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Pharmacological evaluation of α and β human tachykinin NK_2 receptor splice variants expressed in CHO cells

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Abstract

In the present study, we have investigated, by binding and functional experiments, the pharmacological profile of a new human tachykinin NK_2 receptor splice variant named β isoform. Neurokinin A, nepadutant, SR48968 [(*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl) butyl]benzamide] and substance P have been tested for binding on the receptor expressed in whole CHO transfected cells. Only SR48968 binds, but with an affinity about sixfold lower in respect to the α isoform. Moreover, neurokinin A was unable to inhibit the [^{3}H]SR48968 binding to the β isoform up to μM concentrations. In cells expressing the human tachykinin NK_2 receptor β isoform, contrary to those expressing the α isoform, natural or selective tachykinin receptor agonists (1 μM) were unable to produce a significant activation of inositol phosphate (IP) production or increase of intracellular calcium concentration $[Ca^{2+}]_i$. The recently discovered tachykinins, endokinins C and D, did not activate IP production or $[Ca^{2+}]_i$ increase in cells expressing the α or β isoform of the human tachykinin NK_2 receptor. The present data indicate that the human tachykinin NK_2 receptor β isoform is poorly or not expressed on the cell membrane surface and that it may possibly act as a regulator of tachykinin NK_2 receptor function. We cannot exclude the possibility that this receptor could interact with other presently unknown ligands.

Keywords: Tachykinin receptor NK_2 α and β isoform; RNA splicing; Tachykinin; CHO cell

1. Introduction

Tachykinins are a family of closely related peptides whose best known members in mammals are substance P, neurokinin A and neurokinin B. Substance P and neurokinin A are encoded by the preprotachykinin-A gene (Nawa et al., 1984; Carter and Krause, 1990). Transcription of this gene generates a pre-mRNA that could be spliced into four different mRNA isoforms (α, β, γ and δ). Substance P is encoded by all four isoforms whereas the neurokinin A sequence is present in β and γ preprotachykinin-A mRNAs (Nawa et al., 1984; Kawaguchi et al., 1986; Krause et al., 1987; Harmar et al., 1990). Neurokinin B is the only tachykinin derived from the preprotachykinin-B gene (Kotani et al., 1986; Page et al., 2000). A new tachykinin named hemokinin-1 encoded by a third preprotachykinin gene, preprotachykinin-C, has recently been identified (Zhang et al., 2000; Kurtz et al., 2002). Moreover, a recent study by Page et al. (2003) has shown that transcription of the human preprotachykinin-C gene can also generate four distinct mRNAs (α, β, γ and δ) as a result of the alternate

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splicing of the preprotachykinin-C primary transcript. This gives rise to four different peptides which have been named endokinin A, endokinin B, endokinin C and endokinin D.

For many years, tachykinins have been considered almost exclusively as peptides of neuronal origin. In particular, neurokinin B has been considered to be present in the central nervous system (Moussaoui et al., 1992; Goubillon et al., 2000; Patacchini and Maggi, 2001) whereas substance P and neurokinin A were found both in the central enteric and peripheral nervous systems, including capsaicin-sensitive primary afferent neurons (Holzer, 1988; Lundberg, 1996; Patak et al., 2000; Patacchini and Maggi, 2001). From this latter neuronal source, substance P and neurokinin A are released from nerve endings at both the spinal cord and the peripheral level and play a role as excitatory neurotransmitters (Lembeck and Holzer, 1979; Otsuka and Yoshioka, 1993; Lecci and Maggi, 2003). Nevertheless, the consideration of tachykinins exclusively as neuropeptides is being challenged because substance P, neurokinin A and neurokinin B are also present in nonneuronal cells and in noninnervated tissues (Chiwakata et al., 1991; Khan and Collins, 1994; Ho et al., 1997; Page et al., 2000; Maghni et al., 2003; Pintado et al., 2003). Moreover, the recently identified mammalian tachykinins hemokinin-1, endokinin A, endokinin B, endokinin C and endokinin D are primarily expressed in nonneuronal cells, suggesting a widespread distribution and important role for these peptides as intercellular signalling molecules (see Zhang et al., 2000; Kurtz et al., 2002; Page et al., 2003; Patacchini et al., 2004 for review).

Tachykinins interact with specific membrane receptors belonging to the family of G protein-coupled receptors (GPCRs; Gerard et al., 1993; Krause et al., 1994). This family includes a large number of membrane proteins which share the same structural motif, a bundle of seven hydrophobic transmembrane domains (TM 1–7) with three extracellular loops (EC 1–3), three intracellular loops (IC 1–3), an extracellular amino-terminus and a cytoplasmic carboxy-terminus (Krause et al., 1994). Until presently, three distinct tachykinin receptors, NK_1, NK_2 and NK_3, have been cloned in different species including humans (Gerard et al., 1990, 1991; Takeda et al., 1991; Takahashi et al., 1992; Buell et al., 1992).

Majority of human genes is characterized by the presence of introns in their genomic structures. Conversely, and for still unknown reasons, most genes coding for proteins of the GPCR family are intronless. In this context, the tachykinin receptors constitute an exception as the genes encoding the tachykinin NK_1, NK_2 and NK_3 receptors have a similar structural organization and contain five exons, with introns interrupting the protein-coding sequence in identical positions (Gerard et al., 1993; Krause et al., 1994). The presence of exons permits an expansion of the tachykinin receptor family and suggests a functional role for at least some of the splicing isoforms of these receptors as each of them could show a distinct conformation and affinity for the different endogenous ligands. Several studies have indicated the existence of isoforms of the tachykinin NK_1 receptor, differing in the length of the C-terminal tail (Fong et al., 1992; Baker et al., 2003). In addition, a splice variant of the tachykinin NK_2 receptor has been recently identified (Candenas et al., 2002). The aim of the present study was to investigate the pharmacological profile of this splice variant of the human tachykinin NK_2 receptor named β isoform, which is found in different human and rat tissues that also express the wild-type tachykinin hNK_2 α isoform (Candenas et al., 2002). For this purpose, binding experiments with the radioligands [^{125}I]neurokinin A (tachykinin NK_2 receptor agonist), [^{3}H]nepadutant (Renzetti et al., 1998) and [^{3}H]SR48968 (Emonds-Alt et al., 1993; tachykinin NK_2 receptor antagonists) and [^{3}H]substance P (tachykinin NK_1 receptor agonist) were performed on whole cells and membrane preparations of dihydrofolate reductase deficient ($dhfr^-$) CHO DUKX-B11 cells (referred to as CHO $dhfr^-$ cells) transfected with either the hNK_2 receptor β or α isoform. Moreover, we also tested the activity of a subset of agonist ligands on the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and the production of inositol phosphates (IP) on whole CHO cells expressing the human tachykinin NK_2 receptor β or α isoform.

2. Materials and methods

2.1. Receptor expression in CHO cells

The cDNAs for the full-length human tachykinin NK_2 receptor and its splice variant, named NK_2α (clone #H24) and NK_2β (clone #L64), respectively, were cloned in pEF6/V5–His–TOPO (Invitrogen). The clones were excised using *Bam*HI, blunted ending using standard Klenow polymerase reaction and *Xba*I and ligated into the polylinker region of *Eco*RV+*Xba*I-digested pMRS182 vector under the transcriptional control of the murine cytomegalovirus major immediate early promoter. pMRS182 was constructed by removing G-CSF cDNA sequences from pmCMVβG-CSFSV1dhfr (Rotondaro et al., 1997) and insertion of unique *Eco*RV, *Not*I, *Sal*I, *Xba*I and *Sma*I cloning sites by means of a synthetic linker. The structure of all constructs was confirmed by restriction analysis. The complete coding sequence of cDNAs for full-length and splice variant NK_2 receptors was confirmed by DNA sequencing and is shown in Fig. 1.

Large-scale preparation of vector DNA for transfection experiments was carried out using a Qiagen maxi-preparation column (Qiagen, Hilden, Germany). Full-length and splice variant NK_2 receptor cDNAs in pMRS182 were introduced by lipofection as described (Rotondaro et al., 1997) into CHO $dhfr^-$ cells (Urlaub and Chasin, 1980). Stable $dhfr^+$ transformants were selected into nucleoside-free alpha-modification Eagle's Minimum Essential Medium (α-MEM) containing 10% dialyzed fetal calf serum; 12–14 days after transfection,

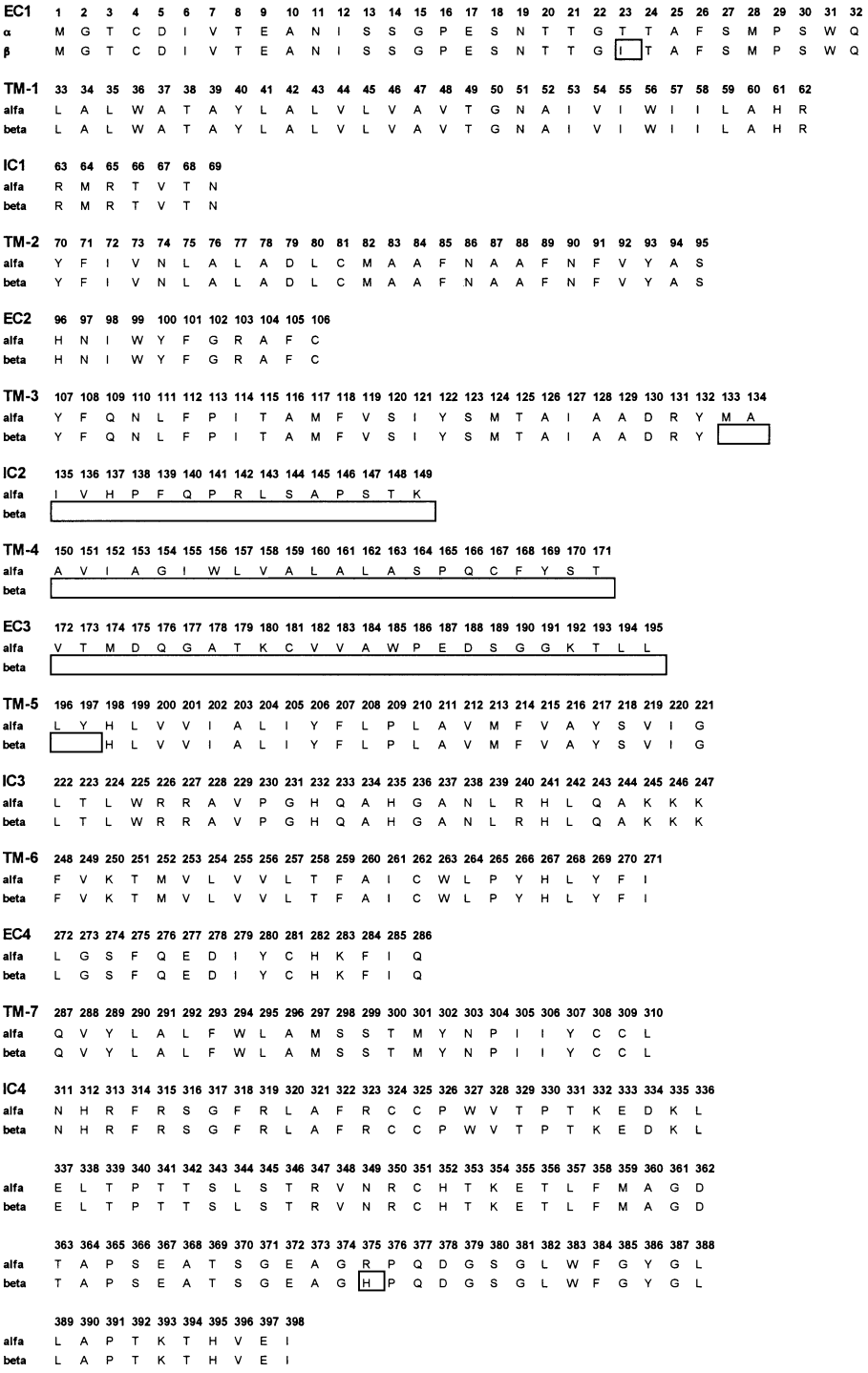

Fig. 1. Amino acid sequence alignment of human tachykinin NK_2 receptor α and β isoforms. The differences between the α and β isoform sequences are indicated by boxes. Empty and full boxes evidence the omitted or replaced amino acids, respectively, in comparison with the α isoform.

more than 100 individual $dhfr^+$ clones were pooled, grown to mass culture and used for ligand binding and receptor activation studies.

$CHOdhfr^-$ cells were grown in α-MEM containing ribo- and deoxyribonucleosides, and supplemented with 10% fetal calf serum.

Stable CHO transfectants were cultured in nucleoside-free α-MEM supplemented with 10% dialyzed fetal calf serum.

2.2. Radioligand binding studies

2.2.1. Whole cells

Cells were grown in 6-well tissue culture clusters until confluence, then the culture medium was replaced with the binding buffer containing phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS, 135 mM), Na/HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid], 10 mM),

$CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), glucose (5 mM), bovine serum albumin (1 g/l), 1,10 phenanthroline (1 mM) and bacitracin (140 μg/ml) at pH 7.4. Nonspecific binding was determined in the presence of 1 μM concentration of the appropriate unlabeled ligand. Binding assay was performed in a final volume of 1.5 ml/well and with an incubation time of 60 min at 4 °C. Each experiment was performed in triplicate. All incubations were terminated by aspiration of the binding buffer and by washes with cold PBS (1 ml×3). Cells were then solubilized (1 ml NaOH, 0.3 M) and added with 20 ml of CytoScint ES scintillation liquid (ICN Biomedicals) for counting in a β-scintillation counter (2200 CA, Packard).

2.2.2. Cell membranes

Cells at confluence were rinsed with ice-cold phosphate buffered saline without Ca^{2+} and Mg^{2+} and pelleted by centrifugation at 200×*g* for 10 min at 4 °C. The pellet was homogenized with a Polytron (PT 3000, Kinematica) at 15000 r.p.m. for 30 s in 30 ml of 50 mM Tris–HCl, pH 7.4, containing bacitracin (0.1 mg/ml), chymostatin (0.01 mg/ml), leupeptin (5 μg/ml) and thiorphan (10 μM; buffer A) to avoid peptide degradation. The homogenate was centrifuged at 25,000×*g* for 1 h at 4 °C and the pellet was resuspended in the binding buffer composed of buffer A supplemented with 150 mM NaCl, 5 mM $MnCl_2$ and 0.1% bovine serum albumin to obtain 5 mg/ml membrane protein concentration and frozen immediately in 1 ml of aliquots by immersion in liquid nitrogen and then stored at −80 °C until use.

Binding assay was performed at room temperature in a final volume of 0.5 ml and an incubation time according to the used radioligand: 30 min was used for [^{125}I]neurokinin A and [^{3}H]SR48968 or (*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide whereas 60 min was used for [^{3}H]nepadutant. Each radioligand was used at a concentration which was comparable to the calculated K_d value ([^{125}I]neurokinin A 0.2 nM, [^{3}H]SR48968 0.2 nM and [^{3}H]nepadutant 0.4 nM) of binding at the human tachykinin NK_2 receptor (α isoform) less than 10% of the total added radioligand concentration and a specific binding representing approximately 70–80% of the total bound. Nonspecific binding was defined as the amount of labeled ligand bound in the presence of the appropriate unlabeled ligand (neurokinin A, SR48968 or nepadutant, 1 μM).

Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard) which had been presoaked for at least 2 h in polyethylenimine 0.6% using a MicroMate 96 Cell Harvester (Packard Instrument Company). The tubes and filters were then washed five times with 0.5 ml aliquots of Tris buffer (50 mM, pH 7.4, 4 °C). Filters were dried and soaked in 50 μl/well of Microscint 40 (Packard Instrument Company), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company).

2.3. Functional studies

2.3.1. Inositol phosphates measurement

Cells were grown in 24-well tissue culture clusters and labeled for 24 h with *myo*-[1,2-^{3}H] inositol (0.5 ml/well, 1 μCi/ml) in Iscove Modified Dulbecco' s medium (IMDM) and Ham' s F12 Medium (F12; 1:1) containing dialyzed fetal bovine serum (1%) and L-glutamine (2 mM). After a 15-min preincubation period at 37 °C in a buffer consisting of PBS (135 mM), HEPES (20 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), EGTA (1 mM), glucose (11.1 mM), bovine serum albumin 0.05% and LiCl (25 mM; IP buffer), cells were incubated for 30 min a 37 °C in 0.5 ml of IP buffer added with 1 μM concentration of different agonists. The reaction was stopped by 1 ml of ice-cold mixture methanol and HCl 0.1 N (1:1, v/v), and samples were applied to a Bio-Rad AG1X8 column. The columns were washed twice with 6 ml of ammonium formate (0.06 M) in sodium tetraborate (0.005 M) to remove free inositol. After these washing steps, the total [^{3}H]IP was eluted with two 3 ml aliquots of ammonium formate (1.2 M) in formic acid (0.1 M). The radioactivity in the eluates was determined by a liquid β-scintillation counter (2200 CA, Packard). Determinations were made in triplicate.

2.3.2. Intracellular calcium transients measurements

Cytosolic free calcium ($[Ca^{2+}]_i$) was measured in cell suspensions using the Ca^{2+} fluorescent dye, Fura-2/AM. Confluent cells were washed twice with Dulbecco's phosphate buffer saline without Ca^{2+} and Mg^{2+} and harvested by treatment with a nonenzymatic cell dissociation solution. After centrifugation at 500×*g* for 10 min, the cells were incubated in oxygenated Krebs-buffer saline (pH 7.4; composition in mM: NaCl 119; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4$ 1.5; KCl 4.7; $CaCl_2$ 2.5 and glucose 11) supplemented with 0.1% bovine serum albumin and 5 μM Fura-2/AM for 30 min at 37 °C.

Thereafter, the cells were washed with Krebs-buffer saline, plus 0.5% bovine serum albumin and 0.5 mM probenecid, and the incubation was prolonged for 35 min at room temperature to allow complete hydrolysis of the dye by the intracellular esterases. The Fura-2/AM loaded cells were washed again and finally resuspended in Krebs-buffer plus 0.5 mM probenecid for immediate measurement of Ca^{2+}. For measurements, cells were transferred in a water-jacketed thermostated cuvette (37 °C) with continuous stirring, and fluorescence was monitored at an emission wavelength of 509 nm using a Perkin-Elmer LS-50B spectrofluorimeter with excitation wavelenghts switching continuously between 340 and 380 nm. The obtained fluorescence intensities were analysed by the customised software Winlab provided by Perkin-Elmer. The changes of the ratio of Fura-2/AM fluorescence intensities, ΔF340/F380, obtained after substraction of the respective background, were used as an index of

the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. The values of $[Ca^{2+}]_i$ were calculated (Grynkiewicz et al., 1985) as:

$$\left[Ca^{2+}\right]i = K_d \times \ (R - R\min)/(R\max - R) \times \ \beta$$

where K_d is the dissociation constant for Fura-2/AM (224 nM); Rmax and Rmin are the ratios for bound and unbound forms of Fura-2/AM, respectively; and β is the ratio between maximum and minimum fluorescence intensities of Fura-2/AM at 380 nm excitation. For the calibration procedure, maximum and minimum fluorescence signals were obtained by adding ionomycin (10 μM) and EGTA (6.25 mM) sequentially at the end of the experiment. The autofluorescence value was less than 15% of the Fura-2/AM signals at either excitation wavelength.

2.4. Data analysis

All values in the text and figures are the mean±S.E.M. of the mean or mean and 95% confidence limits (c.l.) for K_i and K_d.

Binding data were fitted by nonlinear regression using GraphPad Prism 3.0 (San Diego, CA) in order to determine the equilibrium dissociation constant (K_d) from homologous competition experiments and the ligand concentration inhibiting the radioligand binding of the 50% (IC_{50}) from heterologous competition experiments. K_i values were calculated from IC_{50} using the Cheng–Prusoff equation (K_i=IC_{50}/(1+[radioligand]/K_d) according to the concentration and K_d of the used radioligand.

2.5. Drugs

[^{125}I]neurokinin A (specific activity 2000 Ci/mmol) and [^{3}H]substance P (specific activity 41 Ci/mmol) were provided by Amersham. [^{3}H]SR48968 (specific activity 25.5 Ci/mmol) and *myo*-[1,2-^{3}H] inositol (specific activity 65 Ci/mmol) were provided by Perkin-Elmer Life Science (Zaventem, Belgium) and [^{3}H]nepadutant (specific activity 30 Ci/mmol; MEN11420 or (cyclo–{[Asn(β–D–GlcNAc)–Asp–Trp–Phe–Dpr–Leu]cyclo(2β–5β)}) was synthesized by SibTech (Newington, CT, USA).

SR48968 ((*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) was a kind gift of Sanofi.

Leupeptin was obtained from Boehringer Mannheim (Germany), Mergetpa, ionomycin, Fura-2/AM were from Calbiochem (La Jolla, CA, USA). All salts were purchased from Merck (Darmstadt, Germany).

Substance P, neurokinin A, neurokinin B, [Sar^9]substance P sulfone, septide, [βAla^8]neurokinin A(4–10), [Nleu^{10}]neurokinin A(4–10) were from Bachem (Bubendorf, Switzerland). Endokinin C and endokinin D were synthesized by the conventional solid phase method by Chemistry Department of Menarini Ricerche, Florence.

All other compounds and materials were from Sigma (St. Louis, MO, USA).

3. Results

3.1. Binding studies

All experiments were performed concomitantly at the human tachykinin NK_2 receptor α and β isoforms.

Binding experiments on whole cells expressing the human tachykinin NK_2 receptor α isoform revealed a specific binding of 3797±123 sites/cell for 0.12 nM of [^{125}I]neurokinin A, 39670±3357 sites/cell for 1.5 nM of [^{3}H]nepadutant and 48287±7931 sites/cell for 0.2 nM of [^{3}H]SR48968; no specific binding was observed with [^{3}H]substance P (1 nM). None of the used radioligands, in the same experimental conditions, specifically bound to cells expressing the human tachykinin NK_2β receptor isoform.

Binding experiments were performed in membrane preparation at different protein concentrations: only [^{3}H]SR48968 was able to specifically bind the β isoform of the human tachykinin NK_2 receptor although at a lesser extent as compared to the α isoform (Fig. 2).

At the human tachykinin NK_2α receptor, SR48968 displayed the same affinity values both in competing for the binding site of [^{125}I]neurokinin A and for that of [^{3}H]SR48968, K_i being 0.3 (0.2–0.5, 95% c.l.) nM, and K_d 0.6 (0.4–0.8, 95% c.l.) nM, respectively (Fig. 3A and B). At the human tachykinin NK_2β receptor, the affinity of SR48968 was about sixfold lower than that measured at the NK_2α receptor, the K_d values being 3.8 (2.3–6.4, 95% c.l.) nM (Fig. 3C). Neurokinin A displayed a K_d of 3.8 (3.3–4.3, 95% c.l.) nM and a K_i of 55.1 (32.1–94.5, 95% c.l.) nM when measured at the [^{125}I]neurokinin A and [^{3}H]SR48968 binding to the human tachykinin NK_2α receptor (Fig. 3A and B) whereas it did not inhibit the [^{3}H]SR48968 binding to the β isoform up to μM concentration (Fig. 3C).

3.2. Functional studies

3.2.1. IP production

IP production was evaluated in the absence (basal) or in the presence of some agonist ligands at 1 μM concentration (Fig. 4). No differences were observed in basal IP production between cells expressing the human tachykinin NK_2α or β receptor isoform. The natural tachykinins, substance P, neurokinin A and neurokinin B, and the synthetic NK_2 receptor selective agonists, [βAla^8]neurokinin A(4–10) and [Nleu^{10}]neurokinin A(4–10), were able to produce a significant IP increase over

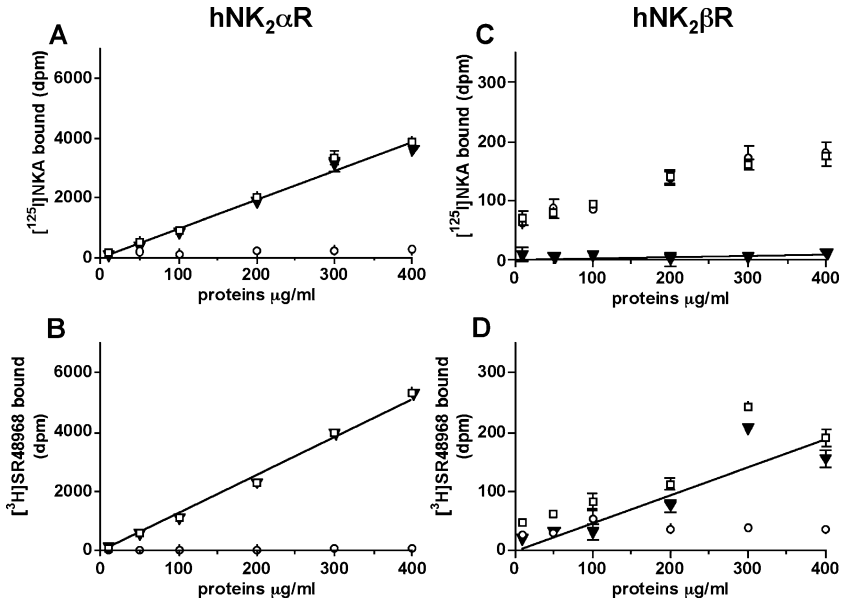

Fig. 2. [^{125}I]neurokinin A and [^{3}H]SR48968 binding related to protein concentrations of membranes from cells expressing the α (A, B) or β (C, D) isoform of the human tachykinin NK_2 receptor. Specific (▼), total (□) and nonspecific (○) bindings are plotted versus different membrane protein concentrations. Radioligand concentrations are reported under Methods. Results are expressed as the mean±S.E.M. of three independent experiments performed in duplicate.

the basal in the cells expressing the human tachykinin NK_2 receptor α receptor isoform only (Fig. 4). The selective tachykinin NK_1 and NK_3 receptor selective agonists, [Sar^{9}]substance P sulfone, septide and senktide, or the newly discovered endokinin C and endokinin D, did not produce a significant effect on IP production in cells expressing the human tachykinin NK_2α isoform.

None of the agonists used led to a significant increase of IP production in cells expressing the human tachykinin NK_2 receptor β isoform (Fig. 4).

3.2.2. Intracellular calcium measurement

In cells expressing the human tachykinin NK_2 receptor α isoform, neurokinin A (1 μM) stimulated a rapid and transient increase in intracellular calcium level ($[Ca^{2+}]_i$) from a basal level of 70±9 nM to a maximum level of 163±8 nM 15 s after agonist application. Then, $[Ca^{2+}]_i$ slowly declined remaining above basal levels for at least 2 min. As shown in Fig. 5, the effect of neurokinin B (1 μM) was similar to that obtained with neurokinin A while substance P (1 μM) produced only a partial $[Ca^{2+}]_i$ increase (42±7 versus 85±4 and 108±9 nM for substance P, neurokinin B and neurokinin A, respectively). Neither septide nor endokinins C or D were able to produce any effect although the cells responded to a successive application of neurokinin A. In cells expressing the human tachykinin NK_2 receptor β isoform, the basal level of $[Ca^{2+}]_i$ was 54±2 nM and none of the mentioned peptides (1 μM) were able to produce a significant increase in the $[Ca^{2+}]_i$ level over a period of at least 2 min after application.

4. Discussion

An extensive search of the genome databases presently available indicates that tachykinin receptors have been highly conserved throughout evolution and are present in most species along *Bilateria*, from invertebrates to mammals. Moreover, the gene structure has also been conserved throughout evolution (Fig. 6). In this context, five is the lower number of exons present in any tachykinin receptor and is also the number of exons found in mammalian receptors (see the *Homo sapiens* NK_2 receptor in Fig. 6). The tachykinin NK_2 receptor from the cordate *Fugu rubripens* has six introns, the tkr-1 receptor from the urochordate *Ciona intestinalis* has seven introns and the DTK receptor from the insect *Drosophila melanogaster* has eight introns. However, the exon–intron boundaries present in mammals are also found in these species (Fig. 6). The conservation of the genomic structure of the tachykinin receptors in species that diverged millions of years ago suggests that these genes are under selective pressure to maintain introns, possibly reflecting important functional differences between different splice variants (Minneman, 2001). It seems therefore important to analyse the localization and pharmacological properties of the different known isoforms of tachykinin receptors.

In agreement with previous data, [^{125}I]neurokinin A, [^{3}H]nepadutant and [^{3}H]SR48968 displayed a high affinity in binding assays performed in CHO cells expressing the human tachykinin NK_2 α receptor isoform (Giolitti et al., 2000, 2002). [^{3}H]substance P showed the lower affinity among the tested radioligands. In functional studies,

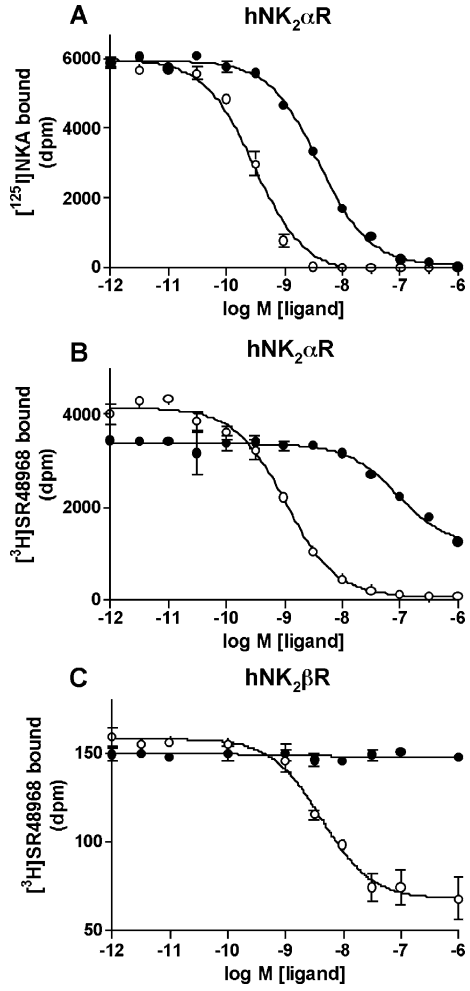

Fig. 3. Homologous and heterologous competition binding curves by the peptide agonist neurokinin A (●) and the nonpeptide antagonist SR48968 (○) at the α (A, B) and β (C) isoforms of the human tachykinin NK_2 receptor. Results are expressed as the mean±S.E.M. of three independent experiments performed in duplicate.

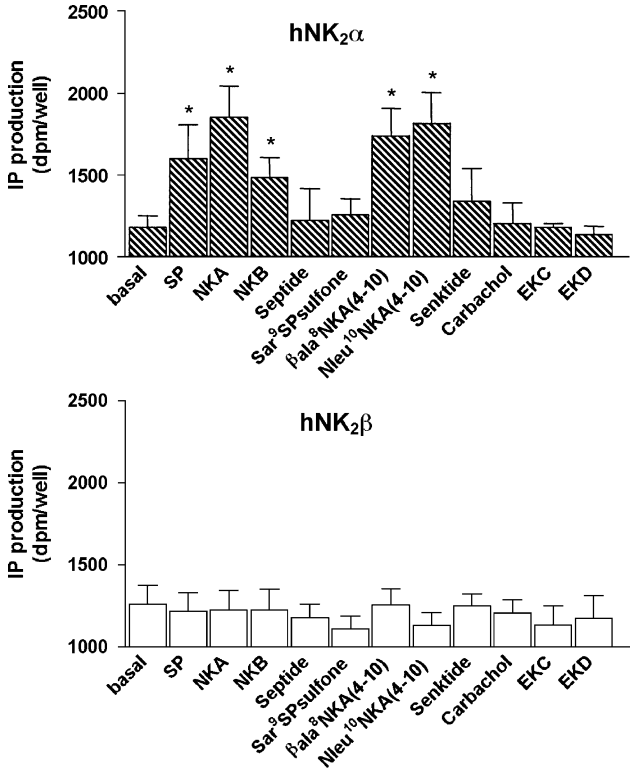

Fig. 4. Agonist-induced IP production in cells expressing the α (upper panel) and β (lower panel) isoforms of the human tachykinin NK_2 receptor. Cells were incubated for 30 min at 37 °C in an IP buffer (as described under Methods) added with 1 μM concentration of tachykinin NK_2 receptor agonists (neurokinin A, [βAla^8]neurokinin A(4–10) and [Nleu^{10}]neurokinin A(4–10)), tachykinin NK_1 receptor agonists (SP, [Sar^9]substance P sulfone and septide), tachykinin NK_3 receptor agonists (neurokinin B and senktide), carbachol and endokinins C and D (EKC, EKD). Results are expressed in total dpm per well as the mean±S.E.M. of three independent experiments performed in triplicate.

substance P, neurokinin A and neurokinin B increased $[Ca^{2+}]_i$ and intracellular IP levels in whole CHO cells expressing the α isoform, confirming that these three endogenous tachykinins are able to activate, albeit with different potencies, the wild-type NK_2 receptor (Lecci and Maggi, 2003).

Compared to the human tachykinin NK_2α receptor isoform, the sequence of the β splice variant would produce a protein whose sequence lacks IC2-TM4 (see Fig. 1). It has been suggested that five TM domains are sufficient to obtain a functional GPCR (Ling et al., 1999). In this context, it can be speculated that it is more probable for a five TM to be active than for a six TM, due to the following reasons: (a) an odd number of TMs would facilitate the presence of an extracellular N-terminal and an intracellular C-terminal, as what usually occurs in GPCRs and (b) each α-helix has a dipole moment, i.e., a driving force that is needed for the correct folding of the receptor. In other words, when TM segments are in odd number, the structure has an overall dipole moment while a six-TM receptor will not have a net dipole. This and the loss of the S–S bridge between Cys106 and Cys181 (see Fig. 1) suggest that the human tachykinin NK_2 receptor β isoform would probably be unable to be exposed on the membrane surface. In agreement with this reasoning, none of the radioligands used in the present study displayed a specific binding in whole CHO cells transfected

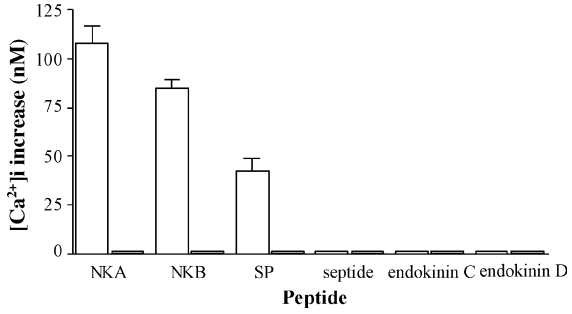

Fig. 5. Peptide-induced $[Ca^{2+}]_i$ increase in cells expressing the α and β isoforms. Net $[Ca^{2+}]_i$ increase induced by neurokinin A, neurokinin B, substance P, septide and endokinins C and D (1 μM each) in cells expressing the α (open bars) or β (closed bars) isoform of the human tachykinin NK_2 receptor.

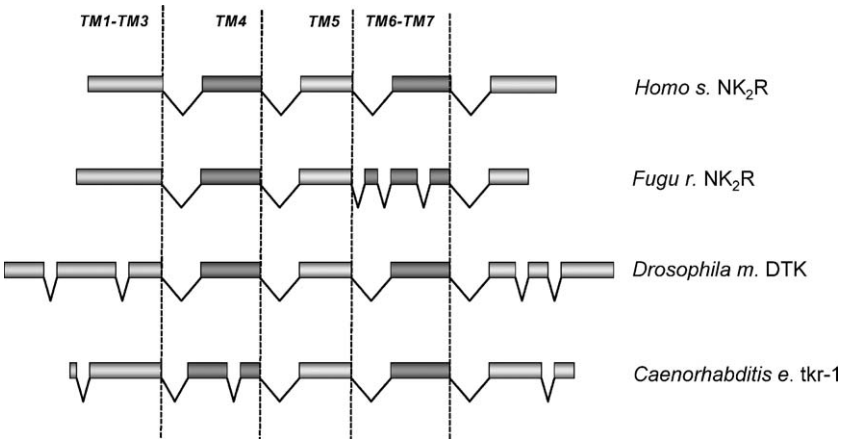

Fig. 6. Schematic structure of the genes encoding tachykinin receptors in different species of *Metazoa*. The protein-coding region of these genes is divided into exons (indicated by boxes) interrupted by a different number of introns (indicated by continuous lines) that depends on the species considered. Four of the exon–intron boundaries are conserved in all species, as shown by dashed lines. TM1-7 indicates the relative position of the seven hydrophobic transmembrane domains.

with the human tachykinin NK_2 receptor β isoform. It was also observed that although the β isoform maintains the amino acidic sequence involved in the interaction with G proteins (see Fig. 1), none of the agonists used were able to produce a functional response, at least in terms of IP production and $[Ca^{2+}]_i$ mobilization.

Among the investigated radioligands, both agonists, neurokinin A, substance P and antagonists, nepadutant (Renzetti et al., 1998) and SR48968 (Emonds-Alt et al., 1993), only the latter was able to bind to the β isoform of the human tachykinin NK_2 receptor, albeit at a lesser extent (about 4%) as compared to the binding detected at the α isoform. The fact that the SR48968 binding was detectable in the membrane preparation only may suggest that the β isoform receptor is not able to be fully targeted to the extracellular membrane because of its poor folding. On the other hand, the affinity of the selective tachykinin NK_2 receptor antagonist was in the nM range. This finding is in line with the conservation in the β isoform receptor sequence of the previously identified molecular determinants for SR48968 binding, i.e., residues in the transmembrane region defined by helices 4, 5, 6 and 7 (Giolitti et al., 2000).

Recent evidence demonstrates that certain membrane receptors have truncated forms that are not inserted in the membrane but remain in solution and are able to interact with ligands (Rose-John and Heinrich, 1994; Dannies, 2001). In our study, the endogenous tachykinins assayed were unable to bind to the truncated NK_2 receptor, even in membrane preparations. However, this does not exclude the possibility that the human tachykinin NK_2 receptor β could interact with other presently unknown ligands. In this context, it has been shown that substance P binds to distinct proteins that mimic the tachykinin NK_1 receptor, such as fibronectin in the serum (Rameshwar et al., 2003). The existence of GPCR protein isoforms having an unusual localization and recognizing ligands different from their usual ones is an interesting possibility and suggests that members of the GPCR superfamily may have further physiological roles than presently thought.

Endokinin C and endokinin D, tachykinin-like peptides recently cloned in humans (Page et al., 2003), possess a C-terminal pentapeptide different from the sequence Phe–X–Gly–Leu–Met–NH_2 that is commonly found in mammalian tachykinins (Otsuka and Yoshioka, 1993; Severini et al., 2002).

To our knowledge, this is the first report where the functional effects of endokinin C and endokinin D on the human tachykinin NK_2 receptor have been studied. The possibility that these new mammalian tachykinins might activate the α or β isoform of the human tachykinin NK_2 receptor can be ruled out by the present results.

The tachykinin NK_2 receptor β splice variant is present in different human and rat tissues and was only visualized in tissues also expressing the wild-type tachykinin NK_2 receptor α isoform (Candenas et al., 2002). In addition, the ratio of tachykinin $NK_2α/NK_2β$ receptor mRNA levels varied among different tissues (Candenas et al., 2002). These findings suggest that independent of its functional role per se, the β isoform may be of physiological relevance and may act as a regulator of tachykinin NK_2 receptor function. In this way, an increase in the abundance of truncated mRNA, relative to the wild-type isoform, would produce a concomitant decrease in functional levels of the tachykinin NK_2 receptor.

The existence of an isoform of the tachykinin NK_2 receptor could explain some unexpected experimental data, such as the lack of precise correlation between mRNA levels and the functional response observed following activation of the tachykinin NK_2 receptor in a particular tissue (Patak et al., 2003) or the presence of the tachykinin NK_2 receptor in sites where the tachykinins are not expressed (Saffroy et al., 2003). The results of the present study have therefore a practical interest: the existence of the

tachykinin NK_2 receptor β isoform must be taken into account, particularly in those studies in which techniques such as Reverse Transcriptase–Polimerase Chain Reaction (RT–PCR) or in situ hybridization are used to analyse the expression and cellular localization of the mRNA of this tachykinin receptor.

In conclusion, the present data show that the human tachykinin NK_2 receptor β isoform gives rise to a protein that is poorly or not at all expressed on the cell membrane surface. The protein does not bind to substance P or neurokinin A but retains its ability to recognise the selective tachykinin NK_2 receptor antagonist SR48968 in membrane preparations from CHO transfected cells. The existence of the tachykinin NK_2 receptor β isoform must necessarily be considered in those assays that analyse the mRNA expression of the tachykinin NK_2 receptor.

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