British Journal of Pharmacology (1999) 128, 627–636 © 1999 Stockton Press All rights reserved 0007–1188/99 $15.00
http://www.stockton-press.co.uk/bjp

Molecular and pharmacological characterization of a functional tachykinin NK_3 receptor cloned from the rabbit iris sphincter muscle

*,[1]Andrew D. Medhurst, [1]Warren D. Hirst, [1]Jeffery C. Jerman, [1]Jacqueline Meakin, [1]Jennifer C. Roberts, [2]Tania Testa & [1]Darren Smart

[1]Department of Neuroscience Research, SmithKline Beecham Pharmaceuticals, Third Avenue, Harlow, Essex, CM19 5AW and [2]Department of Biopharmaceutical Research, SmithKline Beecham Pharmaceuticals, Third Avenue, Harlow, Essex, CM19 5AW

1 A functional tachykinin NK_3 receptor was cloned from the rabbit iris sphincter muscle and its distribution investigated in ocular tissues.

2 Standard polymerase chain reaction (PCR) techniques were used to clone a full length rabbit NK_3 receptor cDNA consisting of 1404 nucleotides. This cDNA encoded a protein of 467 amino acids with 91 and 87% homology to the human and rat NK_3 receptors respectively.

3 In CHO-K1 cells transiently expressing the recombinant rabbit NK_3 receptor, the relative order of potency of NKB > > NKA ⩾ SP to displace [^{125}I]-[MePhe^7]-NKB binding and to increase intracellular calcium, together with the high affinity of NK_3 selective agonists (e.g. senktide, [MePhe^7]-NKB) and antagonists (e.g. SR 142801, SB 223412) in both assays was consistent with NK_3 receptor pharmacology. In binding and functional experiments, agonist concentration response curves were shallow (0.7–0.8), suggesting the possibility of multiple affinity states of the receptor.

4 Quantitative real time PCR analysis revealed highest expression of rabbit NK_3 receptor mRNA in iris sphincter muscle, lower expression in retina and iris dilator muscle, and no expression in lens and cornea. *In situ* hybridization histochemistry revealed discrete specific localization of NK_3 receptor mRNA in the iris muscle and associated ciliary processes. Discrete specific labelling of NK_3 receptors with the selective NK_3 receptor agonist [^{125}I]-[MePhe^7]-NKB was also observed in the ciliary processes using autoradiography.

5 Our study reveals a high molecular similarity between rabbit and human NK_3 receptor mRNAs, as predicted from previous pharmacological studies, and provide the first evidence that NK_3 receptors are precisely located on ciliary processes in the rabbit eye. In addition, there could be two affinity states of the receptor which may correspond to the typical and 'atypical' NK_3 receptor subtypes previously reported.

Keywords: Tachykinin NK_3 receptors; rabbit iris sphincter muscle; ciliary processes; cloned receptor

Abbreviations: bp, base pair; CHO-K1 cells, Chinese hamster ovary-K1 cells; cDNA, complementary deoxyribonucleic acid; ds, double stranded; FLIPR, fluorescent imaging plate reader; i.v., intravenous; mRNA, messenger ribonucleic acid; NK_3, neurokinin$_3$; NKA, neurokinin A; NKB, neurokinin B; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription-polymerase chain reaction; SP, substance P

Introduction

The rabbit eye provides a novel system for the study of tachykinin NK_3 receptors. For example, the detailed *in vitro* pharmacology of NK_3 receptors mediating smooth muscle contraction in the rabbit iris sphincter muscle has been previously described (Medhurst *et al*., 1997b). In addition, activation of ocular NK_3 receptors *in vivo* by intravenous (i.v.) administration of specific NK_3 receptor agonists has been shown to induce pupillary constriction (miosis) in the conscious rabbit, an effect that can be inhibited by i.v. administration of selective NK_3 receptor antagonists (Medhurst *et al*., 1997a). The antagonist pharmacology of ocular NK_3 receptors in the rabbit (particularly *in vitro*) appears more similar to that of human (Oury-Donat *et al*., 1995; Suman-Chauhan *et al*., 1994) and guinea-pig (Nguyen *et al*., 1994; Emonds-Alt *et al*., 1995) NK_3 receptors than to that of rat (Nguyen *et al*., 1994; Patacchini *et al*., 1995) NK_3 receptors; in the latter species lower affinity of certain NK_3 receptor antagonists (e.g. SR 142801) compared to the receptor in other species has been demonstrated. Indeed, site-directed mutagenesis studies have demonstrated that two variant residues in the second transmembrane domain of the human receptor (Met134 and Ala146) compared to the rat receptor contribute to the species variation in affinity of such compounds (Wu *et al*., 1994; Chung *et al*., 1995). There is also some pharmacological evidence for the presence of 'typical' and 'atypical' NK_3 receptor subtypes in rabbit iris sphincter muscle in the presence of NK_1 receptor blockade (Medhurst *et al*., 1997b), but this has yet to be confirmed at the molecular level. Interestingly a recent study of recombinant human NK_3 receptors suggests the existence of different affinity states of the receptor which are sensitive to Zn^{2+} (Rosenkilde *et al*., 1998). Whilst the molecular nature of both human (Huang *et al*., 1992) and rat (Shigemoto *et al*., 1990; Buell *et al*., 1992) NK_3 receptors is well documented, there is no information on the molecular structure of any rabbit tachykinin receptors.

The aim of the present study was to clone an NK_3 receptor from the rabbit iris sphincter muscle using standard PCR techniques, and to study the distribution of this receptor in

*Author for correspondence;
E-mail: Andy_Medhurst-1@sbphrd.com

ocular tissues using *in situ* hybridization histochemistry, TaqMan PCR analysis (Lie & Petropoulos, 1998) and receptor autoradiography. Pharmacological characterization of the cloned rabbit iris NK_3 receptor transiently expressed in CHO-K1 cells using radioligand binding and Fluorescent Imaging Plate Reader (FLIPR) technology (Schroeder & Neagle, 1996), would allow a comparison with the pharmacology of the endogenous iris NK_3 receptor previously described (Medhurst *et al*., 1997a,b). In addition, these experiments would allow further investigation into the existence of putative NK_3 receptor subtypes. A preliminary account of this work was presented at the Tachykinins in Health and Disease meeting, Cairns, Australia (1997), and the rabbit iris NK_3 receptor cDNA sequence identified was submitted to the GenBank database (Accession No. AF133908).

Methods

Rabbit NK_3 receptor cloning

The cloning strategy used is outlined in Figure 1 (numbers for primers, letters for cDNA sequences). Rabbit cDNAs were synthesized from iris sphincter muscle total RNA (prepared in house with Trizol reagent, Life Technologies) or brain polyA$^+$ mRNA (Clontech), using SuperScript II reverse transcriptase (GibcoBRL). To obtain cDNA encoding a central core region of the rabbit NK_3 receptor, cDNA synthesis was primed with an antisense primer 5′-GAGAGCAGCCATTGAAAC (2), corresponding to nucleotides 1447–1464 of the human NK_3 receptor cDNA sequence ([A], GenBank Accession No. M89473). This cDNA was amplified using AmpliTaq polymerase (Perkin Elmer) with a sense primer 5′-ATCCCTTGAAACCCAGAC (1) corresponding to nucleotides 709–726 of the human sequence, and primer (2) above, with the following touchdown PCR conditions: 94°C for 1 min, 10 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, reducing annealing temperature by 1°C per cycle, then 25 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, and finally 72°C for 5 min. The resulting 756 bp fragment [B] was subcloned (into pCR2.1, Invitrogen) and sequenced. Since the sequences of iris and brain cDNA fragments were identical, and there was limited iris RNA available, brain polyA$^+$ mRNA was utilized for Marathon cDNA synthesis (Clontech), to allow 5′- and 3′-ends of rabbit NK_3 receptor cDNA to be generated using RACE long distance PCR (Chenchik *et al*., 1996). In addition, Marathon cDNAs were made from rabbit lung and liver polyA$^+$ mRNA (Clontech).

First and second strand cDNA was synthesized using manufacturers protocols (Clontech) and following blunt ending, the double stranded cDNA was ligated to the Marathon cDNA Adaptor (Gubler & Hoffmann, 1983). This uncloned 'library' of adaptor-ligated ds cDNA was then diluted 1/50 for Marathon RACE reactions. Both 5′- and 3′-RACE cDNA ends were amplified with primers specific to the partial rabbit NK_3 receptor sequence and Marathon adaptor primers AP-1 (3) and AP-2 (5; Clontech) following manufacturers protocols and using the following PCR conditions: 94°C for 1 min, 5 cycles of 94°C for 30 s, 72°C for 4 min, then 5 cycles of 94°C for 30 s, 70°C for 4 min, followed by 25 cycles of 94°C for 20 s, 68°C for 4 min. To obtain the 3′-end of the rabbit NK_3 receptor, brain Marathon ds cDNA was amplified in first round PCR with (3) and sense primer 5′-CAAGACCACCAGGTTTCACCCAACTC (4), followed by second round PCR with nested primers (5) and sense primer 5′-ACCCAAGTGACGCAGACAACACCAGA (6). The resulting 300 bp fragment [D] was subcloned and sequenced to confirm presence of an in frame stop codon.

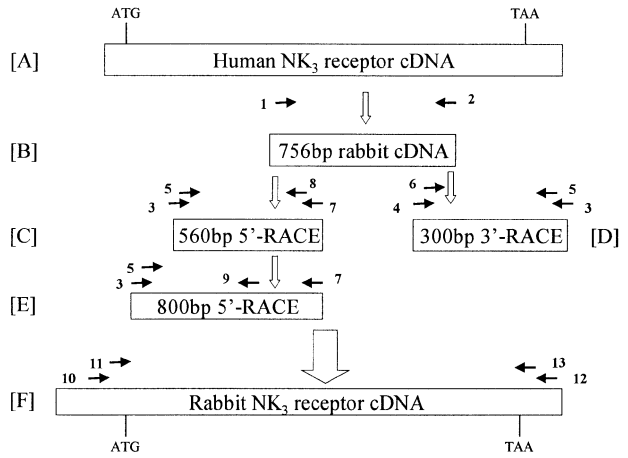

Figure 1 Cloning strategy for isolating the rabbit NK_3 receptor. Primers designed to a core sequence of the human NK_3 receptor were utilized to amplify a rabbit NK_3 receptor cDNA fragment. RACE–PCR with nested primers was then used to obtain 5′ and 3′ cDNA ends of the rabbit NK_3 receptor. Numbered arrows represent primers, sequences of which are shown in Methods section. ATG represents initiation codon and TAA the stop codon.

To obtain the 5′-end of the rabbit NK_3 receptor, first round PCR amplification of brain Marathon ds cDNA was performed with (3) and antisense primer 5′-AGCGTGATTCCAACAATGGTGTATG (7), followed by second round PCR amplification with nested primer (5) and another antisense primer 5′-TAAAGACACTGAGGCAAAGCAAGTAGA (8). This resulted in a 560 bp RACE fragment [C] which did not yield the full 5′-end. Consequently, a further round of 5′-RACE reactions were performed using the Advantage-GC^{TM} KlenTaq polymerase mix (Clontech) with primers (3) and (7), nested with (5) and another antisense primer 5′-CGTCGGAGAAAGCCAGGTTCACGAGGA (9) designed from the 560 bp fragment generated previously. The resulting 800 bp 5′-RACE product [E] was subcloned and sequenced, to confirm that the full 5′-end had been obtained.

Full length rabbit NK_3 receptor was amplified (using *PfuTurbo* DNA polymerase, Stratagene) from the 'library' of adaptor-ligated ds brain cDNA and also from iris sphincter, iris dilator, lens, cornea, retina, lung and liver cDNA using primers at the 5′ and 3′ untranslated regions. First round PCR amplification was performed with 5′-AGAGGAACTTCGGGCGATTCG (10) and 5′-GGGACTGTGATAGCTTTACACTCATC (12). Second round PCR was performed on 2 μl of first round PCR template using nested primers 5′-ATGGACTCTTTCGCCGCCGC (11, sense including initiation codon) and 5′-TTAAGAGTATTCCTCCATAGAGGTATAG (13, antisense ending with stop codon). In both rounds of PCR the cycling conditions were 95°C for 1 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 2 min, ending with 72°C for 7 min. The resulting 1404 bp product [F] was subcloned into the cytomegalovirus (CMV) promoter-based plasmid pcDNA3 (Invitrogen) for sequence confirmation and expression studies.

Transient transfection of the rabbit NK_3 receptor into CHO-K1 cells

As the sequences for the NK_3 receptor cloned from the iris/lung and brain/liver differed by two amino acids, both NK_3 receptor cDNAs were transfected separately into CHO-K1

cells for pharmacological characterization. Cells were grown to approximately 80% confluency in T175 flasks containing Ham's F12 nutrient media supplemented with 10% foetal calf serum and 2 mM glutamine. All cells were incubated at 37°C, in an atmosphere of 5% CO_2. For each transfection 5 μg rabbit NK_3/pcDNA3 DNA was diluted into 750 μl OPTI-MEM (serum-free medium) and 20 μl PLUS reagent (Life Technologies) and left for 15 min. This was then added to a second tube containing 750 μl OPTIMEM and 30 μl Lipofectamine (Life Technologies), and left for a further 15 min to allow formation of DNA-liposome complexes. While the complexes were forming the cells were rinsed with 5–10 ml OPTIMEM. The transfection complex was then added to each flask containing 5 ml OPTIMEM and incubated for 3–4 h, after which the solution was removed and replaced with 25 ml of fresh growth medium. The cells were cultured for a further 24–48 h.

Radioligand binding

Membrane preparation and radioligand binding assays were performed as previously described (Sarau *et al*., 1997). Briefly, cells were homogenized in a solution containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and protease inhibitors. Samples were then rapidly frozen and thawed three times, and homogenized again.

Membranes (15–20 μg of protein) were incubated with 0.15 nM [^{125}I]-[MePhe^7]-NKB in 150 μl buffer containing 50 mM Tris-HCl, pH 7.4, 4 mM $MnCl_2$, 1 μM phosphoramidon and 0.1% ovalbumin, in the presence and absence of tachykinin receptor ligands (10 pM–3 μM); 90 min incubations (room temperature) were terminated by rapid filtration through GF/C filters which had been pre-soaked in 0.5% BSA. Non-specific binding was determined as the binding in the presence of 1.5 μM [MePhe^7]-NKB. The concentration of drug inhibiting specific [^{125}I]-[MePhe^7]-NKB binding by 50% (IC_{50}) was determined by iterative curve fitting (Bowen & Jerman, 1995). pK_i values (−log of the inhibition constant) for receptor binding were then calculated from the IC_{50} values as described by Cheng & Prusoff (1973) using a K_d value of 0.3 nM.

Measurement of intracellular calcium using FLIPR

FLIPR (Fluorescent Imaging Plate Reader, Molecular Devices; Schroeder & Neagle, 1996) assays measuring changes in intracellular calcium were carried out to assess functional activity of NK_3 receptor agonists and antagonists at the rabbit NK_3 receptor. CHO-K1 cells expressing recombinant rabbit NK_3 receptors were seeded into Costar, black wall, clear base, 96 well plates at a density of 30,000 cells per well in MEM-Alpha medium supplemented with 10% foetal calf serum. Twenty-four hours later cells were incubated in Tyrodes solution, containing 10 mM HEPES, with the cytoplasmic Ca^{2+} indicator, FLUO-3AM (2 μM; Molecular Probes, The Netherlands) and 2.5 mM probenecid to reduce dye leakage, at 37°C for 60 min in 5% CO_2/95% O_2. The cells were then washed four times with, and finally resuspended in, Tyrodes containing 10 mM HEPES and 2.5 mM probenecid. The plate was then placed into the FLIPR to monitor cell fluorescence (λ_{Ex} = 488 nm, λ_{Em} = 540 nm) before and after the addition of various agonists and/or antagonists (10 pM–1 μM for tachykinin ligands and 3–100 μM for $ZnCl_2$). Responses were recorded as peak fluorescence intensity (FI) minus basal FI and data were analysed using iterative non-linear regression in Excel (Bowen & Jerman, 1995). Agonist potency was expressed as pD_2 values (−log concentration of agonist producing 50% of the maximum response). Apparent pK_B values for antagonists were calculated using the Gaddum-Schild equation ($-\log K_B = pK_B = -\log[B] + \log[CR-1]$) where [B] represents 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.

TaqMan PCR analysis

A more quantitative analysis of NK_3 receptor mRNA expression was undertaken in ocular tissues using TaqMan PCR technology (Lie & Petropoulos, 1998). OligodT-primed cDNA was amplified in triplicate using rabbit NK_3 specific primers (sense primer 5′-GGCAAAGCAGCCTGTACACC; antisense primer 5′-GTCTGCGTCACTTGGGTCAAA) and TaqMan fluorogenic probe (antisense 5′-CACTGTCATG-GACTCCATCCTGGTCA) designed using Primer Express software and synthesized by PE Applied Biosystems. Minus reverse transcriptase controls were run for each RNA to assess genomic DNA contamination. TaqMan PCR using TaqMan universal PCR Mastermix (PE Applied Biosystems) was performed following manufacturers protocols on an ABI Prism 7700 (PE Applied Biosystems). To correct for differences in RNA quantity and quality, parallel TaqMan reactions were run with rat/mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (sense 5′-GAACATCATCCCTG-CATCCA; antisense 5′-CCAGTGAGCTTCCCGTTCA) and probe (5′-CTTGCCCACAGCCTTGGCAGC) which were 95% identical to the rabbit GAPDH sequence. Serial dilutions of rabbit genomic DNA (Clontech) were used to generate a standard curve relating threshold cycle to known copy number of genomic DNA (Lie & Petropoulos, 1998). Data was expressed as arbitrary units or normalized as relative expression to GAPDH.

In situ *hybridization histochemistry*

Avoiding homology to other known receptors, especially NK_1 and NK_2 receptors, and using the sequence data generated from the specific NK_3 receptor cDNA obtained from the rabbit iris sphincter muscle, specific oligonucleotide probes for *in situ* hybridization histochemistry (ISHH) were designed and obtained from Genosys (Cambridge, U.K.):- antisense, 5′-CTTCTGGCCATTGCACATAGCAAAGAGTACGTCCA-GGC; sense, 5′-GCCTGGACGTACTCTTTGCTATGTG-CAATGGCCAGAAG. Probes were 3′ end-labelled with [^{35}S]-dATP and purified using a commercially available kit (Dupont NEN). Twenty μm cryostat sections of frozen eye tissue were cut at −18°C and collected on glass slides coated with 2% 3-aminopropyltriethoxylene.

Standard ISHH methodology was utilized as described elsewhere (Najlerahim *et al*., 1990). Sections were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7) for 5 min, rinsed twice in PBS and then soaked for 10 min in triethanolamine (TEA). The slides were then dehydrated in a graded series of ethanol washes (70, 80, 95 and 100%) for 1 min each, except 95% (2 min). After 10 min in chloroform, slides were washed with 100% ethanol and 95% ethanol for 1 min each. Sections were covered with 100 μl hybridization buffer (Najlerahim *et al*., 1990) containing 10^6 c.p.m.) [^{35}S]-dATP-labelled antisense or sense probe. Each section was then covered with a nescofilm coverslip and hybridizations were performed for 18 h at 35°C. Following hybridization, the coverslips were removed by floatation in 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate) and the slides washed in

$1 \times SSC$ for 3×20 min at 55°C followed by two 1 h washes at room temperature. Slides were finally rinsed briefly in water to remove excess salt and left to dry thoroughly. Visualization was carried out using a phosphoImager (Molecular Dynamics).

Receptor autoradiography

The methods used for autoradiography were as described previously (Medhurst *et al*., 1997b), but in this study were carried out on whole eye sections (20 μm) rather than iris sphincter muscle sections. Total binding was defined by incubating the eye sections in assay buffer containing 1 nM [^{125}I]-[MePhe^7]-NKB (specific activity 2200 Ci mmol^{-1}; New England Nuclear, Dreieich, Germany) for 90 min at room temperature. Non-specific binding was 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 for 90 min at room temperature. The effects of the selective NK_1 receptor antagonist CP 99994 (McLean *et al*., 1993; 1 μM) on binding of 1 nM [^{125}I]-[MePhe^7]-NKB was also determined. Results were also quantified using an image analysis system (Optimas) with densities of receptor expressed as nCi mg^{-1} tissue.

Materials

Senktide, neurokinin B (NKB), [MePhe^7]-neurokinin B, neurokinin A (NKA) and substance P 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-phenylepipiperidin-4-yl)-N-methylacetamide), SR 48968 ((S)-N-methyl-N[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide), SB 223412 ((−)-(S)-N-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide), SB 222200 ((−)-(S)-N-(α-ethylbenzyl)-3-methyl-2-phenylquinoline-4-carboxamide), SB 218795 ((−)-(R)-N-(α-methoxycarbonylbenzyl)-2-phenylquinoline-4-carboxamide) and CP 99994 ((+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine) were synthesized by colleagues in the Department of Medicinal Chemistry at SmithKline Beecham Pharmaceuticals (Milan, Italy). [^{125}I]-[MePhe^7]-NKB (specific activity 2200 Ci mmol^{-1}) was obtained from New England Nuclear, Dreieich, Germany and $ZnCl_2$ from Sigma (U.K.).

Results

Rabbit NK_3 receptor cDNA cloning

Using primers designed to a core sequence of the human NK_3 receptor cDNA sequence, a 756 bp cDNA fragment was isolated from the rabbit iris sphincter muscle and rabbit brain (Figure 1). These cDNAs encoded a 251 amino acid protein with 96 and 94% homology to a region spanning the IVth–VIIth transmembrane domains and part of the intracellular C-terminus of the human (GenBank Accession No. M89473) and rat (GenBank Accession No. J05189) NK_3 receptor respectively. 3′-RACE of the rabbit brain Marathon adaptor-ligated cDNA library yielded a fragment of approximately 300 bp, of which 160 bp was 3′-untranslated region (3′-UTR). The remaining nucleotides encoded a sequence with high homology to the human NK_3 receptor. The first attempt at 5′-RACE in brain yielded a product of 560 bp, also with high homology to human and rat NK_3 receptors. However, full 5′-sequence was not obtained, possibly due to the high GC content at the 5′-end as predicted from human and rat sequences. A second 5′-RACE assay using the Advantage-GC Klentaq polymerase system yielded a product of 800 bp, including 298 bp of 5′-UTR. Despite greatest divergence at the 5′-end, consistent with differences in human and rat NK_3 receptor sequences, the initiation ATG codon was identified, as well as three in frame upstream stop codons (at positions −54, −234 and −288 with the A of ATG at position 1).

Using primers targeting 5′- and 3′-UTR the full length 1404 bp coding sequence of the rabbit NK_3 receptor cDNA was isolated from iris sphincter muscle, and has been deposited in the GenBank database (Accession No. AF133908). The deduced amino acid sequences of the full length rabbit NK_3 receptor indicates that this protein consists of 467 amino acids, two residues longer than human (Pro56 and Pro60 of rabbit protein sequence) and 15 residues longer than rat NK_3

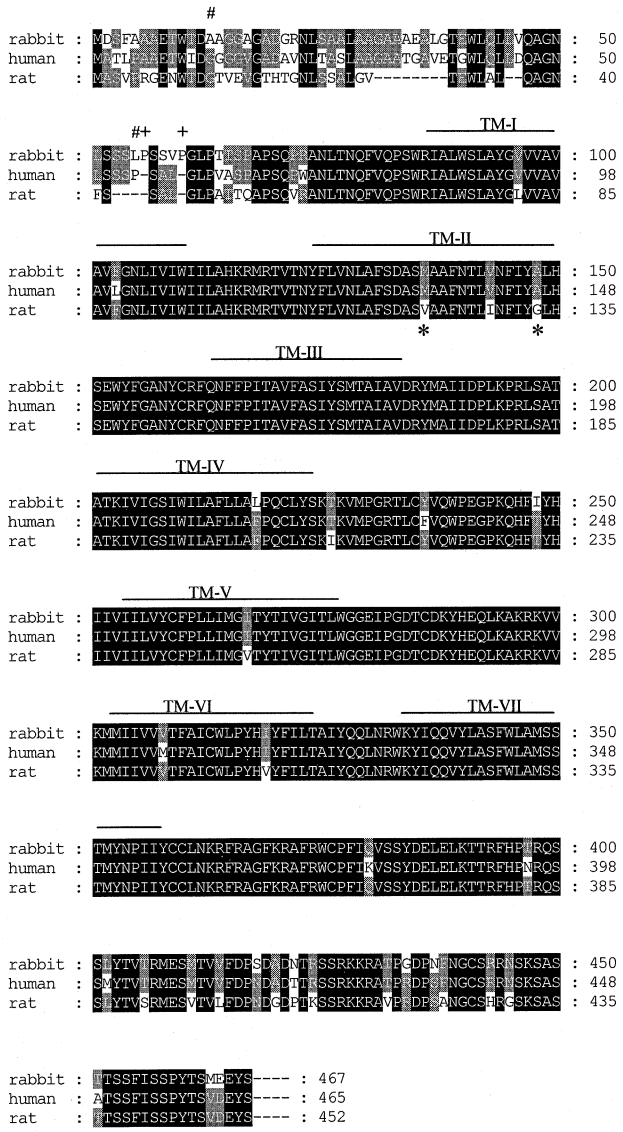

Figure 2 Alignment of protein sequences for the rabbit iris NK_3 receptor with the human and rat NK_3 receptors (Genbank Accession numbers AF133908, M89473 and J05189 respectively). Amino acid residues identical in all species are boxed in black, those conserved in two species boxed in grey, and those unique to one species are shown in white. Hydrophobic transmembrane domains (TM) I–VII are shown by the horizontal bars. + indicates two additional residues in the rabbit compared to human sequences, # shows two amino acids that differ between iris and brain clones, and * shows two amino acid residues confering species differences between human and rat pharmacology.

receptor, respectively (Figure 2). These additional two residues were also confirmed in clones from rabbit brain, lung and liver RACE 'libraries'. Interestingly, two single point mutations were observed between iris/lung and brain/liver NK_3 sequences. Residues Ala-13 and Leu-55 in the iris (GenBank Accession No. AF133908) and lung sequences were substituted to Gly-13 and Pro-55 respectively in both the brain and liver sequences. Therefore, both iris and brain NK_3 receptors were transiently expressed for receptor binding analysis and functional evaluation using FLIPR, to determine whether these mutations affected receptor pharmacology. There were only three other nucleotide changes between all rabbit NK_3 clones but none of these resulted in any amino acid changes.

The rabbit NK_3 receptor amino acid sequence showed high homology to human (91%) and rat (87%) NK_3 receptor sequences, with greatest divergence at the extracellular N-terminus (Figure 2). In this N-terminal region (1–60 of the rabbit sequence) there was 62% (37/60) amino acid identity with human, but only 40% (24/60) identity with rat, while in the remaining coding region 96% (389/407) and 93% (379/407) of the amino acid residues were conserved in human and rat respectively.

Radioligand binding

To confirm that the rabbit tachykinin receptors cloned and expressed in the present study displayed typical characteristics of the NK_3 receptor subtype, competition binding studies were carried out using the highly selective NK_3 receptor agonist [^{125}I]-[MePhe^7]-NKB. Non-specific binding accounted for less than 20% of total counts in the presence of unlabelled [MePhe^7]-NKB and there was no [^{125}I]-[MePhe^7]-NKB binding to mock transfected cells. Preliminary experiments with selective NK_3 receptor ligands revealed no differences in pharmacology between iris or brain rabbit NK_3 receptor clones transiently expressed in CHO-K1 cells (unpublished observation). In accordance with NK_3 receptor pharmacology, competition studies showed that the selective NK_3 receptor agonists senktide and [MePhe^7]-NKB, as well as the endogenous ligand NKB possessed high affinity for the recombinant rabbit iris sphincter muscle NK_3 receptor expressed in CHO-K1 cells (Table 1). In contrast, substance P and NKA possessed much lower affinity for this receptor (Table 1), again consistent with NK_3 receptor pharmacology. Binding affinities for non-peptide antagonists SB 223412, SB 222200 and SB 218795, and the structurally unrelated antagonists SR 142801 and SR 48968 showed similar affinity for the cloned rabbit NK_3 receptor as for the human NK_3 receptor (Table 1). Interestingly, the slope factors for the NK_3 receptor agonists were 0.7–0.8, suggesting the possibility of different affinity states of the receptor, whilst the slopes for selective NK_3 receptor antagonists were close to unity. All compounds tested completely inhibited specific binding of [^{125}I]-[MePhe^7]-NKB.

FLIPR assay

Functional responses to NK_3 receptor agonists in CHO-K1 cells expressing rabbit NK_3 receptors were investigated in detail using FLIPR. Mock transfected wild type CHO-K1 cells failed to respond to all tachykinin agonists tested (10 pM–1 μM). In contrast, all tachykinin receptor agonists tested produced concentration-dependent increases in intracellular calcium in CHO-K1 cells transiently expressing rabbit NK_3 receptors (Figure 3). Both iris and brain clones displayed classical NK_3 receptor agonist pharmacology in the FLIPR but had low Hill slope factors, again suggesting the possibility of two affinity states. [MePhe^7]-NKB ($pD_2 = 9.6 \pm 0.08$; $nH = 0.7 \pm 0.01$; $n = 5$), senktide ($pD_2 = 9.7 \pm 0.1$; $nH = 0.8 \pm 0.05$; $n = 5$) and NKB ($pD_2 = 9.0 \pm 0.09$; $nH = 0.7 \pm 0.04$; $n = 4$) were highly potent agonists at the iris receptor, whilst NKA ($pD_2 = 7.26 \pm 0.09$; $nH = 0.7 \pm 0.04$; $n = 4$) and SP ($pD_2 = 6.7 \pm 0.09$; $nH = 1.2 \pm 0.17$; $n = 4$) were much less potent. pD_2 values and slope factors for the brain clone were not significantly different to those for the iris clone ($P < 0.05$, Student's unpaired t-test).

The effects of several NK_3 receptor antagonists on responses to senktide and [MePhe^7]-NKB were then assessed in CHO-K1 cells expressing either iris or brain NK_3 receptor cDNAs. pK_B estimates for SB 223412, SB 222200 and SB 218795, as well as for SR 142801 and SR 48968 (Table 2), were comparable with affinity values expected at human NK_3 receptors.

Preliminary radioligand binding studies revealed that $ZnCl_2$ increased the specific binding of [^{125}I]-[MePhe^7]-NKB to the rabbit NK_3 receptor by 80% (data not shown) consistent with previous observations with the human NK_3 receptor (Rosenkilde *et al*., 1998). Interestingly, in FLIPR experiments $ZnCl_2$ (0.3–1 μM) caused a concentration-dependent rightward shift in the concentration-effect curves to senktide and [MePhe^7]-NKB (single concentration pA_2 estimates of 6.0 ± 0.11 and

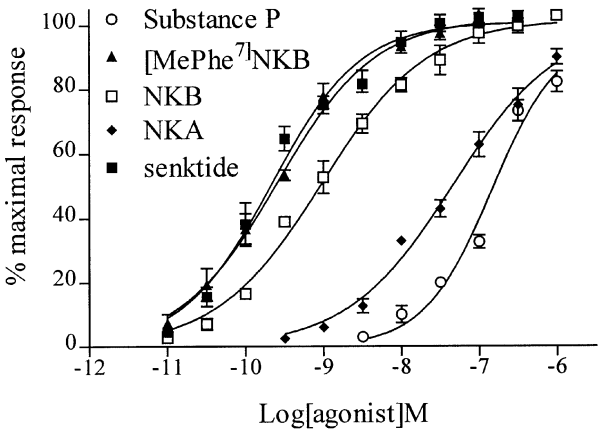

Figure 3 Concentration effect curves to senktide ($n = 5$), [MePhe^7]-NKB ($n = 5$), NKB ($n = 4$), NKA ($n = 4$) and SP ($n = 4$) in CHO-K1 cells transiently expressing the rabbit NK_3 receptor. Increases in intracellular calcium induced by each agonist were measured using FLIPR. Results are expressed as a percentage of the maximum response and data are mean with vertical lines showing s.e.mean.

Table 1 Receptor binding affinity of tachykinin agonists and antagonists for the rabbit recombinant NK_3 receptor

	pK_i	*Hill slope*	n
[MePhe^7]-NKB	8.5±0.09	0.8±0.05	9
senktide	7.7±0.16	0.7±0.06	7
NKB	7.3±0.06	0.8±0.08	4
NKA	5.5±0.10	0.5±0.10	3
Substance P	5.3±0.07	1.0±0.30	3
SR 142801	8.0±0.10	0.9±0.06	7
SR 48968	6.0±0.01	0.8±0.10	3
SB 223412	8.6±0.07	1.0±0.08	7
SB 222200	7.2±0.10	1.0±0.07	5
SB 218795	7.1±0.06	1.1±0.06	3

The rabbit NK_3 receptor was transiently expressed in CHO-K1 cells and competition binding studies were carried out using [^{125}I]-[MePhe^7]-NKB.

Table 2 pK_B estimates for NK_3 receptor antagonists at the rabbit recombinant NK_3 receptor versus Ca^{++} mobilization induced by senktide and [MePhe^7]-NKB

	Iris (pA_2)	*Iris* (pK_B)	*Iris* (pK_B)	*Brain* (pK_B)	*Brain* (pK_B)
	Senktide	Senktide	[MePhe^7]-NKB	Senktide	[MePhe^7]-NKB
SR 142801	8.9	8.94±0.03	8.96±0.02	8.93±0.02	9.02±0.03
SR 48968	6.1	6.56±0.03	6.80±0.15	6.83±0.15	6.80±0.18
SB 223412	8.4	8.45±0.12	8.49±0.11	8.71±0.12	8.74±0.04
SB 222200	7.9	8.71±0.05	8.55±0.09	8.80±0.07	8.35±0.13
SB 218795	7.4	7.93±0.11	7.69±0.14	8.08±0.04	7.86±0.06

Calcium influx was measured using FLIPR. *pA_2 estimates for the endogenous receptor are shown for comparison (Medhurst *et al.*, 1997a,b).

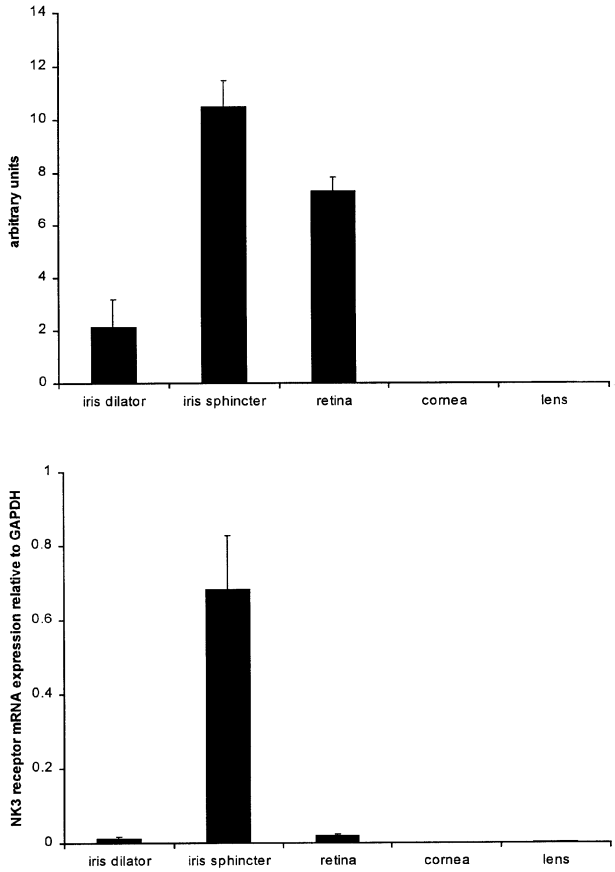

Figure 4 TaqMan PCR analysis of NK_3 receptor mRNA expression in ocular tissues. (a) expressed as arbitrary units, and (b) expressed as a proportion of GAPDH to correct for RNA quantity and quality.

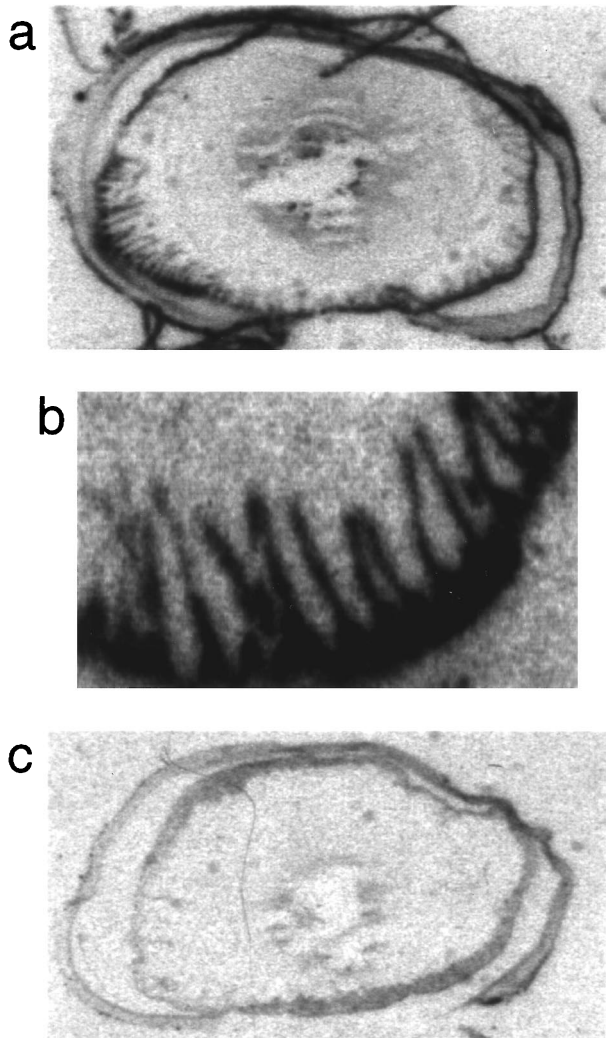

Figure 5 (a) NK_3 receptor mRNA distribution in a whole rabbit eye section (×5.8) labelled with 5′-CTTCTGGCCATTGCACATAG-CAAAGAGTACGTCCAGGC antisense probe, showing discrete hybridization to iris muscle and associated ciliary processes; (b) higher magnification (×10) showing discrete localization of NK_3 receptor mRNA to ciliary processes, and (c) sections (×5.8) exposed to 5′-GCCTGGACGTACTCTTTGCTATGTGCAATGGCCA-GAAG sense probe showing minimal hybridization throughout the eye.

6.41±0.05 respectively), but did not inhibit UTP-induced activation of endogenous P_{2Y} purinoceptors at this concentration. However at higher concentrations of $ZnCl_2$ (3 and 10 μM), maximum responses to senktide, [MePhe^7]-NKB and uridine triphosphate (UTP; 10 μM) were significantly reduced, suggesting non-specific quenching of fluorescent signal.

Rabbit NK_3 receptor mRNA expression

Full length NK_3 receptor cDNA was detected using RT–PCR in iris sphincter, iris dilator and retina, but not in cornea and lens. A more quantitative analysis using TaqMan PCR showed higher levels of NK_3 receptor mRNA in iris sphincter than in other ocular tissues (Figure 4). mRNA levels of the housekeeping gene GAPDH were comparable in iris sphincter, lens and cornea suggesting that it was not differences in RNA quality that prevented detection of NK_3 receptor mRNA in the latter two tissues.

In situ *hybridization histochemistry*

Thirty-eight-mer antisense and sense probes were used for *in situ* hybridization histochemistry on sections of whole rabbit eye. Discrete localization of NK_3 receptor mRNA was observed in the iris muscle and associated ciliary processes

with the antisense probe (Figure 5a,b), whilst minimal labelling was seen in sections exposed to the sense probe (Figure 5c).

Receptor autoradiography

In whole eye sections, [^{125}I]-[MePhe^7]-NKB labelled a population of NK_3 receptors that was discretely localized to the iris muscle and associated ciliary processes (Figure 6a); the discrete binding to ciliary processes is shown at higher magnification in Figure 6b. This specific binding was inhibited by unlabelled [MePhe^7]-NKB (Figure 6c), but not by the selective NK_1 receptor antagonist CP 99994. Some quantitation of autoradiograms was carried out using image analysis (Figure 7). Dense binding was observed in iris sphincter and ciliary processes, moderate binding was seen in iris dilator muscle and low, non-specific binding was observed in sclera.

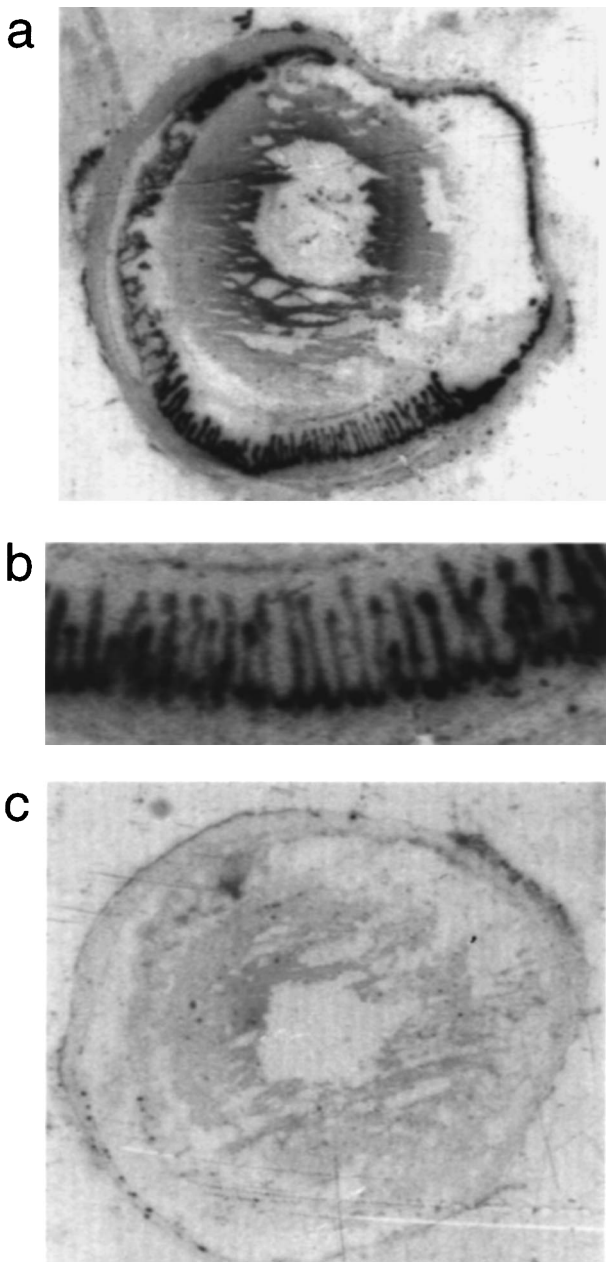

Figure 6 (a) autoradiogram of [^{125}I]-[MePhe^7]-NKB binding in a whole rabbit eye section (×5.8), (b) high magnification (×10) showing the discrete localization of [^{125}I]-[MePhe^7]-NKB binding to ciliary processes, and (c) autoradiogram of [^{125}I]-[MePhe^7]-NKB binding in an adjacent rabbit eye section (×5.8) in the presence of 1 μM [MePhe^7]-NKB.

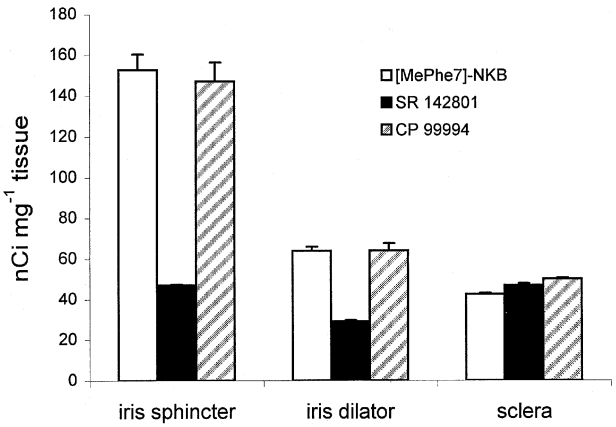

Figure 7 Quantitative analysis of the NK_3 receptor distribution in rabbit eye using autoradiography. Results were quantified using an image analysis system (Optimas) with densities of receptor expressed as nCi mg^{-1} tissue.

Discussion

Ocular NK_3 receptors in the rabbit have been well characterized *in vitro* (Medhurst *et al*., 1997b) and *in vivo* (Medhurst *et al*., 1997a), but there is no previous information on the molecular nature of these receptors. In the present study we have cloned and expressed a functional rabbit NK_3 receptor from the iris sphincter muscle and brain, providing the first molecular information on any tachykinin receptor in the rabbit. In addition, we have provided the first evidence for the distinct localization of NK_3 receptors to the ciliary processes in the rabbit eye using *in situ* hybridization and receptor autoradiography.

Previous data with selective NK_3 receptor agonists and antagonists suggested that the NK_3 receptors mediating contraction in the rabbit iris sphincter muscle (Medhurst *et al*., 1997a) were pharmacologically more similar to the NK_3 receptors in guinea-pig ileum (Nguyen *et al*., 1994; Emonds-Alt *et al*., 1995) and human, cloned NK_3 receptors expressed in Chinese Hamster Ovary cells (Oury-Donat *et al*., 1995; Suman-Chauhan *et al*., 1994), than NK_3 receptors in rat portal vein (Nguyen *et al*., 1994; Patacchini *et al*., 1995). Therefore we chose the human NK_3 receptor cDNA sequence (Huang *et al*., 1992) to design primers for PCR amplification of a core rabbit NK_3 receptor sequence and then used RACE–PCR to obtain 5′- and 3′-ends. The sequence of the full length rabbit NK_3 receptor cDNA obtained in the present study was highly homologous to human (91%) and rat (87%) NK_3 receptor cDNA sequences, with greatest divergence in the first 60 amino acids of the extracellular N-terminal domain. This is consistent with previous observations showing that tachykinin receptors are highly conserved between species, except at the extracellular N-terminus (Shigemoto *et al*., 1990). Interestingly the rabbit NK_3 receptor cDNA also had some homology (18% at N-terminus and 85% in remainder) to the human putative NK_4 receptor or novel human NK_3 receptor 'homologue' (previously known as a human putative opioid receptor, GenBank Accession No. M84665; Donaldson *et al*., 1996; Krause *et al*., 1997), but this homology was lower than for human and rat NK_3 receptors. Importantly, the rabbit NK_3 receptor lacked the two stretches of amino acid deletions (18

and 7 residues) seen in the NK_4 receptor sequence compared to the human NK_3 receptor sequence. In addition, there is no pharmacological evidence at present to suggest NK_4-like receptors exist in the rabbit eye, and we failed to identify such a gene in the present study.

Previous pharmacological studies on human and rat NK_3 receptors showed that the affinity of some selective NK_3 receptor antagonists was lower for rat than for human NK_3 receptors (Chung *et al*., 1995). These differences were attributed at the molecular level to specific changes in amino acid residues of the receptor located in the second transmembrane domain, i.e. Met134 and Ala146 in human changed to Val and Gly respectively in rat (Wu *et al*., 1994). The sequence data generated in the present study shows that these Met and Ala residues are conserved in the rabbit, and this, together with the present functional data generated for the cloned rabbit receptor, support previous pharmacological data showing that antagonist affinities (e.g. of SR 142801) for human and native rabbit iris NK_3 receptors are comparable, but higher than for the rat (Medhurst *et al*., 1997a,b).

There were some other notable features about the rabbit NK_3 receptor sequence. Firstly, the rabbit NK_3 receptor has the same three putative N-glycosylation sites as the human receptor, compared to four sites in the rat receptor. Secondly, an additional two amino acids were identified in the rabbit sequence compared to the human sequence (Pro56 and Pro60). These extra residues were confirmed in four different rabbit tissues and several independent RNA preparations. It is unlikely that these additional residues would affect function of the receptor, as they occur near a region where several amino acids have been deleted from the rat NK_3 sequence compared to the human sequence (Shigemoto *et al*., 1990). They are also not in a region of the receptor previously shown to be important in determining agonist or antagonist pharmacology, e.g. transmembrane domains III and IV important for senktide recognition (Gether *et al*., 1993), third extracellular loop and transmembrane domain VII for determining affinity of NKB (Gether *et al*., 1993), or transmembrane domains V and VII responsible for fundamental recognition of the common C-terminal tachykinin sequence (Yokota *et al*., 1992).

The nucleotide sequences of iris, brain, lung and liver NK_3 clones were very consistent with six nucleotide differences in the full length 1404 bp sequence, of which four were silent changes. The predicted amino acid sequences of several clones from each of these tissues differed by only two residues, with Ala-13 and Leu-55 in iris (GenBank Accession No. AF133908) and lung sequences being substituted by Gly-13 and Pro-55 in brain and liver sequences. These substitutions were not due to sequencing or PCR errors, as the differences in relevant nucleotides were observed with double-stranded sequencing of multiple independent clones, derived from independent RNA samples prepared from two different tissue types (i.e. iris and lung, or brain and liver). This more likely reflects the fact that both 'in house' and commercial RNAs used in the present study were prepared by pooling tissues from multiple animals, suggesting the possibility of genuine single nucleotide polymorphisms between individual rabbits. However, as the pharmacology of the iris and brain clones were virtually indistinguishable, these putative polymorphisms were not studied further. Interestingly, the amino acids Gly-13 and Pro-55 of the rabbit brain NK_3 sequence are conserved in the human sequence, which was originally cloned from human brain libraries (Huang *et al*., 1992; Buell *et al*., 1992).

Our results from both radioligand binding and functional FLIPR assays confirmed that the rabbit tachykinin receptor cloned in the present study was the NK_3 receptor. The relative order of potency for endogenous tachykinins to displace [^{125}I]-[MePhe^7]-NKB binding and to increase intracellular calcium (i.e. NKB$>>$NKA$\geqslant$SP), together with the high affinity of both NK_3 selective agonists (e.g. senktide, [MePhe^7]-NKB) and antagonists (e.g. SR 142801, SB 223412) for the rabbit NK_3 receptor, was comparable with the binding and functional properties of the human NK_3 receptor described previously (Maggi *et al*., 1993; Sarau *et al*., 1997; Giardina *et al*., 1996; Suman-Chauhan *et al*., 1994; Oury Donat *et al*., 1995; Jordan *et al*., 1998). Whilst the affinity estimates from binding studies were in the range consistent with NK_3 receptors, they were sometimes lower than affinity estimates obtained from functional assays such as FLIPR (this study) and previous organ bath studies (Medhurst *et al*., 1997a,b), possibly reflecting amplification steps downstream from receptor binding or differences in methodology.

Agonist pD_2 values, and antagonist affinity estimates obtained for the cloned rabbit NK_3 receptor in the FLIPR (this study), were similar to those previously reported for the endogenous rabbit iris receptor (Table 2, Medhurst *et al*., 1997a,b). In addition, the affinity of antagonists such as SR 142801 for the rabbit receptor, as for the human receptor, were higher than those observed for the rat NK_3 receptor, as expected from the conservation of Met134 and Ala146 in the human and rabbit sequences (as discussed earlier). In general, the antagonist pharmacology of iris and brain clones were remarkably similar, apart from a small but significant ($P<0.05$, Student's unpaired *t*-test) increase in the affinity of SB 223412 for the brain receptor. pK_B values for SB 222200 and SB 218795 were significantly ($P<0.05$, Student's unpaired *t*-test) higher when calculated against senktide than against [MePhe^7]-NKB, an observation less dramatic but consistent with previous observations in the organ bath studies showing that relatively higher concentrations of antagonists were required to block [MePhe^7]-NKB-induced responses compared to those induced by senktide (Medhurst *et al*., 1997b).

Potential agonist-dependent differences in antagonist affinity were proposed as preliminary evidence for putative NK_3 receptor subtypes, although the effect was more dramatic in the organ bath experiments (Medhurst *et al*., 1997b). Another intriguing observation from the rabbit isolated iris sphincter studies was the observed biphasic nature of [MePhe^7]-NKB and NKB concentration response curves, reflected by shallow slopes of 0.45–0.76 (Medhurst *et al*., 1997a,b). By cloning and expressing the rabbit NK_3 receptor from the iris sphincter muscle we were able to reinvestigate this phenomenon in a recombinant receptor system. The present study conclusively shows that shallow concentration effect curves to NK_3 receptor agonists were still obtained with the cloned receptor, inconsistent with the existence of NK_3 receptor subtypes. Shallow agonist slopes (around 0.7) have also been observed with the human NK_3 receptor, both functionally (Jordan *et al*., 1998), and with radioligand binding studies (Rosenkilde *et al*., 1998). The latter studies of Rosenkilde *et al*. (1998) described the possible existence of two affinity states of the NK_3 receptor that are sensitive to modulation by zinc through a bis-His site in transmembrane domain V (Rosenkilde *et al*., 1998). Therefore, different affinity states may be an alternative explanation for the previous data implying the existence of putative NK_3 receptor subtypes (Medhurst *et al*., 1997b), a situation very similar to the NK_1 receptor where subtypes were often proposed but never identified at the molecular level (Maggi & Schwartz, 1997). Moreover, recent evidence suggests that a single antagonist can show different affinities at a single NK_1

receptor population, depending on the agonist with which it competes (Jenkinson *et al*., 1999). These shallow agonist concentration effect curves are also unlikely to be due to peptidase activity since peptidase inhibitors did not affect NKB responses (Jordan *et al*., 1998), and responses to [MePhe^7]-NKB were unaffected by various peptidase inhibitors in rabbit iris sphincter muscle (Hall *et al*., 1991; see also Medhurst *et al*., 1997b for further discussion).

We also investigated ocular tissue distribution of rabbit NK_3 receptors. Full length NK_3 receptor mRNA was detected with RT–PCR in iris sphincter muscle, iris dilator muscle and retina, but not in lens or cornea. The more quantitative technique of TaqMan RT–PCR revealed high levels of NK_3 receptor mRNA in iris sphincter, lower levels in iris dilator and retina, and barely detectable signals in lens and cornea. Since stimulation of ocular NK_3 receptors *in vivo* results in profound miosis *via* contractile NK_3 receptors on the iris sphincter muscle (Medhurst *et al*., 1997a), it is possible that the small amount of NK_3 receptor mRNA detected in the dilator muscle results from poor resolution between the two structures during dissection. Identification of NK_3 receptor mRNA in rabbit retina is consistent with previous observations in other species (Mantyr *et al*., 1989), although its significance at present is unknown.

Autoradiographical studies carried out previously on strips of rabbit iris sphincter muscle revealed specific high affinity NK_3 binding sites which were inhibited by [MePhe^7]-NKB, but not by the selective NK_1 receptor antagonist CP 99994 (Medhurst *et al*., 1997b). However, more precise localization was not reported. In the present study we have obtained the first evidence for discrete localization of NK_3 receptors to ciliary processes as well as the iris muscle in the whole eye. This discrete localization was confirmed at the mRNA level by our *in situ* hybridization results showing specific NK_3 receptor mRNA also discretely localized to ciliary processes.

The role of NK_3 receptors in the rabbit eye is unknown. Given the precise localization of these receptors to the ciliary processes, they may be involved in accommodation and aqueous humour secretion, as well as influencing intraocular pressure. Indeed, in addition to the profound miosis reported previously (Medhurst *et al*., 1997a), topical application of senktide also induces an immediate increase in intraocular pressure, an effect that can be blocked by intravenous, but not topical administration of the selective NK_3 receptor antagonist SB 222200 (personal communication, Professor Neville Osborne). In rabbits, the ciliary body is poorly developed resulting in negligible power of accommodation. Well developed ciliary processes are thought to help compensate for this and may play a role in ocular accommodation (Peiffer *et al*., 1994). Constriction and dilatation of microvessels in these folds of connective tissue may result in some accommodative power. The discrete localization of both NK_3 receptor mRNA and NK_3 receptors in the ciliary processes suggests that NK_3 receptors could play a role in accommodation in the rabbit, perhaps by affecting the microvasculature. In addition, these receptors 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).

In conclusion, the present study supports previous evidence that NK_3 receptors in the rabbit are similar to those in humans, both functionally (based on selective NK_3 receptor agonist and antagonist pharmacology) and at the molecular level. In addition, our data suggests that there may be two affinity states of the receptor which may correspond to the putative subtypes previously reported. Ocular NK_3 receptors in the rabbit provide a rare example where *in vitro* and *in vivo* pharmacological studies, mRNA and receptor localization, and cloning and functional expression of a receptor have all been achieved in the same physiological system.

The authors wish to thank Dr Richard Newton (University of Bristol) and Dr David Chambers (UMDS Guys) for assistance with preliminary RACE–PCR work, Jennie Ranson, Jenifer Powles and Shabina Nasir (SmithKline Beecham, Harlow) for help with cell culture, and Roseanna Muccitelli, SmithKline Beecham Pharmaceuticals, U.S.A. for advice on primers. Thanks also go to Dr Douglas Hay for reviewing the manuscript.

References

BOWEN, W.P. & JERMAN, J.C. (1995). Non-linear regression using spreadsheets. *Trends Pharmacol. Sci.,* **16,** 413–417.

BUELL, G., SCHULZ, M.F., ARKINSTALL, S.J., MAURY, K., MISSOTTEN, M., ADAMI, N., TALABOT, F. & KAWASHIMA, E. (1992). Molecular characterisation, expression and localisation of human neurokinin-3 receptors. *FEBS. Lett.,* **299,** 90–95.

CHENCHIK, A., DIACHENKO, L., MOQADAM, F., TARABYKIN, V., LUKYANOV, S. & SIEBERT, P.D. (1996). Full length cDNA cloning and determination of mRNA 5′ and 3′ ends by amplification of adaptor-ligated cDNA. *Biotechniques,* **21,** 526–534.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CHUNG, F.-Z., WU, L.-H., TIAN, Y., VARTANIAN, M.A., LEE, H., BIKKER, J., HUMBLET, C., PRITCHARD, M.C., RAPHY, J., SUMAN-CHAUHAN, N., HORWELL, D.C., LALWANI, N.D. & OXENDER, D.L. (1995). Two classes of structurally different antagonists display similar species preference for the human tachykinin Neurokinin$_3$ receptor. *Mol. Pharmacol.,* **48,** 712–716.

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.

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.

GETHER, U., JOHANSEN, T.E. & SCHWARTZ, T.W. (1993). Chimeric NK_1 (Substance P)/NK_3 (Neurokinin B) receptors – identification of domains determining the binding specificity of tachykinin agonists. *J. Biol. Chem.,* **268,** 7893–7898.

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., VASSALO, M., VECCHIETI, 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.

GUBLER, U. & HOFFMANN, B.J. (1983). A simple and very efficient method for generating complimentary DNA libraries. *Gene,* **25,** 263–269.

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.

HUANG, R.C., CHEUNG, A.H., MAZINA, K.E., STRADER, C.D. & FONG, T.M. (1992). cDNA sequence and heterologous expression of the human neurokinin-3 receptor. *Biochem. Biophys. Res. Commun.,* **184,** 966–972.

JENKINSON, K.M., SOUTHWELL, B.R. & FURNESS, J.B. (1999). Two affinities for a single antagonist at the neuronal NK_1 tachykinin receptor: evidence from quantitation of receptor endocytosis. *Br. J. Pharmacol.,* **126,** 131–136.

JORDAN, R.E., SMART, D., GRIMSON, P., SUMAN-CHAUHAN, N. & MCKNIGHT, A.T. (1998). Activation of the cloned human NK3 receptor in Chinese Hamster Ovary cells characterised by the cellular acidification response using the Cytosensor microphysiometer. *Br. J. Pharmacol.,* **125,** 761–766.

KRAUSE, J.E., STRAVETEIG, P.T., NAVE MENTZER, J., SCHMIDT, S.K., TUCKER, J.B., BRODBECK, R.M., BU, J.-Y. & KARPITSKIY, V.V. (1997). Functional expression of a novel human neurokinin-3 receptor homolog that binds [^{3}H]senktide and [^{125}I-MePhe^7]-neurokinin B, and is responsive to tachykinin peptide agonists. *Proc. Natl. Acad. Sci.,* **94,** 310–315.

LIE, Y.S. & PETROPOULOS, C.J. (1998). Advances in quantitative PCR technology: 5′ nuclease assays. *Curr. Opin. Biotech.,* **9,** 43–48.

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.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & GIAHETTI, A. (1993). Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.,* **13,** 23–93.

MAGGI, C.A. & SCHWARTZ, T.W. (1997). The dual nature of the tachykinin NK1 receptor. *Trends Pharmacol. Sci.,* **18,** 351–355.

MANTYR, P.W., GATES, T., MANTYR, C.R. & MAGGIO, J.E. (1989). Autoradiographic localisation and characterisation of tachykinin receptor binding sites in the rat brain and peripheral tissues. *J. Neurosci.,* **9,** 258–279.

MEDHURST, A.D., HAY, D.W.P., PARSONS, A.A., MARTIN, L.D. & GRISWOLD, D.E. (1997a). *In vitro* and *in vivo* characterisation of NK_3 receptors in the rabbit eye using selective non-peptide NK_3 receptor antagonists. *Br. J. Pharmacol.,* **122,** 469–476.

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

NAJLERAHIM, A., HARRISON, P.J., BARTON, A.J.L., HEFFERNAN, J. & PEARSON, R.C. (1990). Distribution of messenger RNAs encoding the enzymes glutaminase, aspartate aminotransferase and glutamic acid decarboxylase in rat brain. *Mol. Brain Res.,* **7,** 317–333.

NGUYEN, Q.T., JUKIC, D., CHRETIEN, L., GOBEIL, F., BOUSSOUGOLU, M. & REGOLI, D. (1994). Two NK-3 receptor subtypes: demonstration by biological and binding assays. *Neuropeptides,* **27,** 157–161.

OURY-DONAT, F., CARAYON, P., THURNEYSSEN, O., PAILHON, V., EMONDS-ALT, X., SOUBRIE, P. & LE FUR, G. (1995). Functional characterisation of the nonpeptide neurokinin3 (NK_3) receptor antagonist, SR 142801 on the human NK_3 receptor expressed in Chinese hamster ovary cells. *J. Pharmacol. Exp. Ther.,* **274,** 148–154.

PATACCHINI, R., BARTHO, L., HOLZER, P. & MAGGI, C.A. (1995). Activity of SR 142801 at peripheral tachykinin receptors. *Eur. J. Pharmacol.,* **278,** 17–25.

PEIFFER Jr, R.L., POHM-THORSEN, L. & CORCORAN, K. (1994). Models in ophthalmology and vision research. In: Manning, P.J., Ringler, D.H. & Newcomer, C.E. (eds). *The biology of the laboratory rabbit* Academic Press Inc., pp. 409–431.

ROSENKILDE, M.M., LUCIBELLO, M., HOLST, B. & SCHWARTZ, T.W. (1998). Natural agonist enhancing bis-His zinc-site in transmembrane segment V of the tachykinin NK_3 receptor. *FEBS Lett.,* **439,** 35–40.

SARAU, H.M., GRISWOLD, D.E., POTTS, W., FOLEY, J.J., SCHMIDT, D.B., WEBB, E.F., 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. Pharm. Exp. Ther.,* **281,** 1303–1311.

SCHROEDER, K.S. & NEAGLE, B.D. (1996). FLIPR: a new instrument for accurate, high throughput optical screening. *J. Biomol. Scr.,* **1,** 75–80.

SHIGEMOTO, R., YOKOTA, Y., TSUCHIDA, K. & NAKANISHI, S. (1990). Cloning and expression of a rat neuromedin K receptor cDNA. *J. Biol. Chem.,* **265,** 623–628.

SUMAN-CHAUHAN, N., GRIMSON, P., GUARD, S., MADDEN, Z., CHUNG, F-Z, WATLING, K., PINNOCK, R. & WOODRUFF, G. (1994). Characterisation of [^{125}I][MePhe^7]neurokinin B binding to tachykinin NK_3 receptors: evidence for interspecies variance. *Eur. J. Pharmacol.,* **269,** 65–72.

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

WU, L-H., VARTANIAN, M.A., OXENDER, D.L. & CHUNG, F-Z. (1994). Identification of methionine134 and alanine146 in the second transmembrane segment of the human tachykinin NK_3 receptor as residues involved in species-selective binding to SR 48968. *Biochem. Biophys. Res. Commun.,* **198,** 961–966.

YOKOTA, Y., AKAZAWA, C., OHKUBO, H. & NAKANISHI, S. (1992). Delineation of structural domains involved in the subtype specificity of tachykinin receptors through chimeric formation of substance P/substance K receptors. *EMBO J.,* **11,** 3585–3591.

(Received May 21, 1999
Revised July 16, 1999
Accepted July 28, 1999)