RESEARCH COMMUNICATION

Functional characterization by heterologous expression of a novel cloned tachykinin peptide receptor

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An orphan receptor resembling the neurokinin 3 tachykinin receptor (NK3), initially claimed to be an atypical opioid receptor, is shown herein to respond potently to the physiological NK3 receptor ligand, neurokinin B. This 'NK4' receptor did not give functional responses in *Xenopus* oocytes to opioid agonists. However, NK4 receptor activation was inhibited by nanomolar concentrations of dynorphin. The NK4 receptor is therefore a tachykinin receptor which is functionally antagonized by an endogenous opioid peptide.

INTRODUCTION

The three mammalian tachykinin receptors, termed NK1 (or 'SPR'), NK2 (or 'SKR') and NK3 (or 'NKR') are pharmacologically matched to a family of three distinct cognate ligands: Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) [1]. However, there is the potential for physiological 'cross-talk' at the three known receptors, complicating the analysis of the minimum number of distinct physiological tachykinin receptors. Thus new tachykinin receptors have been suggested [2], but have not been identified by molecular cloning.

Recently Xie and colleagues [3] cloned a novel human orphan receptor (termed 'hK1R'), which was described as an atypical kappa opioid receptor based exclusively on ligand binding. Specifically, the approach was the use of an immobilized dynorphin analogue in a panning method to select cells expressing kappa-opioid-binding sites. In the subsequent characterization of radioligand binding, hK1R was reported to have an atypical pharmacology for opioid drugs; for example, it exhibited little binding selectivity between kappa and mu ligands. Surprisingly, this receptor exhibited remarkable sequence similarity to that of both the rat and human NK3 receptors (80 % at the amino acid level; see Figure 1), but was reported as incapable of binding [^{3}H]eledoisin, a selective NK3 radioligand probe. We therefore have hypothesized that hK1R might encode a novel functional tachykinin receptor, but with an unusual pharmacology responsive to opioid ligands. The results presented here support this hypothesis, and we have renamed the receptor 'neurokinin receptor 4' ('NK4') to reflect this functional assignment.

EXPERIMENTAL

Materials

All reagents were obtained from Sigma unless otherwise noted. Peptides were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), and non-peptide opioid agonists from RBI (Natick, MA, U.S.A.). Media and supplements were supplied through GIBCO–BRL (Gaithersburg, MD, U.S.A.). The human Northern blot was purchased from Clontech (Palo Alto, CA, U.S.A.). All reagents used in Northern-blot analysis were of the highest molecular-biology grade. Restriction enzymes were from NEB (Beverly, MA, U.S.A.), and *Taq* polymerase was from Pharmacia (Alameda, CA, U.S.A.). Plasmids pRc/CMV and pcDNA3 were obtained from Invitrogen (San Diego, CA, U.S.A.). The hK1R cDNA was a gift of Dr. A. Miyajima (DNAX Research Institute, Palo Alto, CA, U.S.A.).

Expression-vector construction

The NK4 open reading frame was subcloned from the full-length insert (4.4 kb *Hin*dIII restriction fragment; see [3]) into pRc/CMV (pRc/CMV.NK4) for initial receptor characterization of potencies of tachykinins. The open reading frames of the rat NK3 receptor and NK4 (hK1R) were also amplified by PCR from longer cDNA templates from the original cDNA clones. There were no detectable differences between responses elicited by either full-length cDNAs, subcloned restriction fragments containing the open reading frames or PCR-generated open reading frames lacking untranslated regions.

PCR constructions were generated as follows: 10 ng of vector DNA as template, 1.5 mM $MgCl_2$, 200 mM each of the dinucleotides and 100 pmol of each of the primers. The upstream primers used for each reaction encompassed the start codon and contained a Kozak sequence.

The downstream primers also encompassed the stop codons. The primers used had the following sequences: NK3 upstream primer: 5′-AACTCAAGCTTCGCCACC-ATGGCCTCAGTCCCCAGGGGCGAAA-3′; downstream primer: 5′-TTTTGAATTCTCTAGATTAGGAATATTCATC-CACAGA-3′; NK4 upstream primer: 5′-ACTCAAGCTTG-GCCACCATGGCCTCGCCCG-3′; downstream primer: 5′-T-GACGAATTCTCTAGATCAAGAGCCTTCCACCGACAT-3′. Coding sequences within the primers are underlined. 10 % (v/v) DMSO was used in the reaction for NK4 amplification owing to the high GC content in the NK4 upstream primer. 2.5 units of *Taq* polymerase was used for NK3 amplification, and this amount was doubled in NK4 reactions, since DMSO inhibits *Taq* polymerase [14]. PCR conditions were as follows: (1) for NK3: denaturation temperature, 95 °C; annealing temp-

Abbreviations used: NK1/SPR, Substance P receptor; NK2/SKR, Substance K/neurokinin A receptor; NK3/NKR, neuromedin K/neurokinin B receptor; NK4, neurokinin receptor 4; NKA, neurokinin A; NKB, neurokinin B; OR2, oocyte Ringer; poly(A)$^+$, polyadenylated RNA.

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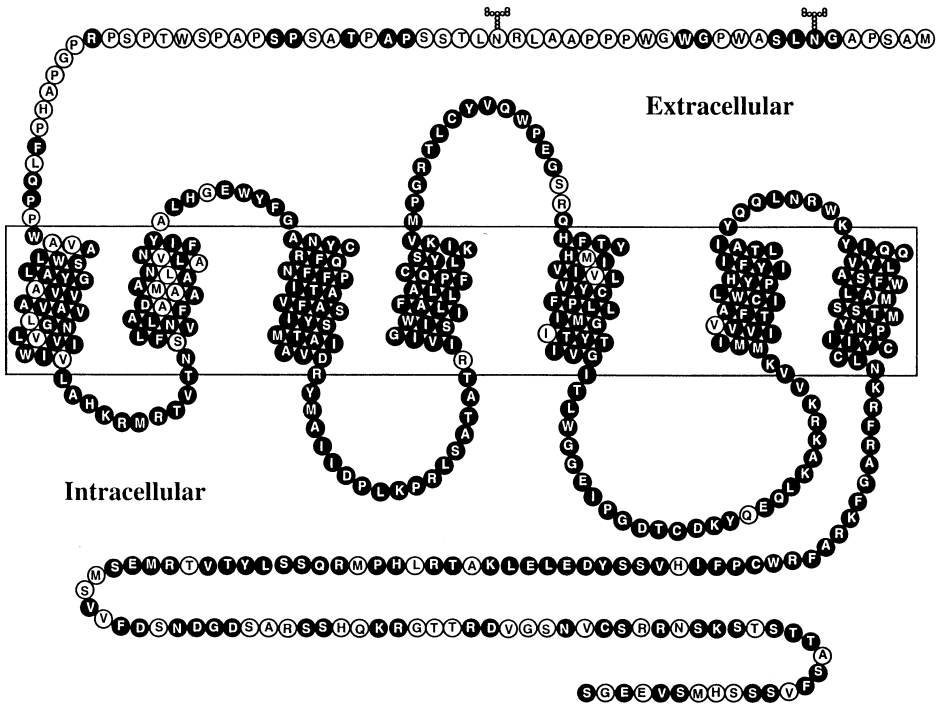

Figure 1 Amino acid alignment of NK4 and NK3 receptors

The sequence of the NK4 receptor is shown, with dark circles indicating identity with the rat NK3 receptor. Sites of potential N-linked glycosylation are represented by the smaller circle structures. Sequence alignment inserted two gaps, both in the N-terminal extracellular domain. Alignment was performed with the 'gap' algorithm on the Genetics Computer Group software (Madison, WI, U.S.A.), and the default settings were as follows: gapweight, 5.0; gaplengthweight, 0.05.

erature, 50 °C; extension temperature, 72 °C for 30 cycles; (2) for NK4: denaturation temperature, 95 °C; annealing temperature, 60 °C; extension temperature, 72 °C for 30 cycles. Following PCR amplification, the products were gel-purified and then subjected to cleavage with appropriate restriction endonucleases (*Hin*dIII and *Xba*I for NK3; *Hin*dIII and *Eco*RI for NK4) with subsequent directional insertion into the previously restricted vector. NK3-coding DNA was inserted into pRc/CMV (pRc/CMV.NK3) and NK4 into pcDNA3 (pcDNA3.NK4). The vectors are essentially identical except for the restriction sites in the multiple cloning site.

Electrophysiological recording of *Xenopus laevis* oocytes

Xenopus laevis oocytes were obtained by ovarectomy, and were prepared for DNA injection as previously described [4]. Following nuclear injection of plasmid DNA and incubation in modified L15 (3 days) to allow expression of receptors, oocytes were analysed with a two-electrode voltage clamp [5] under continuous perfusion of oocyte Ringer (OR2). To stimulate the expressed receptor, flow was shifted to another source reservoir, containing OR2 with tachykinin or opioid agonist (15 s). Oocytes showing no response to application of agonist other than NKB were tested with NKB (1 μM) to verify functional expression of the receptor. Desensitization was assessed by restimulation of the same oocyte with NKB after a 10 min incubation under constant OR2 perfusion. Control oocytes exhibited no responses to tachykinin or opioid peptides.

For antagonism studies, all oocytes were initially screened for receptor expression with NKB application (10–30 nM). Oocytes expressing NK3/4 receptors were allowed to recover in L15 (at least 2–3 h at 18 °C) before co-perfusion with antagonist and NKB. Oocytes were perfused with putative antagonists (30 s) prior to the second addition of NKB. For kappa-agonist experiments, both acute co-perfusion with high dose (5 μM) and long exposure to low dose (2–3 h) kappa agonist were tested. Oocytes were re-clamped after recovery and again tested with the same concentration of NKB. Each experiment included control oocytes which were stimulated twice with NKB, with no other treatment. For elucidation of the nature of the dynorphin antagonism at the NK4 receptor, an additional experiment was performed using the same experimental outline as above, but using a higher concentration of NKB for the second stimulation, to determine whether the inhibition of the second response by dynorphin could be overcome. All antagonism data are paired responses in the same oocyte and are analysed using paired Student's *t* tests.

Northern-blot analysis

A single-stranded NK4 DNA probe was internally labelled by means of a Perkin–Elmer thermocycler, the downstream primer and [α-^{32}P]dCTP, under the same cycling conditions as in the legend to Figure 2 (below). A human polyadenylated-RNA [poly(A)$^+$] Northern blot was hybridized with the probe in hybridization buffer [5 × SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 5×10^{-3} M EDTA), 10 × Denhardt's solution (0.2 % Ficoll, 0.2 % polyvinylpyrrolidone, 0.2 % BSA), 2 % (w/v) SDS and 100 μg/ml salmon sperm DNA] at 65 °C overnight, and then washed to a maximum stringency of 0.1 × SSC/0.1 % SDS at 70 °C (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). The blot was exposed to X-ray film (Reflection™; NEN Research Products) for 6 days.

Expression of NK4 receptor in NIH3T3 cells and functional assays: NIH3T3 clonal cell lines

Stable clones of pRc/CMV.NK4 were generated using NIH3T3 rat fibroblasts. Cells, split 1:15 the day before, were transfected with plasmid DNA (10 μg) using the calcium phosphate protocol. Stable transfectants were selected in the presence of 400 μg/ml G418 (Life Technologies, Grand Island, NY, U.S.A.), and clones were generated using limiting dilution. Clonal lines expressing NK4 receptor were identified by functional responses to NKB, as measured by an increase in calcium. Mock-transfected NIH3T3 cells exhibited no responses to tachykinin or opioid peptides. Clonal cells lines expressed NK4 receptor mRNA as confirmed by Northern-blot analysis (results not shown).

Measurement of cytosolic [Ca^{2+}] by intracellular loading of Fura-2

Cells were assayed for functional response to NKB with the use of the calcium-sensitive fluorescent dye Fura-2 (Molecular Probes, Eugene, OR, U.S.A.). Cells were rinsed twice in Dulbecco's modified Eagle's medium media and then loaded with 33 μg/ml Fura-2 acetoxymethyl ester in Dulbecco's modified Eagle's medium for 20 min. Cells were then washed twice in the assay buffer (Hanks Balanced Salt Solution, 10 mM Hepes, pH 7.4, and 100 units/ml penicillin and streptomycin). Measurement of population responses to changes in [Ca^{2+}] were performed in a Hitachi F2000 instrument (Hitachi, San Jose, CA, U.S.A.). During the collection time, the cell solution was maintained at a temperature of 37 °C, and the solution was continuously stirred with a magnetic 'flea' stir bar. Ligand was added with a Hamilton syringe at 1 μM final concentration through an injection port. Methods were as described previously [6]. In brief, maximum and minimum [Ca^{2+}] values were calibrated by the addition of saturating ionomycin and EGTA respectively. Cells were excited at wavelengths of 340 nm and 380 nm, and emission was measured at 510 nm. Results are given as a change in ratio (340/380) of the two wavelengths. Fluorescent ratio measurements were not calibrated for [Ca^{2+}].

RESULTS

Neurokinin B (10–300 nM) consistently elicited functional current responses in oocytes expressing the NK4 receptor (Figure 2A). Comparison of the dose–response curves to NKB at the NK3 and NK4 receptors showed similar responses (Figure 2A), with EC_{50} values estimated to be 40 nM (NK3) and 10 nM (NK4). Comparison of NK3 and NK4 receptors using a variety of tachykinin peptides and tachykinin analogues showed similar patterns of agonist responses, with the exception that NK3 was unresponsive to Substance P (Table 1). Both NK3 and NK4 receptors showed similar desensitization responses to a second application of NKB (e.g. NK3, $60 \pm 4\%$; NK4, $69 \pm 3\%$ of the first response to 10 nM NKB). The results for the NK3 receptor are comparable with those in previous reports on the desensitization of the NK3 receptor [7].

The NK3-selective receptor antagonist [Tyr^7,β-Ala^8]NKA-(4–10)-peptide (1 μM) significantly reduced the responses of oocytes expressing NK3 receptors, but not NK4 . Values for first and second applications of NKB at NK3 and NK4 receptors were: NK3: first application of NKB (10 nM), 3200 ± 102 nA; second application of NKB + [Tyr^7,β-Ala^8]NKA-(4–10)-peptide, 1880 ± 193 nA ($P = 0.01$); NK4: first application, 2420 ± 534 nA; second application + [Tyr^7,β-Ala^8]NKA-(4–10)-peptide, 2890 ± 433 nA ($n = 5$ per group).

To rule out the possiblity that these results were unique to functional expression in oocytes, stable transfectant NIH3T3 clones expressing the NK4 receptor were also tested. They were stimulated by NKB, giving robust calcium responses at doses of 10 nM (Figure 2C).

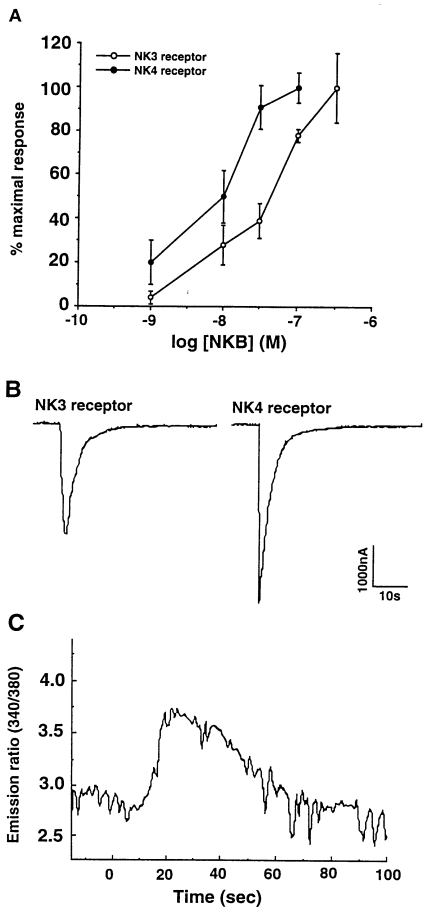

Figure 2 Functional responses of the NK4 receptor to NKB application

(**A**) Dose–response curves to bath application of NKB (1–300 nM) at the NK3 and NK4 receptors measured as Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes ectopically expressing either receptor. Results are means $\pm$ S.E.M. ($n = 3$ per point). (**B**) Representative voltage-clamp traces for the NK3 and NK4 receptors in response to 10 nM NKB. (**C**) Clonally selected NIH3T3 fibroblasts were assayed for functional response to NKB with the fluorescent Ca^{2+}-sensitive ratio dye fura-2. Shown is a representative trace of cells on coverslips in response to 10 nM NKB. The trace shown is one representative experiment ($n = 10$). Results shown in (**A**) and (**B**) were obtained with both pRc/CMV.NK4 (full-length cDNA) and pcDNA3.NK4 (open reading frame only).

As the NK4 (hK1R) receptor was initially isolated and subsequently characterized as a receptor with affinity for kappa-opioid agonists, we examined several possibilities for kappa-opioid interactions. First, we tested for direct effects. Four compounds [bremazocine, dynorphin A, Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol ('DAGO') and U-50488] used in the original characterization of the receptor were tested for current responses in *Xenopus* oocytes. No opioid agonist (1 μM concentration) tested elicited any detectable Ca^{2+}-dependent Cl^- current in oocytes expressing the NK4 receptor (results not shown).

Simultaneous co-perfusion of dynorphin A (5 μM) with NKB did not significantly alter responses in oocytes expressing either the NK3 or NK4 receptor. However, we hypothesized that dynorphin peptides might act as functional antagonists with relatively slow kinetics. Thus, following pre-treatment of oocytes (2–3 h) with low concentrations of dynorphin A, a substantial and reproducible decrease ($> 70\%$) in the response to NKB was

Table 1 Action of tachykinin agonists on *Xenopus* oocytes expressing NK3 and NK4 receptors

Oocytes expressing NK3 and NK4 receptors were voltage-clamped, and Ca^{2+}-dependent Cl^- currents evoked by bath application of 1 μM of each agonist were recorded. Values given are means ± S.E.M. (*n*) in nA. Abbreviations used: SP, Substance P; NKA, neurokinin A; NKB, neurokinin B; diMe-C7 {[Glu^5,MePhe^8,Sar^9]Substance P-(5–11)-peptide, where Glu is pyroglutamic acid and Sar is sarcosine}. The agonist responses are not significantly different (Student's *t*-test) with the exception of SP.

	Response (nA)	
Agonist	NK3 receptor	NK4 receptor
SP	0 (4)	537 ± 309 (4)
NKA	1036 ± 663 (4)	1854 ± 856 (4)
NKB	2176 ± 355 (4)	2120 ± 583 (4)
Eledoisin	1865 ± 415 (4)	2809 ± 659 (4)
Hylambatin	0 (4)	0 (4)
diMe-C7	1950 ± 269 (4)	2923 ± 528 (4)

observed (Table 2 and Figure 3). This effect was seen with concentrations of dynorphin in the range 0.1–10 nM, but not with lower concentrations, when responses were not significantly different from controls (Figure 3). This inhibition of the second response to NKB was not seen with prolonged exposure to a non-peptide kappa agonist, bremazocine (10 nM) (Table 2). Significantly, this functional antagonism by dynorphin at the NK4 receptor was not seen at the NK3 receptor. In order to assess whether the dynorphin-induced block could be overcome with higher NKB concentrations, oocytes were stimulated with a supramaximal dose of NKB (1 μM). The dynorphin inhibition of NKB responses was not overcome by a saturating concentration of NKB, suggesting a non-competitive mechanism (Figure 3, ●).

To examine whether the NK4 receptor was physiologically expressed, Northern-blot analysis of human poly(A)$^+$ RNA from various tissues was undertaken. In all cases, positive tissues showed a single band of 4.4 kb. Highest levels were observed in skeletal muscle, liver, lung and heart, with a lower level of expression in pancreas. However, absolute levels of expression appeared to be low, as long exposure times were required (Figure 4). These results are unlike the pattern reported for the NK3 receptor, in that the NK3 receptor is absent from several NK4-expressing tissues, such as skeletal muscle and liver [8]. Thus NK4 is not a pseudogene and is widely expressed in human tissues with a distribution distinct from identified tachykinin receptors.

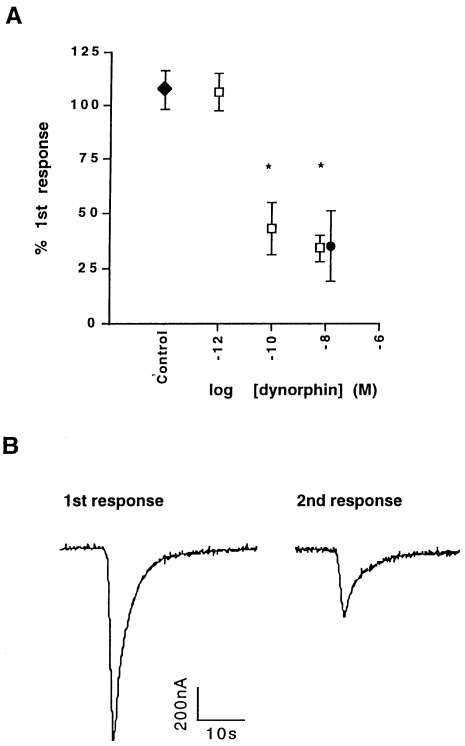

Figure 3 Antagonism of NKB-evoked current responses at the NK4 receptor by dynorphin A

(**A**) Dose–response curve for dynorphin A antagonism at the NK4 receptor. Oocytes were stimulated with 30 nM NKB, incubated in L-15 (control: ◆) or L-15 containing dynorphin A for at least 3 h (□). Oocytes were re-clamped and again stimulated with 30 nM NKB. Data are second responses expressed as a percentage of the first response elicited by NKB. In an additional experiment, oocytes were initially stimulated with 30 nM NKB, incubated in 10 nM dynorphin and stimulated a second time with 1 μM NKB (●), in an attempt to overcome the dynorphin antagonism. Statistical analysis was performed by comparison of first and second responses using a paired Students *t* test. $^*P < 0.05$ (the number of oocytes used for each data point is 4, except for the 10 nM point where $n = 24$). (**B**) Voltage-clamp traces of responses to 30 nM NKB from an individual oocyte before (first response) and after (second response) prolonged treatment with dynorphin (10 nM).

DISCUSSION

On the basis of the sequence similarity to the NK3 receptor, the NK4 receptor is herein hypothesized to represent a novel tachykinin receptor, and not, as originally claimed, an atypical opioid receptor. This hypothesis is further reinforced by the

Table 2 Antagonism of NKB-evoked current responses at the NK4 receptor by dynorphin A

Responses of *Xenopus* oocytes expressing NK4 receptors, to sequential application of NKB (30 nM). Oocytes were first stimulated with 30 nM NKB then allowed to recover in L-15 medium with or without 10 nM kappa opioid agonist as described in the text. Statistical analysis of data was performed using paired Student's *t* test, comparing first and second responses to NKB. $^{**}P = 0.02$. Data are means ± S.E.M. (*n*) of one representative experiment (five experiments were performed)

Treatment	None	10 nM Dynorphin exposure	10 nM Bremazocine exposure
NKB response before treatment (nA)	2227 ± 634 (5)	2301 ± 1056 (5)	2122 ± 581 (5)
NKB response after treatment (nA)	1922 ± 672 (5)	549 ± 93 (5)**	2260 ± 1186 (5)
NK3 receptor responses to NKB after treatment (nA)	2040 ± 380 (5)	1423 ± 259 (5)	–

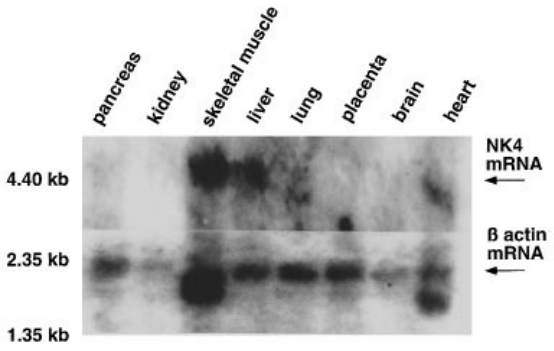

Figure 4 Expression of NK4 mRNA in human tissues

A human Northern blot was probed with a single-stranded internally labelled DNA probe complementary to NK4 mRNA and was exposed to film for 6 days. The probe hybridized to a transcript of length 4.4 kb, in pancreas, skeletal muscle, liver, lung and heart. The signal generated by a β-actin cDNA probe is also shown.

cloning of a family of opioid receptors, including at least one identified kappa subtype, because all members of the opioid peptide receptor family have proved to be highly divergent from NK4 [9]. It is important to emphasize that a functional evaluation of NK4 has not been reported previously.

Herein, we have shown by ectopic expression in both *Xenopus* oocytes and mammalian cells that the NK4 receptor responds to the tachykinin NKB at concentrations similar to those needed to activate the NK3 receptor. The estimated EC_{50} values for NKB are 10 nM and 40 nM at the NK4 and NK3 receptors respectively, suggesting that NKB is unexpectedly *more* potent at this novel receptor. Overall, the NK4 receptor exhibited a pharmacological profile very like that of the NK3 receptor, but with some important differences, such as insensitivity to an NK3-selective antagonist. Thus it remains an important issue as to whether this receptor should be considered an NK3 receptor subtype or a distinct tachykinin receptor. However, we cannot explain why previous studies did not detect tachykinin binding to this receptor, although it is possible that the unusual choice of radioligand (e.g. [^{3}H]eledoisin) may have led to unacceptable background levels of non-specific binding.

As we were unable to elicit agonist responses to opioids in NK4-expressing oocytes, we concluded that the NK4 receptor is not an authentic kappa-opioid receptor. However, its apparent ability to recognize dynorphin-related peptides remained unexplained. In this regard our most interesting finding was that exposure of oocytes expressing the NK4, but not the NK3, receptor to a low concentration of an endogenous opioid peptide, dynorphin A, resulted in a graded (30–90 %) reduction in responses to NKB. This result suggests that dynorphin A may act as a functional antagonist, rather than as an agonist, on the expressed NK4 receptor. Thus it is not surprising that there do not appear to be any agonist domains in the NK4 receptor similar to the reported kappa-opioid-binding site [10]. The dynorphin antagonism appears to be specific to peptides, as it was not seen with the non-peptide agonist bremazocine. However, on the basis of previously obtained results [3], bremazocine may nevertheless bind to the NK4 receptor, albeit non-productively.

In this regard there has been a long-standing and intriguing suggestion of a direct interaction of opiates with a tachykinin receptor in spinal cord [11]. This will be an important topic for further study. However, there is an important precedent for functional antagonism at a G-protein-coupled receptor by a related endogenous peptide, in that a parathyroid-hormone-related peptide blocked the action of parathyroid hormone on its cognate receptor in cardiomyocytes [12].

Northern analysis of NK4 tissue distribution in human tissues confirmed that NK4 was an expressed gene and not a pseudogene or cloning artefact. The distribution of NK4 mRNA expression in human tissues showed important differences from the reported distribution of NK3 transcripts [8]. NK4 mRNA levels were highest in liver and lung, but NK4 mRNA was undetectable in brain, the site of highest expression of NK3 mRNA. However, calibrated levels of loaded RNA appeared very low for brain, so this issue will have to be systematically addressed for different brain regions. In this regard, although radioligand binding initially failed to identify NK3 receptors in human brain [13], human NK3 mRNA has subsequently been demonstrated throughout the brain. Thus demonstration of NK4 mRNA in brain may require more sensitive methods, such as reverse transcription PCR, that were required for the demonstration of human NK3 mRNA in brain. Similar concerns are also applicable to the apparent absence of the transcript from placenta.

In summary, we have used functional responses in heterologous cells to identify the hK1R orphan receptor as a novel member of the tachykinin peptide receptor family. It is unclear whether the tachykinin NKB is its physiological agonist, and this result may motivate the search for new mammalian tachykinins.

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