the presence of SB 223412 (30 and 300 nM), log concentration-effect curves to [MePhe[7]]-NKB became steeper and monophasic ($n_H=0.93\pm0.08$ and 1.38 ± 0.10, respectively, $n=4$), but the maximum responses to [MePhe[7]]-NKB were unchanged (Figure 1b). Thus, responses to lower concentrations ($\leqslant1$ nM) of [MePhe[7]]-NKB appeared to be inhibited to a greater extent than responses to higher concentrations (>1 nM) of [MePhe[7]]-NKB.

SB 222200 had a similar antagonist profile to SB 223412 but was approximately 2.5 fold less potent. Concentration-effect curves to senktide were antagonized in a surmountable manner by SB 222200 (30, 100 and 300 nM, $n=4$) with log concentration-ratios of 0.69 ± 0.05, 1.26 ± 0.08 and 1.99 ± 0.07, respectively (Figure 1c). The pA_2 value was calculated to be 7.89 with a slope of 1.4 ± 0.06 ($P<0.05$). In the presence of SB 222200 (0.3 and 3 μM), concentration-effect curves to [MePhe[7]]-NKB became steeper and monophasic ($n_H=1.06\pm0.1$ and 1.34 ± 0.07, $n=4$), implying that SB 222200 inhibits responses to lower ($\leqslant1$ nM) concentrations of [MePhe[7]]-NKB to a greater extent than responses to higher concentrations (>1 nM) of [MePhe[7]]-NKB (Figure 1d).

SB 218795 (0.3 and 3 μM, $n=6$) was the least potent of the three antagonists tested *in vitro* (Figure 1e), and induced concentration-dependent rightward shifts in the concentration-effect curves to senktide with no decrease in maximum responses. A mean apparent pK_B estimate of 7.4 ± 0.06 was calculated for antagonism of senktide-induced contraction. In contrast, concentration-effect curves to [MePhe[7]]-NKB (Figure

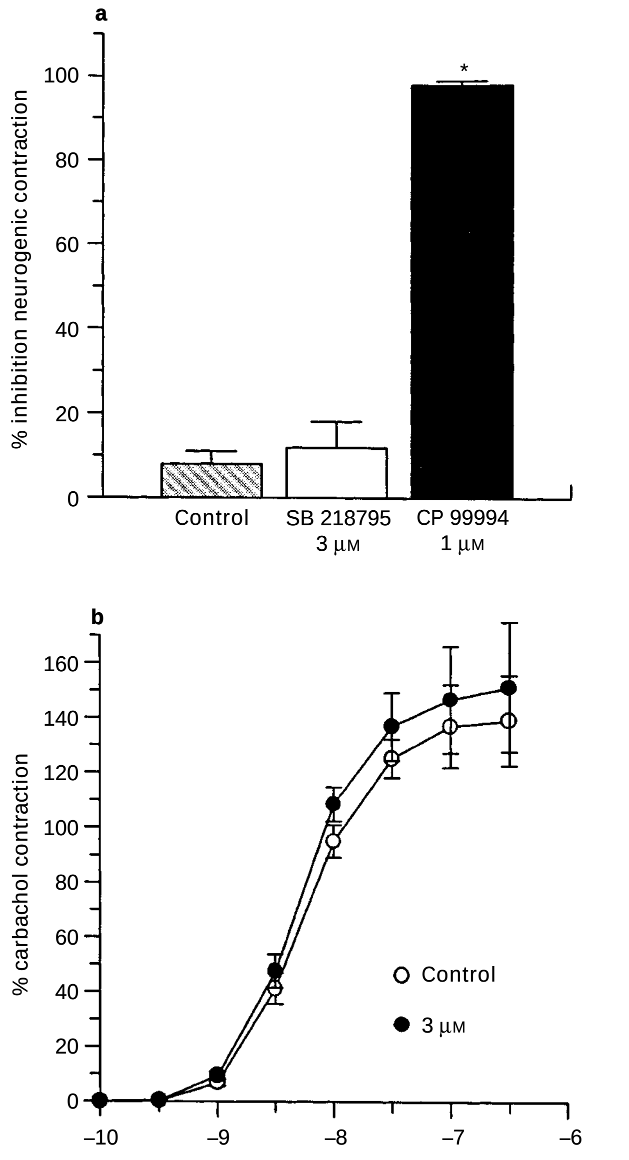

Figure 2 (a) Effects of SB 218795 (3 μM) and CP 99994 (1 μM) on neurogenic contraction ($n=4$), and (b) effects of SB 218795 (3 μM) on substance P-induced contraction ($n=4$) in the rabbit isolated iris sphincter muscle. (a) Tissues were electrically stimulated in the presence of atropine (1 μM) before (S1) and after (S2) the addition of SB 218795 or CP 99994 for 120 min. Mean data, expressed as % inhibition calculated from contraction elicited by (S1−S2)/S1, are shown with vertical lines representing s.e.mean. (b) Concentration-effect curves to substance P were generated in the absence and presence of 3 μM SB 218795. *Significant inhibition ($P<0.05$, Student's unpaired *t* test).

1f) were not inhibited by SB 218795 (0.3 and 3 μM, $n=8$), although the slope of the concentration-effect curve to [MePhe[7]]-NKB became steeper ($n_H = 1.56 \pm 0.16$ and 1.60 ± 0.08).

SB 223412, SB 222200 and SB 218795 had no effect on baseline tension. Electrical stimulation (2 Hz) of the rabbit iris sphincter muscle induced reproducible contractile responses which were unaffected by 3 μM SB 218795 but were significantly ($P<0.05$, Student's unpaired t test) inhibited by 1 μM CP 99994 ($n=4$, Figure 2a). Contractile responses to substance P were also unaffected by 3 μM SB 218795 ($n=4$, Figure 2b).

In vivo*: miosis in conscious rabbits*

Both senktide and [MePhe[7]]-NKB induced bilateral miosis following i.v. administration in conscious rabbits (Figure 3). Senktide (10 and 25 μg, i.v.) induced a significant ($P<0.05$, Student's unpaired t test) pupillary constriction, whereas 1 μg senktide was without significant effect. The mean maximum pupillary constriction observed with senktide (25 μg, i.v.) was 4.25 ± 0.25 mm. A dose of 100 μg senktide induced shivering and no further pupillary constriction, so was not studied further. At least 100 fold higher concentrations of [MePhe[7]]-NKB (i.v.) were required to induce the same degree of pupillary constriction as senktide (i.v.). [MePhe[7]]-NKB (125, 250 and 500 μg, i.v.) induced significant ($P<0.05$, Student's t test) pupillary constriction. The maximum pupillary constriction observed with [MePhe[7]]-NKB (4.13 ± 0.30 mm; 500 μg, i.v., $n=4$) was not significantly different (Student's unpaired t test) to that observed with senktide (25 μg, i.v.).

The miosis induced by senktide was characterized further. The mean timecourse of response to 25 μg senktide is illustrated in Figure 4. Pupillary constriction generally occurred within 2–5 min post-injection and was partially reversed within 20 min. Two i.v. injections of 10 μg senktide 30 min apart showed no evidence of tachyphylaxis, since there was no significant difference ($P<0.05$, Student's unpaired t test) between the first (2.67 ± 0.2 mm) and second (2.33 ± 0.3 mm) pupillary responses.

Topical administration of senktide to the eye also produced miosis, but the pupillary constriction was unilateral, occurring only in the treated eye ($n=4$). Interestingly, there was no significant difference between the mean pupillary constriction induced by 25 μg senktide when administered topically (3.6 ± 0.4 mm) or i.v. (3.7 ± 0.2 mm). Topical administration of atropine (1%) failed to inhibit responses to i.v. or topically administered senktide (25 μg) and actually, significantly ($P<0.05$, Student's unpaired t test) increased pupillary constriction induced by i.v. senktide. This could be due to the mydriatic effect of atropine, which increased the baseline pupillary diameter from 7.0 ± 0.4 mm to 9.0 ± 0.7 mm ($n=4$).

A summary of the effects of SR 142801, SB 222200, SB 223412 and SB 218795 on senktide-induced miosis is shown in Figure 5. SR 142801 (Figure 5a, $n=3$), at a dose of 1.5 mg kg^{-1} (i.v.) but not 0.75 and 0.375 mg kg^{-1} (i.v.), significantly ($P<0.05$, Student's unpaired t test) blocked pupillary constriction (maximum inhibition 92%) induced by 25 μg senktide (i.v.). SB 222200 (1 and 2 mg kg^{-1}, i.v., $n=3-4$) significantly ($P<0.05$, Student's unpaired t test) inhibited senktide-induced miosis (maximum inhibition 100%), whilst a lower dose (0.5 mg kg^{-1}, i.v., $n=3$) was inactive (Figure 5b). SB 223412 (0.5 and 1 mg kg^{-1}, i.v., $n=3$) also induced significant ($P<0.05$, Student's unpaired t test) inhibition of senktide-induced pupillary constriction (maximum inhibition 100%), whilst the lower dose of 0.25 mg kg^{-1}, i.v. ($n=3$) was

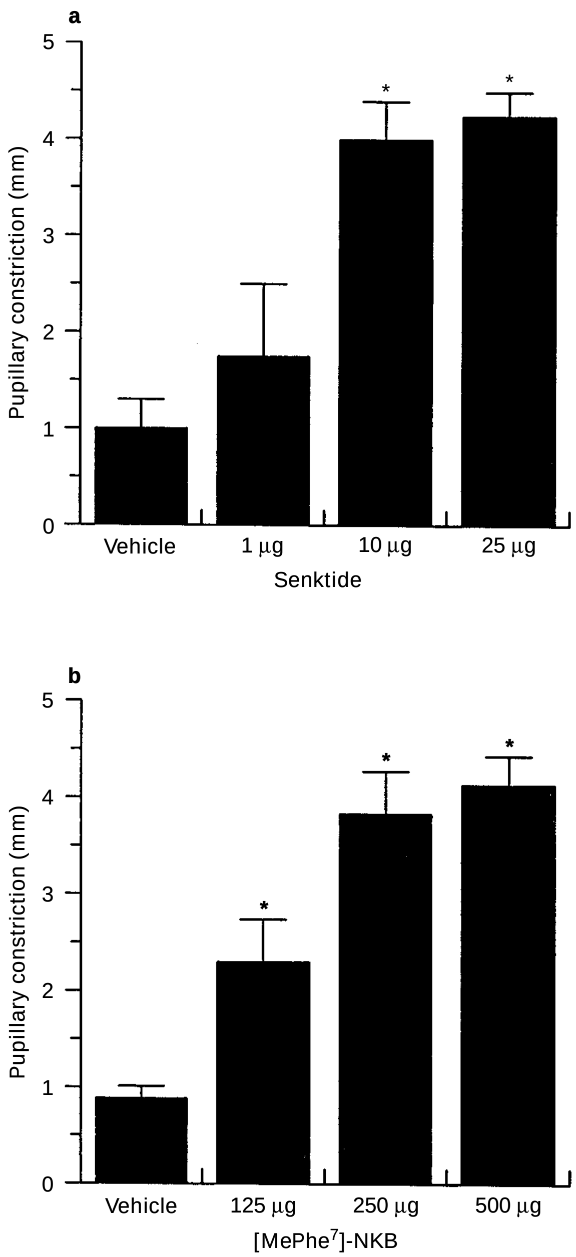

Figure 3 The effects of (a) i.v. senktide and (b) i.v. [MePhe[7]]-NKB on pupillary diameter in the conscious rabbit ($n=3-4$). Senktide 1, 10 or 25 μg or [MePhe[7]]-NKB 125, 250 and 500 μg, was administered in DMSO/saline as a 0.2 ml bolus injection. Responses are expressed as pupillary constriction (mm) and data are mean with vertical lines showing s.e.mean. *Significant pupillary constriction ($P<0.05$, Student's unpaired t test).

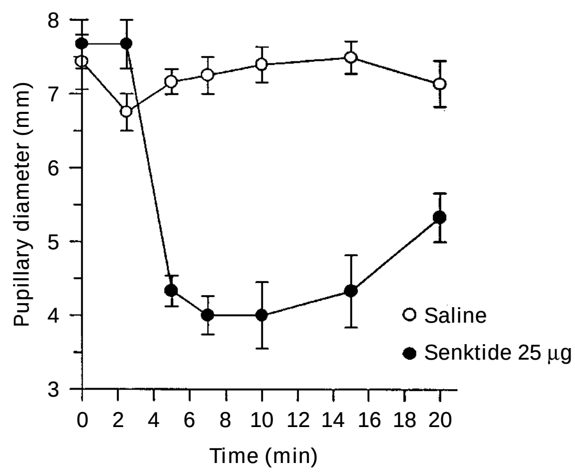

Figure 4 Time course of pupillary constriction in the conscious rabbit following i.v. senktide ($n=3-6$) or vehicle ($n=4-7$). Senktide 25 μg was administered in DMSO/saline as a 0.2 ml bolus injection. Responses are expressed as pupillary diameter (mm) and data are mean with vertical lines showing s.e.mean.

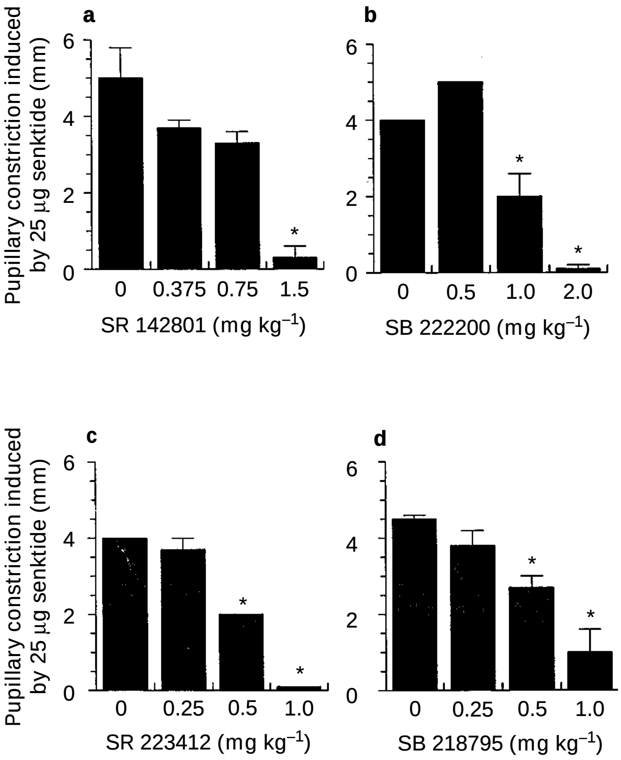

Figure 5 The effects of (a) SR 142801 ($n=3$), (b) SB 222200 ($n=3$–4), (c) SB 223412 ($n=3$), and (d) SB 218795 ($n=3$) on pupillary constriction induced by senktide (25 μg, i.v.) in the conscious rabbit. SR 142801 (0.375, 0.75 and 1.5 mg kg^{-1}), SB 222200 (0.5, 1 and 2 mg kg^{-1}), SB 223412 (0.25, 0.5 and 1.0 mg kg^{-1}) and SB 218795 (0.25, 0.5 and 1.0 mg kg^{-1}) were administered as a 0.2 ml i.v. bolus injection 2.5 min before senktide was injected. Responses are expressed as pupillary constriction induced by senktide (mm) and data are mean with vertical lines showing s.e.mean. *Significant inhibition ($P<0.05$, Student's unpaired t test).

inactive (Figure 5c). Similarly, SB 218795 (0.5 and 1.0 mg kg^{-1}, i.v., $n=3$) induced significant ($P<0.05$, Student's unpaired t test) inhibition of pupillary constriction (maximum inhibition 78%) induced by senktide, whilst 0.25 mg kg^{-1} (i.v., $n=3$) had no effect (Figure 5d). Pupillary constriction (4.13 ± 0.33 mm) induced by [MePhe^7]-NKB (500 μg, i.v.) was also inhibited to 1.33 ± 0.33 mm by SB 222200 (5 mg kg^{-1}, i.v., maximum inhibition 69%; $n=3$), but higher concentrations of antagonist were needed for significant blockade of [MePhe^7]-NKB-induced responses than for senktide-induced responses.

Discussion

The results of the present study show that the 2-phenyl-4-quinolinecarboxamides, SB 223412, SB 222200 and SB 218795 (Giardina *et al.*, 1996), are potent antagonists of NK$_3$ receptor-mediated contractions in the rabbit isolated iris sphincter muscle and have a similar pharmacological profile to the structurally distinct NK$_3$ receptor antagonist SR 142801 (Medhurst *et al.*, 1997). In addition, we have demonstrated, for the first time, that NK$_3$ receptor activation induces miosis in the rabbit *in vivo* and that this pupillary constriction is sensitive to blockade by the NK$_3$ receptor antagonists SB 222200, SB 223412, SB 218795 and SR 142801. Furthermore, differences were apparent in the sensitivity of the selective NK$_3$ receptor agonists senktide and [MePhe^7]-NKB to inhibition by the receptor antagonists.

It has been previously demonstrated *in vitro* that NK$_3$ receptor activation results in direct contraction of the rabbit iris sphincter muscle (Hall *et al.*, 1991; 1993; Wang & Håkanson, 1993; Medhurst *et al.*, 1997). The affinity of the 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) for blocking senktide-induced contraction in the rabbit iris sphincter muscle was very similar to their affinities for human and guinea-pig, but not rat NK$_3$ receptors (Medhurst *et al.*, 1997). We therefore used this system for the secondary functional screening of novel NK$_3$ receptor antagonists, primarily characterized in CHO cells expressing human cloned NK$_3$ receptors (Giardina *et al.*, 1996). In the present study, the affinity values obtained for SB 223412, SB 222200 and SB 218795, as well as the rank order of potency of these antagonists for the NK$_3$ receptor in the rabbit iris sphincter muscle (i.e. SB 223412 > SB 222200 > SB 218795), were comparable to results seen in CHO cells expressing the human cloned NK$_3$ receptor (Giardina *et al.*, 1996). The steep Schild slope obtained with SB 223412 against senktide-induced contraction may be a result of the complex pharmacology of tachykinin receptors in the rabbit iris, complicated by the existence of putative 'atypical' and 'typical' NK$_3$ receptor subtypes (Medhurst *et al.*, 1997). Another possible explanation for the steep Schild slopes could be that the receptor-antagonist complex was not at equilibrium, even after 120 min. This seems unlikely for these antagonists since, in contrast to SR 142801, binding and calcium mobilization studies indicate that the inhibitory effects are reversible by washout and are not time-dependent (Sarau *et al.*, 1997).

The results with [MePhe^7]-NKB are more difficult to interpret, but not unexpected, based on our previous results with SR 142801 which blocked responses to low concentrations but not to higher concentrations of [MePhe^7]-NKB (Medhurst *et al.*, 1997). In a similar way to SR 142801 shown previously (Medhurst *et al.*, 1997), SB 223412 and SB 222200 blocked responses to low concentrations ($\leqslant 1$ nM) of [MePhe^7]-NKB to a greater extent than responses to higher concentrations (>1 nM) and, also, increased the slope of the concentration-effect curve to [MePhe^7]-NKB. In addition, higher concentrations of these antagonists were required to block [MePhe^7]-NKB-induced contractions than contractions elicited by senktide, providing further evidence for putative NK$_3$ receptor subtypes (see Medhurst *et al.*, 1997). The results with SB 218795 were more dramatic, since concentrations that induced large blockade of senktide-induced contractions had no effect on [MePhe^7]-NKB-induced responses. Selectivity of these compounds for NK$_3$ receptors over other tachykinin receptors has not been previously investigated in the rabbit. The fact that SB 218795 had no effect on contraction induced by electrical stimulation or substance P makes it unlikely that SB 218795 acts on 'typical' or 'atypical' NK$_1$ receptors, respectively (Hall *et al.*, 1993; 1994). These results also support our previous observations with selective NK$_1$ and NK$_3$ receptor antagonists, showing that neurogenic contraction is mediated by NK$_1$ and not NK$_3$ receptors in the rabbit iris sphincter muscle (Medhurst *et al.*, 1997). Taken together, our *in vitro* results in the present study suggest that SB 223412, SB 222200 and SB 218795 have a similar pharmacological profile to that of the structurally distinct NK$_3$ receptor antagonist SR 142801 described previously, as well as supporting the possible existence of putative NK$_3$ receptor subtypes (Medhurst *et al.*, 1997).

In contrast to NK$_1$ and NK$_2$ receptors, minimal information on NK$_3$ receptor activation *in vivo* is available and most studies have involved the measurement of behavioural responses induced by administration of selective NK$_3$ receptor agonists. For example, senktide (i.c.v.) induces wet dog shakes in both guinea-pigs (Piot *et al.*, 1995) and rats (Stoessl *et al.*, 1990; Picard *et al.*, 1994) and has also been shown to produce a syndrome mediated by endogenous 5-hydroxytryptamine (5-HT), including forepaw treading and hindlimb splaying (Wormser *et al.*, 1986; Stoessl *et al.*, 1987). Based on our *in vitro* observations, work was therefore initiated to develop an *in vivo* model to mimic the NK$_3$ receptor-mediated contraction seen in the rabbit isolated iris sphincter muscle, which is a direct response involving stimulation of NK_3 receptors on smooth muscle rather than an indirect response through the release of other neurotransmitters.

Senktide and [MePhe^7]-NKB administered i.v. induced bilateral miosis in the conscious rabbit. Behavioural responses induced by both agonists were negligible, except that a high dose of senktide (100 μg) induced shivering. In contrast to *in vitro* studies in the rabbit iris sphincter muscle, where there was little difference in the potency between senktide and [MePhe^7]-NKB, in the conscious rabbit senktide was much more potent than [MePhe^7]-NKB at inducing pupillary constriction, suggesting an active metabolism of this peptide or decreased bioavailability *in vivo*. As predicted from our *in vitro* studies, all the NK_3 receptor antagonists tested inhibited NK_3 receptor agonist-induced miosis in the conscious rabbit, suggesting that pupillary constriction *in vivo* is a useful measure of the efficacy and pharmacodynamic profile of NK_3 receptor antagonists. Interestingly, SB 218795 appeared relatively more potent than predicted from the *in vitro* data, suggesting either a metabolism of this antagonist *in vivo* to a more active derivative, or a relative decrease in the bioavailability of the other antagonists tested.

Since all *in vitro* studies were conducted in the presence of atropine to block any effects of released acetylcholine (Ueda *et al*., 1982; Medhurst *et al*., 1997), we investigated the effects of topically administered atropine on pupillary constriction in the conscious rabbit. Atropine failed to inhibit miosis induced by i.v. senktide, suggesting a direct activation of NK_3 receptors on the iris sphincter muscle *in vivo*. Pupillary constriction induced by i.v. senktide was actually increased by atropine, probably due to the blockade of basal acetylcholine release by atropine (manifested as mydriasis), which in turn increases the capacity for pupillary constriction. Interestingly, topical administration of senktide induced unilateral miosis only in the treated eye, and the miosis was not blocked by topical atropine, providing further evidence for direct activation of NK_3 receptors in the iris sphincter muscle. This is the first demonstration that topical administration of an NK_3 receptor agonist can achieve enough intraocular penetration to result in pupillary constriction.

The present methods described here for the study of miosis are less invasive than other previous studies investigating the effects of tachykinins in the eye. For example, intracameral injection during anaesthesia (Beding-Barnekow *et al*., 1988) is more likely to result in local inflammation, as well as pupillary constriction, since it is more invasive. NKB, the natural preferred ligand for NK_3 receptors, has been previously shown to induce miosis in the rabbit when injected intracamerally, but this could not be attributed to NK_3 receptor activation due to a lack of selective antagonists at the time (Beding-Barnekow *et al*., 1988).

The role of NK_3 receptors in the rabbit eye is unknown. However, by use of autoradiography studies with [^{125}I]-[MePhe^7]-NKB, as previously described (Medhurst *et al*., 1997), the receptors appear to be specifically localized to the iris and associated ciliary processes (unpublished observations). The ciliary body in the rabbit is poorly developed which accounts for the negligible power of accommodation in this species. To compensate, ciliary processes are well developed and are thought to provide a mechanism for some accomodative power in the rabbit (Peiffer *et al*., 1994). Alternatively, NK_3 receptors on ciliary processes may be involved in secretory function and may play a role in regulation of intraocular pressure. In addition, they may mediate the effects of NKB released in ocular inflammation, since this tachykinin peptide has been detected in the rabbit eye (Taniguchi *et al*., 1986). However, the contribution of NK_3 receptors to the ocular inflammatory responses *in vivo* has yet to be determined.

In conclusion, we have described the pharmacology of the novel NK_3 receptor antagonists SB 223412, SB 222200 and SB 218795, which is similar to that of SR 142801 in the rabbit iris sphincter muscle. In addition, the *in vivo* correlate of this phenomenon has been demonstrated in the conscious rabbit, where selective NK_3 receptor agonists induced pupillary constriction, which was sensitive to blockade by the NK_3 receptor antagonists SB 222200, SB 223412, SB 218795 and SR 142801. NK_3 receptor-induced miosis in the conscious rabbit is a simple non-invasive model for characterizing the efficacy and pharmacodynamic profile of NK_3 receptor antagonists.

Note added in proof

There is no pharmacological evidence to suggest that the 'atypical' NK_3 receptors proposed in the rabbit iris sphincter muscle resemble recently described 'putative NK_4 receptors'. (Donaldson, L.F., Haskell, C.A. & Hanley, M.R. (1996). Functional characterisation by heterologous expression of a novel cloned tachykinin peptide receptor. *Biochem. J*., **320,** 1–5).

The authors would like to thank Dr Giuseppe Giardina and colleagues (Department of Chemistry, SmithKline Beecham S.p.A., Via Zambeletti, 20021 Baranzate, Milan, Italy), for the synthesis of SB 223412, SB 222200, SB 218795, SR 142801 and CP 99994, and Dr Henry Sarau (Department of Pulmonary Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, U.S.A.) for reviewing the manuscript.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonism. *Br. J. Pharmacol. Chemother*., **14,** 48–58.

BEDING-BARNEKOW, B., BRODIN, E. & HÅKANSON, R. (1988). Substance P, neurokinin A and neurokinin B in the ocular response to injury in the rabbit. *Br. J. Pharmacol*., **95,** 259–267.

EMONDS-ALT, X., BICHON, D., DUCOUX, J.P., HEAULME, M., MILOUX, B., PONCELET, M., PROIETTO, V., VAN BROECK, D., VILAIN, P., NELIAT, G., SOUBRIE, P., LE FUR, G. & BRELIERE, J.C. (1995). SR 142801, the first potent non-peptide antagonist of the tachykinin NK_3 receptor. *Life Sci*., **56,** 27–32.

GIARDINA, G.A.M., SARAU, H.M., FARINA, C., MEDHURST, A.D., GRUGNI, M., FOLEY, J.J., RAVEGLIA, L.F., SCHMIDT, D.B., RIGOLIO, R., VASSALLO, M., VECCHIETTI, V. & HAY, D.W.P. (1996). 2-Phenyl-4-quinolinecarboxamides: a novel class of potent and selective non-peptide competitive antagonists for the human neurokinin-3 receptor. *J. Med. Chem*., **39,** 2281–2284.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1991). Neurokinin receptors in the rabbit iris sphincter characterised by novel agonist ligands. *Eur. J. Pharmacol*., **199,** 9–14.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1993). Tachykinin receptors mediating responses to sensory nerve stimulation and exogenous tachykinins and analogues in the rabbit isolated iris sphincter muscle. *Br. J. Pharmacol*., **109,** 1008–1013.

HALL, J.M., MITCHELL, D. & MORTON, I.K.M. (1994). Typical and atypical NK_1 tachykinin receptor characteristics in the rabbit isolated iris sphincter. *Br. J. Pharmacol*., **112,** 985–991.

MCLEAN, S., GANONG, A., SEYMOUR, P.A., SNIDER, R.M., DESAI, M.C., ROSEN, T., BRYCE, D.K., LONGO, K.P., REYNOLDS, L.S., ROBINSON, G., SCHMIDT, A.W., SIOK, C. & HEYM, J. (1993). Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *J. Pharmacol. Exp. Ther*., **267,** 472–479.

MEDHURST, A.D., PARSONS, A.A., ROBERTS, J.C. & HAY, D.W.P. (1997). Characterisation of NK_3 receptors in rabbit isolated iris sphincter muscle. *Br. J. Pharmacol*., **120,** 93–101.

PEIFFER JR, R.L., POHM-THORSEN, L. & CORCORAN, K. (1994). Models in ophthalmology and vision research. In *The Biology of the Laboratory Rabbit*. ed Manning, P.J., Ringler, D.H. & Newcomer, D.E. pp. 409–431. New York: Academic Press.

PETITET, F., BEAUJOUAN, J.-C., SAFFROY, M., TORRENS, Y. & GLOWINSKI, J. (1993). The nonpeptide NK_2 antagonist SR 48968 is also a NK_3 antagonist in the guinea-pig but not in the rat. *Biochem. Biophys. Res. Commun.*, **191,** 180 – 187.

PICARD, P., REGOLI, D. & COUTURE, R. (1994). Cardiovascular and behavioural effects of centrally administered tachykinins in the rat: characterisation of receptors with selective antagonists. *Br. J. Pharmacol.*, **112,** 240 – 249.

PIOT, O., BETSCHART, J., GRALL, I., RAVARD, S., GARRET, C. & BLANCHARD, J.-C. (1995). Comparative behavioural profile of centrally administered tachykinin NK_1, NK_2 and NK_3 receptor agonists in the guinea-pig. *Br. J. Pharmacol.*, **116,** 2496 – 2502.

SARAU, H.M., GRISWOLD, D.E., POTTS, W., FOLEY, J.J., SCHMIDT, D.B., MARTIN, L.D., BRAWNER, M.E., ELSHOURBAGY, N.A., MEDHURST, A.D., GIARDINA, G.A.M. & HAY, D.W.P. (1997). Nonpeptide tachykinin receptor antagonists. I. Pharmacological and pharmacokinetic characterisation of SB 223412, a novel, potent and selective neurokinin-3 receptor antagonist. *J. Pharmacol. Exp. Ther.*, **281,** 1303 – 1311.

STOESSL, A.J., DOURISH, C.T. & IVERSON, S.D. (1990). Pharmacological characterisation of the behavioural syndrome induced by the NK_3 tachykinin agonist senktide in rodents: evidence for mediation by endogenous 5-HT. *Brain Res.*, **517,** 111 – 116.

STOESSL, A.J., DOURISH, C.T., YOUNG, S.C., WILLIAMS, B.J., IVERSON, S.D. & IVERSON, L.L. (1987). Senktide, a selective neurokinin B-like agonist, elicits serotonin-mediated behaviour following intracisternal administration in the mouse. *Neurosci. Lett.*, **80,** 321 – 326.

TANIGUCHI, T., FUJIWARA, M., MASUO, Y. & KANAZAWA, I. (1986). Levels of neurokinin A, neurokinin B and substance P in rabbit iris sphincter muscle. *Jpn. J. Pharmacol.*, **42,** 590 – 593.

UEDA, N., MURAMATSU, I. & FUJIWARA, M. (1984). Capsaicin and bradykinin-induced substance P-nergic responses in the iris sphincter muscle of the rabbit. *J. Pharmacol. Exp. Ther.*, **230,** 469 – 473.

UEDA, N., MURAMATSU, I., HAYASHI, H. & FUJIWARA, M. (1982). Trigeminal nerve: The possible origin of substance P-ergic response in isolated rabbit iris sphincter muscle. *Life Sci.*, **31,** 369 – 375.

WANG, Z.-Y. & HÅKANSON, R. (1992). The electrically-evoked, tachykinin-mediated contractile response of the isolated rabbit iris sphincter muscle involves NK_1 receptors only. *Eur. J. Pharmacol.*, **216,** 327 – 329.

WANG, Z.-Y. & HÅKANSON, R. (1993). The rabbit iris sphincter contains NK_1 and NK_3 but not NK_2 receptors: a study with selective agonists and antagonists. *Regul. Peptides*, **44,** 269 – 275.

WORMSER, U., LAUFER, R., HART, Y., CHOREV, M., GILON, C. & SELINGER, Z. (1986). Highly selective agonists for substance P receptor subtypes. *EMBO J.*, **5,** 2805 – 2808.

(Received April 3, 1997
Revised June 20, 1997
Accepted July 2, 1997)