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Occurrence and pharmacological characterization of four human tachykinin NK_2 receptor variants

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ABSTRACT

Tachykinin NK_2 receptor antagonists are potentially beneficial in treating various disorders including irritable bowel syndrome, urinary incontinence, depression and anxiety. The current study evaluates the frequency of single nucleotide polymorphisms (SNPs) in the human NK_2 receptor gene (TACR2). In addition, the potency of the endogenous peptide agonist neurokinin A (NKA), and the small molecule antagonists saredutant (NK_2-selective) and ZD6021 (pan-NK antagonist) at the various NK_2 receptor protein variants were determined. The TACR2 gene was sequenced from 37 individuals. Two amino acid changing SNPs encoding the NK_2 receptor variants Ile23Thr and Arg375His were found. The frequency of the four possible protein variants differed between populations. Site-directed mutagenesis was performed introducing either SNP or both SNPs into the TACR2 gene and the constructs were transfected into CHO cells. NKA-evoked increases in intracellular Ca^{2+} were monitored by FLIPR. The potency of saredutant and ZD6021 was evaluated by their ability to inhibit NKA-induced increases in intracellular Ca^{2+}. NKA evoked increases in intracellular Ca^{2+} with a potency ranging between 1 and 5 nM in CHO cells expressing the different constructs. Saredutant and ZD6021 blocked NKA-evoked increases in intracellular Ca^{2+} with pK_b values ranging between 8.8–9.3 and 7.9–8.7, respectively. The current study demonstrates that polymorphisms leading to the Ile23Thr and Arg375His amino acid exchanges are highly prevalent in the human TACR2 gene. These polymorphisms however do not appear to affect the potency of the endogenous agonist NKA or the small molecule antagonists saredutant and ZD6021 with respect to intracellular Ca^{2+} signalling.

1. Introduction

Tachykinins such as substance P (SP) and neurokinins A (NKA) and B (NKB), exert their biological effects by activating specific G-protein coupled tachykinin (NK) receptors [1]. Three NK receptors have been cloned and characterized and are termed NK_1, NK_2 and NK_3 [2]. The rank order of potency of tachykinins at the NK_2 receptor is NKA > NKB > SP [2]. When activated, the NK_2 receptor interacts with Gq heterotrimeric proteins, resulting in inositol triphosphate formation and increased

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intracellular levels of Ca^{2+} [3,4]. However, Gs-mediated increases in cAMP formation, in response to NK_2 receptor activation, have also been reported [5–8].

The tachykinin NK_2 receptor is widely expressed in organs such as the gastrointestinal [9], urinary [10] and pulmonary [11] tracts. Functional data suggest that NK_2 receptors play a major role in mediating tachykinin-evoked smooth muscle contraction in the respective organs [12–14] and NK_2 receptor antagonists are suggested to be beneficial for indications such as irritable bowel syndrome, asthma and micturition disturbances [15]. Tachykinin NK_2 receptors are not highly abundant in the central nervous system, however NK_2 receptor mRNA has been detected in the human brain [16] and NK_2 receptor binding sites have been identified rat brain [17]. Interestingly, the selective NK_2 receptor antagonist saredutant (SR48968) has demonstrated anti-depressant-like activity in several rodent models [18–21] and is reportedly in phase III for the treatment of depression [22].

Single nucleotide polymorphisms (SNPs) in genes encoding receptors can affect many aspects of receptor function. It has been demonstrated that SNPs can alter receptor-ligand binding, receptor-second messenger coupling and constitutive activity of receptors [23]. In some cases, SNPs may lead to increased disease susceptibility or yield variable responses to therapeutic agents. Thus, evaluating the pharmacogenetics of disease-related targets is an important step in the process of drug development.

Nineteen SNPs in the human tachykinin NK_1 receptor gene were recently identified in a population of 93 individuals [24]. One SNP resulted in an amino acid exchange (tyrosine to histidine at residue 192, Thr192His) in the NK_1 receptor protein. However, this exchange did not appear to affect receptor function or the affinity of evaluated agonists and antagonists [24]. Otherwise, the NK_1 receptor is an excellent example how an exchange in one amino acid (for example Ile290 with the rat counterpart Ser290) can dramatically affect the affinity and potency of antagonists [25,26].

The current study reveals the frequency of polymorphisms in the human NK_2 receptor gene TACR2 and pharmacologically characterizes NK_2 receptor variants. The results indicate that four different NK_2 receptor variants occur in various human populations at a large frequency. However, the NK_2 receptor antagonists saredutant and ZD6021 displayed similar potency at each NK_2 receptor variant. Hence, the likelihood that the identified genetic variations in the human NK_2 receptor would render these compounds ineffective in man is low.

2. Materials and methods

2.1. Research Subjects

The coding sequence of tachykinin NK_2 receptor gene (TACR2) was sequenced in genomic DNA from 37 lymphoblastoid cell lines derived from unrelated individuals of European origin and obtained from the Coriell Institute Repositories. Additional allele frequency data were obtained by sequencing the relevant exons in genomic DNA from 33 UK healthy volunteers, 22 African American, 30 Chinese, 30 Japanese and 30 Hispanic Americans.

2.2. DNA sequencing and analysis

PCR products were generated from each cell line for each of the coding exons of TACR2 using flanking intronic primers. Forward primers were tagged with M13F sequence, reverse primers with M13R sequence. PCR products were sequenced in both directions by dye-terminator sequencing, using standard methods with M13 forward and M13 reverse primers. Sequences traces were analysed and visualised using the Consed automated sequence analysis package [27] and all traces checked manually for polymorphisms.

2.3. Point mutation and cloning

Human NK_2 receptor construct was a gift from Dr. Alexander Graham [28] and was used as a template for subcloning of the human NK_2 receptor into pIRESneo2 (BD Biosciences Clontech, Palo Alto, CA, USA). The NK_2 receptor of the original clone was the Thr23_Arg375 variant. Restriction sites EcoRV and EcoR1 were introduced by PCR with Pfu ultra (Stratgene, La Jolla, CA, USA) and a Kozak sequence was added before the ATG. The desired mutations were introduced using the Quick-Change multi site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers used to introduce the point mutations were AACACCACGGGCA**T**CACAGCCTTCTCC and GGGAGGCGGGG-C**A**TCCCCAGGATGG, resulting in Thr23Ile and Arg375His exchanges, respectively. The underlined sequences indicate the changed bases for nucleotide 68 and 1124, respectively. Plasmids were purified with PhoneIX maxiprep kit (MP Biomedicals, Ilkirch, France).

The four different constructs (Ile23_Arg375, Ile23_His375, Thr23_Arg375, and Thr23_His375) were transfected into Chinese Hamster Ovary (CHO) cells (ATCC, Middlesex, UK) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Clones expressing the four different variants of human NK_2 receptor were selected by growth in 500 μg/ml hygromycin B and tested for functionality in a Ca^{2+} mobilization assay. Cells were cultured at 37 °C in a 5% CO_2 incubator and routinely passaged when 70–80%-confluent for up to 20 passages.

2.4. Intracellular calcium mobilization

Ca^{2+} mobilization was studied using a Fluorometric Imaging Plate Reader, FLIPR^{TM}. Cells were seeded into black-walled clear-base 96-well plates (Costar, #3904) at a density of 35,000 cells per well in culture media and grown for approximately 24 h in a 37 °C CO_2-incubator. In FLIPR experiments, the cells were incubated with the cytoplasmic Ca^{2+} indicator Fluo-4 (TEFLABS 0152) at 4 μM in loading media (Nut Mix F12 (HAM) with Glutamax I, 22 mM HEPES, 2.5 mM probenicid (Sigma, P-8761) and 0.04% Pluronic F-127 (Sigma P-2443)) for 30 minutes in a 37 °C CO_2-incubator. The Fluo-4-loaded cells were then washed three times in assay buffer (Hanks Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenicid and 0.1% BSA). The plates were then placed into the FLIPR^{TM} to monitor cell fluorescence (λ_{ex} = 488 nm, λ_{em} = 540 nm) before and after the addition of antagonists and/or agonists. Antagonists and agonists were dissolved in assay buffer (final DMSO concentration kept below 1%) on 96-well plates and added to the loaded cells by the automated pipettor in the FLIPR^{TM}. Loaded

Table 1 – Allele frequency in different populations

Position in AY322545	SNP	Amino acid change	Minor allele frequency (%)					Database reference
			European	Chinese	Japanese	African-American	Hispanic-American	
68	T–C	Ile23Thr	15	5	Not seen	32	9	rs5030920
1124	G–A	Arg375His	31	12	2	3	30	rs2229170

Table 2 – Haplotype frequency of the four tachykinin NK_2 receptor protein variants in different populations.

Haplotype	European (%)	Chinese (%)	Japanese (%)	African-American (%)	Hispanic-American (%)
Ile23_Arg375	58	84	98	69	62
Ile23_His375	23	11	2	3	29
Thr23_Arg375	16	5	Not seen	28	9
Thr23_His375	3	Not seen	Not seen	Not seen	Not seen

cells were pre-incubated with antagonists for approximately 2 min before addition of NKA at the EC_{50} for each construct (ranged between 1.5 and 7 nM, see also results below). Intracellular Ca^{2+} mobilization responses were measured as peak fluorescence intensity after agonist addition minus basal fluorescence.

2.5. Chemicals

The pan-NK receptor antagonist ZD6021 [29] and the selective tachykinin NK_2 receptor antagonist saredutant [30] were synthesized at AstraZeneca. NKA was purchased from Bachem (Peninsula Laboratories Inc, San Carlos, CA, USA).

2.6. Data and statistical analysis

Data generated *in vitro* were fitted to a four parameter equation using Excel Fit. The NKA potency values are expressed as pEC_{50}-values. The IC_{50} of antagonists was assessed based on their ability to inhibit intracellular Ca^{2+} increases evoked by an EC_{50} concentration of NKA. K_b values were calculated using the Cheng–Prusoff equation [31]. Mean K_b values, along with S.E.M. and 95% confidence intervals, where calculated based on three independent experiments using Excel. The results are expressed as mean $pK_b \pm$ S.E.M.

3. Results

3.1. Genetic variations of the human NK_2 receptor gene

Two SNPs resulting in amino acid substitutions were identified in the human NK_2 receptor gene. One SNP was found in position 68 (T > C) resulting in the amino acid isoleucine being replaced by threonine at residue 23 (Ile23Thr). Residue 23 is located in the N-terminal, extracellular part of the receptor. The second SNP was found in position 1124 (G > A) resulting in the amino acid arginine being replaced by histidine at residue 375 (Arg375His). Residue 375 is located in the intracellular C-terminal part of the receptor.

The allele frequencies of the two SNPs in the five populations studied are indicated in Table 1. Since two amino acid changing SNPs were identified, four possible protein variants could be formed. The frequency of these four haplotypes are indicated in Table 2.

3.2. Conservation of Ile23 and Arg375 among species

Fig. 1A shows that Ile23 is conserved among human, bovine and guinea pig, while position 23 contains the amino acid valine in rat, mouse, rabbit and hamster. At least three different amino acids occur in position 375 amongst the indicated species (Fig. 1B).

3.3. Functional characterization of NK_2 receptor variants

No obvious differences in basal activity between constructs were detected (data not shown). NKA increased intracellular calcium levels with similar potency (EC_{50} values ranging between 1 and 5 nM) at all NK_2 receptor variants (Table 3). The selective NK_2 receptor antagonist saredutant and the pan NK receptor antagonist ZD6021 inhibited NKA-evoked increases in intracellular calcium in a concentration-dependent manner

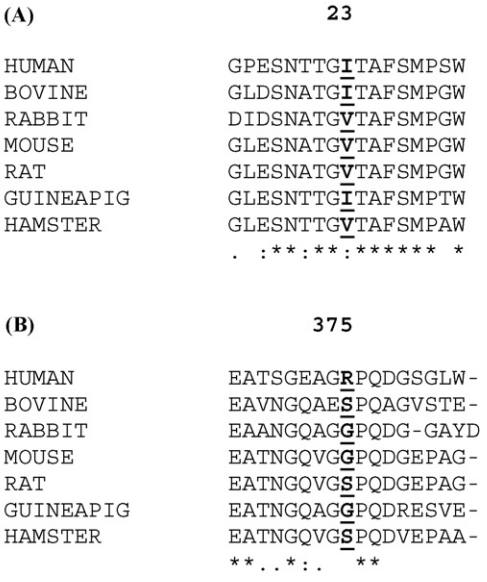

Fig. 1 – Alignment of (A) Ile23 and (B) Arg375 with orthologous NK_2 receptor proteins.

Table 3 – Potency of the endogenous agonist NKA and the antagonists saredutant and ZD6021 at NK_2 receptor variants

Compound	Ile23_Arg375	Ile23_His375	Thr23_Arg375	Thr23_His375
NKA	8.9 ± 0.3 (8.3–9.4)	8.3 ± 0.2 (7.9–8.6)	9.0 ± 0.4 (8.1–9.9)	8.4 ± 0.2 (8.0–8.8)
Saredutant	8.78 ± 0.03 (8.69–8.87)	9.27 ± 0.12 (8.93–9.61)	9.09 ± 0.02 (9.03–9.15)	9.32 ± 0.03 (9.24–9.41)
ZD6021	7.86 ± 0.1 (7.6–8.1)	8.74 ± 0.1 (8.6–8.9)	8.52 ± 0.1 (8.3–8.7)	8.58 ± 0.1 (8.5–8.7)

NKA potency is expressed as pEC_{50} values while the potencies of saredutant and ZD6021 are expressed as pK_b. Data are expressed as mean ± S.E.M. with 95% confidence intervals given in parentheses. $n = 3$.

(representative curves shown in Fig. 2A and B). The potency of ZD6021 and saredutant did not differ to any major extent at the NK_2 receptor variants (Table 3). In addition, ten structural analogues to ZD6021 displayed similar potency at inhibiting NKA-evoked increases in intracellular calcium when comparing all four NK_2 receptor variants (data not shown).

4. Discussion

Tachykinin NK_2 receptor antagonists represent potential therapy in a wide-range of disorders including irritable bowel syndrome, pulmonary and urinary tract disorders and also psychiatric disorders such as depression and anxiety. This is the first study to our knowledge that demonstrates the frequency of amino acid changing SNPs in the human NK_2 receptor gene and also characterizes how these receptor variants affect the potency of the endogenous agonist NKA and the small molecule antagonists saredutant and ZD6021.

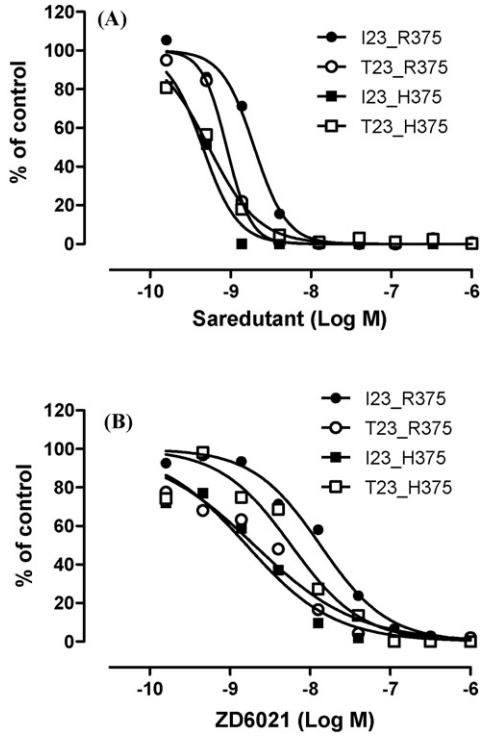

Fig. 2 – Representative curves demonstrating the concentration-dependent inhibition of saredutant (A) and ZD6021 (B) on NKA-evoked increases in intracellular Ca^{2+} in CHO cells expressing the indicated NK_2 receptor variants. An EC_{50} concentration of NKA (ranging between 1.5 and 7 nM) for each construct was used to evoke intracellular increases in Ca^{2+}.

The human NK_2 receptor was cloned almost simultaneously by Gerard et al. [32] from human trachea, by Kris et al. [33] from human jejunum and by Graham et al. [28] from human lung tissue. Interestingly, the NK_2 receptor variant characterized by Gerard et al. was of the Ile23_Arg375 variant while Kris et al. reported the Ile23_His375 variant. Graham et al. characterized the Thr23_Arg375 variant, however variants in positions 23 and 375 were noted. Hence, discrepancies in the human TACR2 sequence were reported already after initial cloning of the receptor. However, studies investigating the occurrence of these variants in different ethnic populations and the comparison of agonist and antagonist potency at the different NK_2 receptor variants have not been performed to our knowledge.

Introduction of point mutations in the human NK_2 receptor has yielded receptors differing in their capability of binding NKA. In mutagenesis studies, it is often difficult to distinguish between receptor sites that participate in direct ligand interactions from those sites that influence receptor conformation indirectly. Nevertheless, the required residues for NKA-binding have been located in the first and second extracellular segments and in the second transmembrane segment [34] while the extracellular third of transmembrane segments 3, 5, 6 and 7 also appear to contribute [35,36]. Detailed modelling of the NKA-NK_2 receptor complex has also recently been reported [37]. In the first extracellular segment, the residues Thr24 and Phe26 are suggested to be involved in mediating the agonistic effects of NKA. Alanine substitution of each residue abolished NKA binding, and functional responses to NKA (phosphatidylinositol hydrolysis) were reduced over a 1000-fold [34]. The affinity of the selective NK_2 receptor antagonist saredutant was not affected by these mutations. The importance of residues Thr24 and Phe26 for NKA binding was confirmed by Labrou et al. [36] using cysteine substitutions. These two residues also play a major role in NKA activation of the rat NK_2 receptor [8]. Indeed, double alanine mutations at positions 24 and 26 in the rat NK_2 receptor reduced the affinity of NKA 10,000-fold. In addition, NKA was 1000-fold less effective at evoking increases in intracellular Ca^{2+} and was unable to stimulate cAMP when the mutations were introduced [8]. The current study demonstrates that a common amino acid changing polymorphism Ile23Thr resides adjacently to these crucial residues. However, the functional data suggest that this polymorphism does not affect the potency of NKA with respect to evoked increases in intracellular Ca^{2+}. The potency of NKA determined in the current study (ranging between 1 and 5 nM) is similar to the degrees of

potency reported by others [8,34]. The affinity of NKA was not evaluated in the current study, however, since the potency of NKA was unchanged, it is unlikely that the affinity of NKA for the different polymorphisms differs to a great degree. The importance of residues Thr24 and Phe26 appear to be fairly specific since the mutation Ser27Ala did not affect NKA affinity either [34]. If the Ile23Thr polymorphism affects NKA-evoked increases in intracellular cAMP or the potency of the lesser active endogenous peptide agonists substance P and NKB remains to be investigated.

Amino acids crucial for binding of saredutant to the human NK_2 receptor reside in transmembrane segments IV, V, VI and VII, which are located relatively far away from the identified polymorphisms evaluated in the current study [38,39]. Although it can not be excluded that substitution of distant residues could evoke allosteric conformation changes in the ligand binding pocket, it was not surprising that the potency of saredutant was preserved when introducing the amino acid exchanging SNPs in the wildtype sequence. The potency values of saredutant in the current study (8.8–9.3) agree well with values previously reported in rabbit pulmonary artery, (pK_b: 9.3) [29]. The binding sites of the peptide-like NK_2 receptor antagonist MEN 11,420 (nepadutant) appear to overlap with saredutant to a certain degree [39]. If the polymorphisms identified in the current study affect the potency of nepadutant remains to be investigated.

Saredutant is a competitive antagonist at NK_2 receptors and also displays slow reversibility *in vitro* [30]. Slow kinetics of recovery after washout (i.e. slow dissociation rates) could contribute to prolonged pharmacological effect duration *in vivo* (see Ref. [40] for a recent review). Indeed, we have also observed slow recovery of human NK_2 receptor signalling after washing out saredutant *in vitro* (von Mentzer et al., paper in preparation). Since these time-course experiments only investigated the human Thr23_Arg375 variant, the reversibility of saredutant from the other human NK_2 receptor variants remains to be investigated. However, saredutant appears to have consistent long-lasting interactions at NK_2 receptors across species (rabbit, hamster and human). Thus major differences between the human NK_2 receptor variants with respect to the kinetics of saredutant binding are not anticipated.

The potency of ZD6021 (or structural analogues to ZD6021) at the four NK_2 receptor variants did not appear to differ to any major extent either. The potency of ZD6021 attained in the current study (7.9–8.7) is consistent with previous reports using rabbit pulmonary artery (pK_b: 8.3) [41].

A splice variant of the human NK_2 receptor has been reported previously [42]. The splice variant consists of a 195 base-pair deletion of the entire exon 2 sequence which predicted a truncated receptor protein containing six transmembrane domains. A subsequent study showed that the truncated receptor was unable to bind endogenous agonists and synthetic antagonists to any significant degree [43]. Interestingly, the report by Bellucci et al. also revealed that Thr23_Arg375 was present in the full length α isoform while Ile23_His375 appeared to be the corresponding residues in the truncated β isoform. The current study extends these observations by showing that these amino acid exchanging polymorphisms occur in full-length receptor protein also, but do not appear to affect receptor function.

This study demonstrated differences in the allele frequencies of the Ile23Thr and Arg375His SNPs between the European, African American, Chinese, Japanese and Hispanic American populations, most notably a virtual absence of both SNPs in the Japanese population. A recent study of a Korean population not only identified the SNP Arg375His, but also a novel SNP Gly231Glu in the TACR2 gene [44] which we did not find when resequencing 37 Europeans. The Ile23Thr polymorphism was not reported by Park et al. providing further evidence for interpopulation variation in this gene. The receptor variants used in the current study all contained Gly at position 231. Whether the Gly231Glu polymorphism, which is located in the third intracellular of the receptor, affects NK_2 receptor function or ligand affinity warrants further investigation. Interestingly, in patients with chronic cough, the 231Glu allele was associated with enhanced cough sensitivity to capsaicin while a similar association was not seen with the Arg375His genotype [44].

In conclusion, amino acid exchanging SNPs are common in the human TACR2 gene and the occurrence differs between populations. However, the current study demonstrates that the Ile23Thr and Arg375His polymorphisms do not affect the potency of NKA, saredutant or ZD6021 with respect to intracellular Ca^{2+} signalling. Thus, major inter-individual differences in the susceptibility of NK_2 receptor blockade by compounds like saredutant or ZD6021 are not anticipated.

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