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Effects of Tyrosine289Phenylalanine Mutation on Binding and Functional Properties of the Human Tachykinin NK_2 Receptor Stably Expressed in Chinese Hamster Ovary Cells

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ABSTRACT. A point mutation was made at position 289 in the transmembrane segment 7 of the human tachykinin NK_2 receptor to yield a tyrosine/phenylalanine (Tyr/Phe) substitution. Chinese hamster ovary cells stably transfected with the wild-type or Tyr289Phe mutant NK_2 receptor both bound neurokinin A (NKA) and the synthetic NK_2 receptor-selective agonists, GR 64349 and [βAla^8]NKA(4–10), with high and even affinities. Neurokinin B (NKB) and substance P (SP) also displayed sizeable binding affinities, albeit with lower affinity as compared to NKA. In a functional assay (production of inositol-1,4,5-trisphosphate, IP_3), NKA, GR 64349, and [βAla^8]NKA(4–10) stimulated IP_3 accumulation via the wild-type and mutant receptors with similar potencies. On the other hand, NKB and SP exhibited a dramatic reduction in their agonist efficacies at the mutant receptor, NKB acting as a partial agonist (maximum effect = 50% of the response to NKA) and SP being totally inactive. The results obtained with phenoxybenzamine inactivation experiments indicated that a large and similar receptor reserve existed for both the wild-type and the mutant receptor. SP, which displayed sizeable binding affinity for the mutant receptor but did not stimulate IP_3 accumulation, antagonized the agonist effect of NKA. The antagonist action of SP at the mutant NK_2 receptor cannot be ascribed to receptor internalization. The Tyr/Phe replacement at position 289 markedly reduced the binding affinity and antagonist potency of the non-peptide ligand, SR 48968, without affecting the binding affinity and antagonist potency of the bicyclic peptide antagonist MEN 11420. The results indicate that the hydroxyl radical function of Tyr289 in transmembrane segment 7 of the human NK_2 receptor is, directly or indirectly, involved in stimulus transduction when the NK_2 receptor is occupied by NKB or SP, but not when using NKA or NK_2 receptor-selective agonists. BIOCHEM PHARMACOL **57**;8:899–906, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. NK_2 receptor; Chinese hamster ovary cells; inositol 1,4,5-trisphosphate; phenoxybenzamine; guanosine 5′-triphosphate

The tachykinin NK_2 receptor recognizes NKA‡ (with high affinity) and NKB and SP (with lower affinity), the natural tachykinins that have an established status of neurotransmitters in mammals [see 1, 2 for reviews]. The human tachykinin NK_2 receptor has been cloned and shown to be a seven transmembrane domain G-protein-coupled receptor. When expressed in CHO cells, the tachykinin NK_2 receptor is able to activate several transduction pathways, including the stimulation of phosphatidylinositol breakdown [3, 4]. There is relatively little information available on the structural motifs that determine the binding affinities of natural tachykinins or antagonists for the tachykinin NK_2 receptor and the molecular determinants of tachykinin NK_2 receptor coupling to G-proteins [5–8]. It has been reported [8] that the Tyr/Phe replacement at position 289 of the human NK_2 receptor induced a considerable reduction in the binding affinity of the non-peptide NK_2 receptor antagonist SR 48968 ((S)-*N*-methyl-*N*[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl]benzamide) [9] without affecting the binding affinity of NKA. In the course of an extensive mutational analysis of the pharmacology of the human NK_2 receptor stably expressed in CHO cells, we observed that the agonist efficacies of NKB and SP, as measured by their ability to induce IP_3 accumulation, are dramatically impaired by the

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‡ *Abbreviations:* NKA, neurokinin A; NKB, neurokinin B; SP, substance P; CHO, Chinese hamster ovary cells; Tyr289Phe, tyrosine 289 phenylalanine; IP_3, inositol 1, 4, 5 - triphosphate; FBS, fetal bovine serum; α - MEM, Eagle's minimum essential medium-α modification; DHFR, dihydrofolate reductase; PBZ, phenoxybenzamine; B_{max}, maximum binding; −OH, hydroxyl radical; and E_{max}, maximum effect.

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Tyr289Phe mutation. The reduced efficacies of these natural tachykinins were detected despite an unimpaired binding affinity, indicating that the −OH function of Tyr289 is important for transduction of the signal initiated by these two tachykinins.

MATERIALS AND METHODS

Materials

Penicillin, streptomycin, and trypsin were purchased from GIBCO. Dialyzed FBS was from Bio-Biowhittaker. α-MEM and GTP were from Sigma Chemical Co. Flasks and Petri dishes were from Falcon (Becton Dickinson). Minisorb tubes for radioimmunoassay were obtained from Nunc. Protein binding dye was from Bio-Rad. PBZ was from ICN Biomedicals. NKA, NKB, GR 64349 [Lys^3, Gly^8-R-lactam-Leu^9]NKA(3–10), [βAla^8]-NKA(4–10) and SP were from Neosystem Laboratoire. SR 48968 was kindly provided by Drs. X. Emonds-Alt and G. Le Fur (Sanofi Recherche, Montpellier, France). The bicyclic peptide antagonist MEN 11420 (c{[(β-D-GlcNAc)Asn-Asp-Trp-Phe-Dpr-Leu]c(2β-5β)}) [10] was synthesized at the Department of Chemistry of Menarini Ricerche, Florence. [^{125}I]NKA (specific activity, 2000 Ci $mmol^{-1}$) and the IP_3 assay system kit were purchased from Amersham. Other reagents were of the highest purity available from commercial sources.

Site-directed Mutagenesis of Human NK_2 Receptor cDNA

Plasmid pBS/hNK_2R (kindly provided by Dr. J. E. Krause, Washington University, St. Louis, MO) contains a 1.2-kb cDNA for the human NK_2 receptor cloned in the *Sma*I site of the pBlueScript II SK(−) phagemid. Site-directed mutagenesis of the NK_2 receptor cDNA was performed by the phosphorothioate technique of Eckstein [11, 12], using an *in vitro* mutagenesis kit (Sculptor™, Amersham) according to the manufacturer's instructions. Wild-type and Tyr289Phe-mutated cDNAs were isolated from *Hin*dIII+*Xba*I-digested pBS/hNK_2R and cloned into the polylinker region of a similarly digested pmCMVβSV1dhfr vector. pmCMVβSV1dhfr was constructed by removing granulocyte colony-stimulating factor cDNA sequences from *Xba*I-digested pmCMVβG-CSFSV1dhfr (plasmid murine cytomegalovirus beta granulocyte colony-stimulating factor SV1dhfr) [13]. The complete coding sequence of cDNAs for wild-type and mutated NK_2 receptors was confirmed by DNA sequencing.

Receptor Expression in CHO Cells

Large-scale preparation of vector DNA for transfection experiments was carried out using a Qiagen maxi-preparation column (Qiagen). Wild-type and (Tyr289Phe-) mutated NK_2 receptor cDNAs in pmCMVβSV1dhfr were introduced by lipofection as described [13] into DHFR-deficient CHO DUKX-B11 cells [14] (referred to as CHOdhfr$^-$). Stable DHFR$^+$ transformants were selected in nucleoside-free α-MEM containing 5% dialyzed FBS; 12–14 days after transfection, more than 100 individual DHFR$^+$ clones were pooled, grown to mass culture, and used for ligand binding and receptor activation studies. Cells stably expressing the human NK_2 receptor (CHO-hNK_2R cells) were cultured in α-MEM, supplemented with 10% dialyzed FBS, in a humidified atmosphere of 5% CO_2–95% air at 37° until slightly confluent. For subcultures, cells were dissociated with 0.25% trypsin/0.5 mM EDTA in saline and transferred at a 1:10 splitting ratio.

Ligand Binding Assay

Confluent cells from four 100-mm Petri dishes were harvested in PBS, pelleted by centrifugation at 200 *g* (4°), and homogenized using a Polytron PT3000 (Kinematica) at 13,000 rpm for 15 sec, in 20 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 10 μM thiorphan (buffer A). The homogenate was centrifuged for 1 hr at 25,000 *g* (4°) and the pellet resuspended in the binding buffer, composed by buffer A supplemented with 150 mM NaCl, 5 mM $MnCl_2$, and 0.1% BSA (buffer B, pH 7.4), at a protein concentration of about 0.35 mg/mL. The membranes (50 μg protein/assay) were incubated for 30 min at 20° with [^{125}I]NKA (120–140 pM) and various concentrations (0.01 nM–1 μM) of cold NKA (saturation experiments) or 10 different concentrations (0.01 nM–10 μM) of the competing compounds (competition experiments), in a final volume of 0.5 mL. Unlabeled NKA (1 μM) was used to define non-specific binding. In the experiments with GTP, the membranes were preincubated for 15 min at 20° with 1 or 10 μM GTP before the addition of the radioligand and of the various concentrations of cold NKA. The reaction was terminated by addition of 4 mL of ice-cold Tris–HCl (50 mM, pH 7.4) followed by rapid filtration through Whatman GF/B filter sheets (presoaked in 0.5% BSA for at least 3 hr) using a Brandel cell harvester. The filters were washed three times with 4 mL of ice-cold buffer. The trapped radioactivity was determined using a γ counter (Cobra, Packard Instruments Co.).

Measurement of Intracellular Levels of IP_3

IP_3 production was quantified using the procedure described by Oldham [15] and Challiss [16]. Cells were cultured in 35-mm Petri dishes until confluent and were then washed twice with α-MEM and preincubated with the vehicle or the antagonist for 30 min. The experiment was initiated by the addition of tachykinin receptor agonists or vehicle, and the incubation was stopped at the indicated time (kinetic experiments) or after 15 sec (concentration–response curves) by rapidly aspirating the medium and adding 1 mL of ice-cold 4% perchloric acid. Cells were kept on ice for 20 min, then the perchloric acid extract was removed while the Petri dishes containing the cell precip-

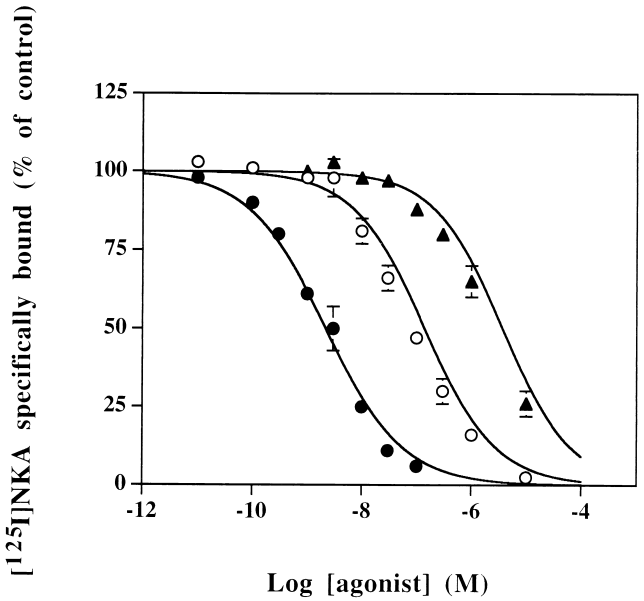

FIG. 1. Competition of [^{125}I]NKA binding to membranes of CHO cells transfected with the wild-type NK_2 receptor by increasing concentrations of NKA (closed circles), NKB (open circles), and SP (closed triangles), as described in Materials and Methods. The values reported are means ± SEM as generated by LIGAND (N = 4–12).

itate were stored for protein determination. The perchloric acid extract was centrifuged at 2000 *g* for 20 min at 4° to precipitate residual denatured proteins, and the resulting supernatant was titrated to pH 7.5 with 5 N KOH and kept on ice for 20 min. After removal of insoluble $KClO_4$ by centrifugation, supernatants were stored at −70° until assay. The IP_3 content was measured with a commercially available radioreceptor assay kit. The cell precipitate was removed from the Petri dishes by adding 0.1 M NaOH. The protein content of the NaOH solubilized samples was determined according to the protein binding dye method of Bradford [17] by measuring the optical density of the samples at 630 nm, using a multiwell plate reader (Ceres 900-UV, Packard Instruments). A standard calibration curve of BSA was run in parallel.

Treatment with PBZ

Confluent cells in 35-mm Petri dishes (for IP_3 formation) or in 75-cm^2 flasks (for binding experiments) were washed twice with PBS and preincubated for 30 min at 37° in a humidified atmosphere of 5% CO_2–95% air, in the presence or in the absence of PBZ, 10 nM for binding experiments and from 30 nM to 30 μM for IP_3 formation. After incubation, the cells were washed twice with PBS and then processed as described above.

Data Analysis

Saturation and competition data, obtained from separate experiments in duplicate, were processed by the method of Munson and Rodbard [18], by sequentially using EBDA [19] and LIGAND (National Institute of Health, Bethesda, MD) programs to determine the B_{max} and K_d for the radioligand and K_i for the competitors. Parameter values are expressed as means and standard error of the mean. Concentration–response curves for IP_3 production were analyzed by the MacAllfit for Macintosh program (Consorzio Mario Negri) in order to determine EC_{50} and IC_{50} values, which are reported as means and standard error of the mean. Means were compared by Student's *t*-test for unpaired samples: a P level <0.05 was considered significant.

RESULTS

Binding Affinities of the Natural Tachykinins for the Wild-type and the Mutant Human NK_2 Receptor

The levels of receptor expression for both the wild-type and Tyr289Phe mutant human NK_2 receptors transfected in CHO cells were comparable, as indicated by the B_{max} values (206 ± 28 and 252 ± 43 fmol/mg^{-1} protein, N=10 and 12, respectively) estimated from saturation binding experiments with [^{125}I]NKA. The natural tachykinins bound to the wild-type human NK_2 receptor with the expected rank order of affinity, NKA > NKB >>> SP (Fig. 1). Neither the rank order nor the affinity values of the three natural tachykinins were significantly affected by the Tyr289Phe mutation (Table 1). In particular, the K_i of NKB was 60-and 42-fold higher than that of NKA at the wild-type and Tyr289Phe mutant NK_2 receptors, respectively. The K_i of SP was 593-and 1183-fold higher than that of NKA at the wild-type and Tyr289Phe mutant NK_2 receptors, respectively (Table 1).

Tachykinin-induced IP_3 Production in CHO Cells Transfected with the Wild-type and the Mutant Human NK_2 Receptor

NKA and SP induced the production of intracellular IP_3 in wild-type cells with similar kinetics, showing a maximum at 15 sec and a relatively fast decay afterwards (Fig. 2, upper panel). A similar kinetic profile was obtained with NKA at

TABLE 1. Effects of tachykinin receptor agonists and antagonists on the binding of [^{125}I]NKA to the wild-type and Tyr289Phe mutant human NK_2 receptor

Ligand	Wild-type*	Tyr289Phe
NKA	2.3 ± 0.32‡ (10)	2.0 ± 0.32‡ (12)
NKB	138 ± 35 (4)	85 ± 5.3 (4)
SP	1366 ± 285 (4)	2366 ± 615 (4)
MEN 11420	2.8 ± 0.03 (4)	3.2 ± 0.38 (6)
SR 48968	0.16 ± 0.07 (6)	466 ± 73 (6)

Results were obtained as described in Materials and Methods. Numbers are means ± SEM of K_i or K_d ‡values (nM). Numbers in parentheses indicate the replicates of concentration–response curves used to generate mean K_i or K_d values.

*B_{max} value was 206 ± 28 fmol mg^{-1} protein.

†B_{max} value was 252 ± 43 fmol mg^{-1} protein.

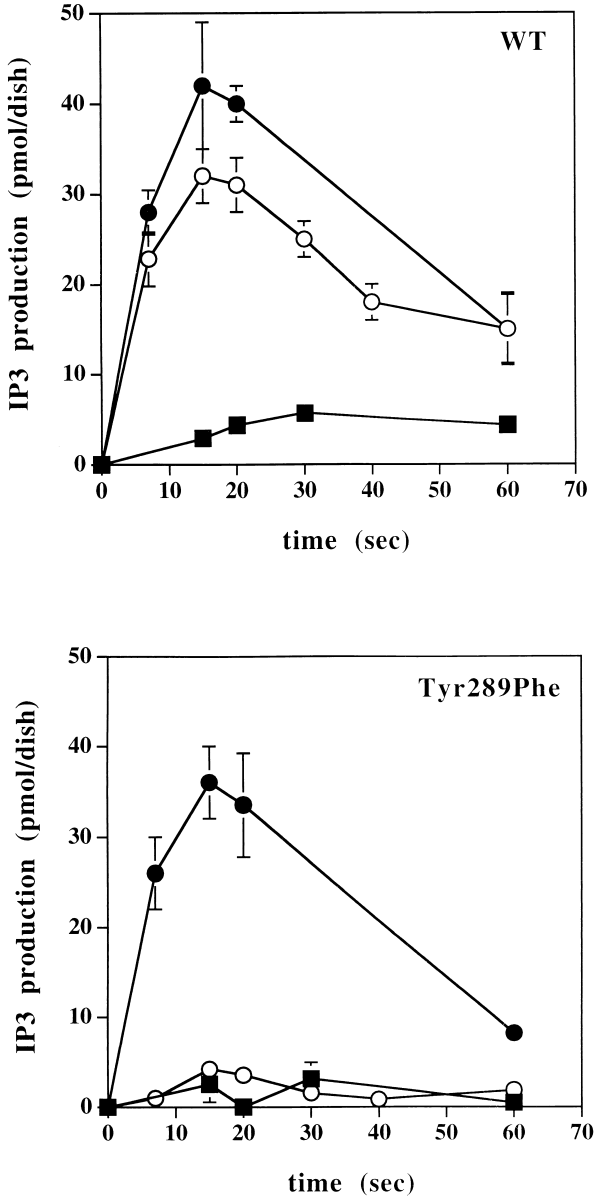

FIG. 2. Time dependence of IP_3 formation in CHO cells transfected with the wild-type NK_2 receptor (upper panel) and with the Tyr289Phe mutant receptor (lower panel). Confluent cells were washed and preincubated for 30 min in α-MEM, and were then exposed for the indicated time with vehicle (closed squares), 1 μM NKA (closed circles), and 10 μM SP (open circles). The amount of IP_3 produced was determined as described in Materials and Methods. For each agonist, the data are expressed as pmol IP_3/dish. Each value is the mean ± SEM of 6–9 measurements.

the Tyr289Phe mutant receptor (Fig. 2, lower panel). Therefore, a contact time of 15 sec was chosen for the further studies on the concentration dependency of IP_3 production.

As shown in Fig. 3, NKA produced a concentration-dependent formation of IP_3 at both the wild-type and the mutant receptor transfected cells. The potency of NKA was slightly affected by the Tyr289Phe mutation (2.8-fold reduction as compared to the wild-type receptor, Table 2), and its agonist efficacy was not significantly affected: in fact, the E_{max} for IP_3 accumulation in response to NKA averaged 171 ± 18 and 124 ± 15 pmol mg^{-1} protein for the wild-type and Tyr289Phe mutant receptors, respectively (N = 12 and 9, respectively, not significant).

The selective tachykinin NK_2 receptor agonists, GR 64349 and [βAla^8]NKA(4–10), proved to be full agonists at both the wild-type and Tyr289Phe mutant NK_2 receptors (Table 2): moreover, the agonist potencies of GR 64349 and [βAla^8]NKA(4–10) at the mutant receptor were not

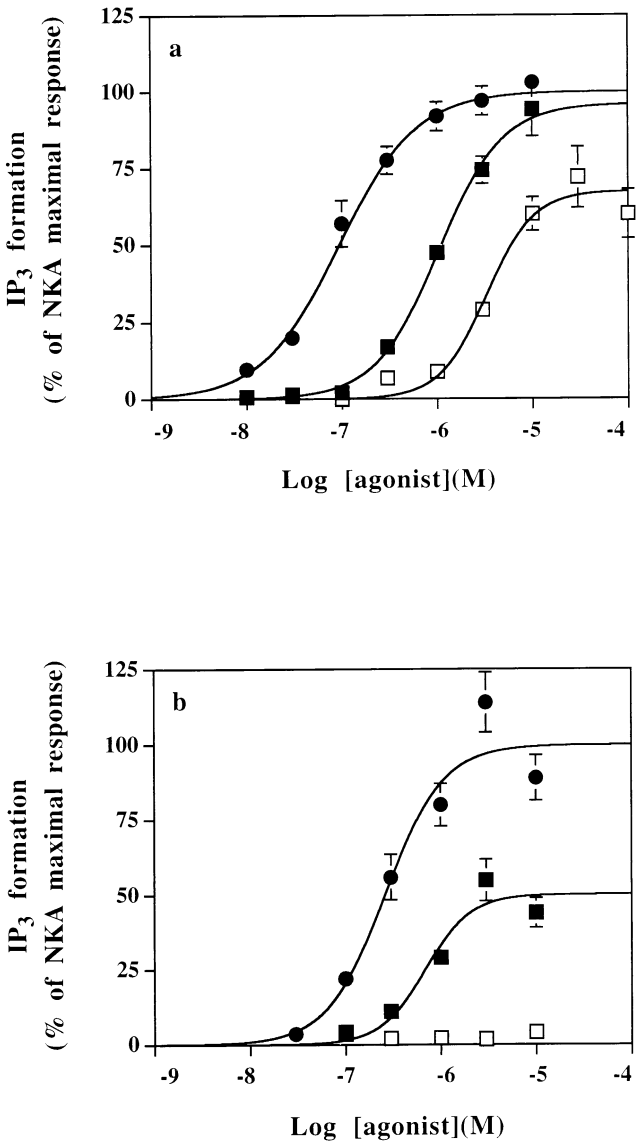

FIG. 3. Concentration dependence of IP_3 formation in CHO cells transfected with the wild-type NK_2 receptor (a) and with the Tyr289Phe mutant receptor (b). Confluent cells were washed and preincubated for 30 min in α-MEM, and were then exposed to increasing concentrations of NKA (closed circles), NKB (closed squares), and SP (open squares). The reaction was stopped after 15 sec and the amount of IP_3 produced was determined as described in Materials and Methods. For each agonist, the data are expressed as percentage of the maximal response to NKA. Each value is the mean ± SEM of 6–12 measurements.

TABLE 2. Effects of tachykinin receptor agonists and antagonists on the IP_3 formation in wild-type and Tyr289Phe mutant human NK_2 receptor

	Wild-type	Tyr289Phe
Agonists		
NKA	97 ± 10 (12)	268 ± 40 (9)*
[β-Ala^8]NKA(4-10)	265 ± 27 (6)	270 ± 36 (6)
GR 64349	72 ± 9 (6)	162 ± 39 (6)
NKB	954 ± 141 (6)	693 ± 100 (9)
SP	3,293 ± 577 (9)	inactive up to 10 μM
Antagonists		
MEN 11420	4.2 ± 0.6 (9)	2.6 ± 0.4 (6)
SR 48968	0.94 ± 0.19 (6)	inactive up to 1 μM

The cells were stimulated with increasing concentrations of agonists, and IP_3 formation was evaluated as described in Materials and Methods. For the antagonist concentration–response curves, cells were preincubated with increasing concentrations of compound and then exposed to NKA 0.3 μM and 1 μM for wild-type and Tyr289Phe receptor, respectively. EC_{50} and IC_{50} values (nM) are reported as means ± SEM. Numbers in parentheses indicate the replicates of concentration–response curves used to generate mean EC_{50} or IC_{50} values.

*$P < 0.05$ as compared to the wild-type receptor.

significantly different from those estimated at the wild-type receptor.

Consistent with their lower binding affinities for the wild-type NK_2 receptor as compared to NKA, the two natural tachykinins, NKB and SP, were less potent agonists (by a factor of about 10 and 34, respectively) than NKA in stimulating IP_3 formation in cells expressing this receptor. While NKB exhibited a full agonist profile, SP was found to be a partial agonist, giving a maximal response which averaged 67 ± 4% that of NKA (Fig. 3a). The efficacy of NKB and SP was markedly affected by the Tyr289Phe mutation. In particular, the E_{max} to NKB in producing IP_3 formation via the mutant NK_2 receptor was reduced to 50% of the maximal response to NKA (Fig. 3b). For this tachykinin, an EC_{50} could be calculated (693 ± 100 nM, Table 2), which, remarkably, was not significantly different from that estimated at the wild-type NK_2 receptor (954 ± 141 nM). We can thus conclude that the Tyr289Phe mutation determined a pure reduction in the efficacy of NKB as an agonist, without affecting its potency.

The effect of Tyr289Phe mutation on agonist efficiency was even more dramatic in the case of SP, which was completely inactive in producing IP_3 accumulation at the mutant NK_2 receptor up to a concentration of 10 μM (Figs. 2 and 3, Table 2). Notably, no agonist effect of SP at the mutant NK_2 receptor was obtained even when IP_3 levels were measured up to 60 sec from its addition (Fig. 2, lower panel).

Effect of GTP on Binding Affinity of NKA

The potencies of agonists in producing IP_3 accumulation was lower than their estimated binding affinities (Tables 1 and 2): for example, the EC_{50} of NKA in producing IP_3 accumulation was 42- and 134-fold higher than the estimated K_d values at the wild-type and mutant NK_2 receptor, respectively. The question was raised as to whether low GTP levels in the membrane preparation could be responsible for this difference. Indeed, the binding of NKA to the membrane preparation was found to be reduced by GTP: in particular, the affinity of NKA was reduced in the presence of GTP by about 3-fold for the wild-type receptor (K_d = 2.3, 7.0, and 6.3 nM in the absence and the presence of 1 or 10 μM GTP, respectively, N = 3–10) and by about 3- to 10-fold for the Tyr289Phe mutant receptor (K_d = 2.3, 12, and 22 nM in the absence and the presence of 1 or 10 μM GTP, respectively, N = 3–12).

SP as an Antagonist of NKA at the Tyr289Phe NK_2 Receptor

The profile of SP action at the Tyr289Phe mutant NK_2 receptor (sizeable binding affinity without appreciable agonist efficacy) is that typical of an antagonist molecule: indeed, when the cells transfected with the mutant Tyr289Phe NK_2 receptor were preincubated with SP (10 μM) for 30 min before the challenge with a submaximally effective concentration of NKA (1 μM), the agonist effect of NKA was markedly inhibited (Fig. 4).

Effect of Pretreatment with PBZ

The observed considerable reduction in the efficacies of certain agonists at the mutant NK_2 receptor may involve a different receptor reserve between the wild-type and mutant NK_2 receptor. We therefore examined whether receptor alkylation with PBZ differentially affected the binding

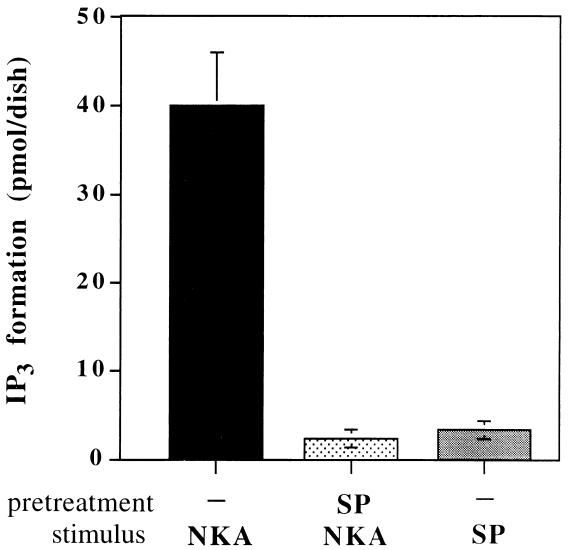

FIG. 4. Effects of SP on IP_3 formation at the Tyr289Phe NK_2 receptor. Confluent cells were washed and preincubated for 30 min in α-MEM in the absence or presence of 10 μM SP, and were then exposed to 1 μM NKA or 10 μM SP. The reaction was stopped after 15 sec and the amount of IP_3 produced was determined as described in Materials and Methods. Data are expressed as the net increase in IP_3 formation (pmol/dish). Each value is the mean ± SEM of 6–12 measurements.

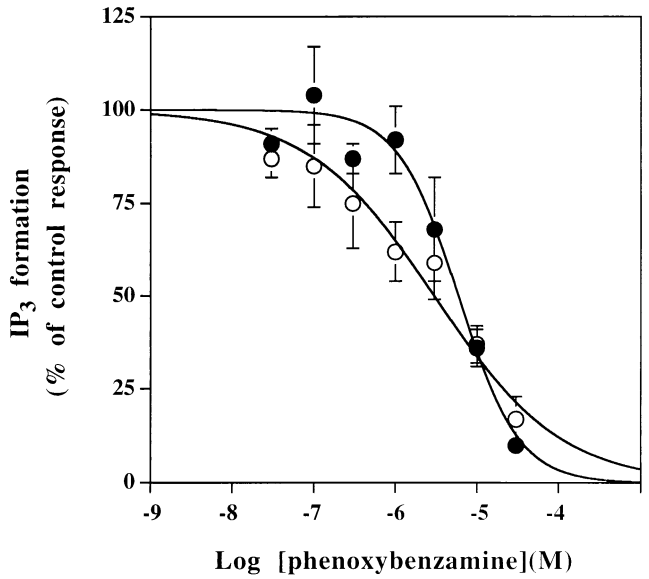

FIG. 5. Effect of PBZ on NKA-induced IP_3 formation. Confluent cells transfected with the wild-type NK_2 receptor (closed circles) or with the Tyr289Phe mutant receptor (open circles) were treated with increasing concentrations of PBZ. After a 30-min incubation, the cells were washed twice with PBS, processed as described in Materials and Methods, and then stimulated with 3 μM NKA. The reaction was stopped after 15 sec and the amount of IP_3 produced was determined as described in Materials and Methods. Each value is the mean ± SEM of 12 measurements.

affinity and agonist efficacy of NKA. At a concentration as low as 10 nM, which did not affect the NKA-induced IP_3 formation, PBZ pretreatment largely reduced the number of binding sites: the B_{max}, estimated by ^{125}I]NKA saturation binding experiments, was reduced to 25 ± 6 and 17 ± 5% of control (N=3 in each case) for the wild-type and mutant NK_2 receptor, respectively. Despite its large inhibitory effect on B_{max}, PBZ pretreatment, up to 100 nM, did not reduce IP_3 formation induced by NKA in either the wild-type or Tyr289Phe mutant NK_2 receptor (Fig. 5). Higher concentrations of PBZ produced a concentration-dependent inhibition of IP_3 formation induced by NKA (3 μM) in cells expressing either receptor form. Although the inhibitory effect of concentrations of PBZ in the range 0.1–0.3 μM appeared to be more intense on the Tyr289Phe mutant than on the wild-type receptor, the overall inhibitory effect on the response produced by activation of the two receptors was largely superimposable: the IC_{50} value of PBZ for inhibition of the NKA-induced IP_3 formation was not significantly different in the two transfected cell lines (IC_{50} = 5.9 ± 1.3 and 3.0 ± 1.0 μM for the wild-type and the mutant, respectively, Fig. 5).

Binding of Peptide and Non-peptide NK_2 Receptor Antagonists

The non-peptide NK_2 receptor antagonist SR 48968 and the bicyclic peptide NK_2 receptor antagonist MEN 11420 both showed high affinity for the wild-type human NK_2 receptor, SR 48968 being about 10-fold more potent than MEN 11420 (Table 1). Confirming the observations of Huang *et al.* [8], the replacement of Tyr289 by Phe dramatically affected the binding affinity of SR 48968: the calculated K_i of SR 48968 for the Tyr289Phe mutant was 1000-fold higher than that for the wild-type NK_2 receptor (Table 1). On the other hand, the binding affinity of MEN 11420 was not significantly affected by replacement of Tyr289 with Phe (Table 1).

Effect of Antagonists on NKA-induced IP_3 Accumulation

When checking the effect of the Tyr289Phe mutation on the potency of antagonists in the functional assay, it was found that MEN 11420 inhibited the IP_3 accumulation induced by a submaximally effective concentration of NKA with a potency similar to that observed at the wild-type NK_2 receptor (IC_{50} = 2.6 and 4.2 nM, respectively, Table 2). SR 48968 potently antagonized the NKA-induced IP_3 accumulation at the wild-type NK_2 receptor (IC_{50} = 0.94 nM), but was ineffective as an antagonist at the Tyr289Phe mutant up to 1 μM, according to its markedly reduced binding affinity.

DISCUSSION

The results obtained with NKA, GR 64349, [βAla^8]NKA(4–10) and MEN 11420 indicate that the Tyr/Phe replacement at position 289 did not produce important alterations in the whole structure of the NK_2 receptor protein. Moreover, the expression of the receptor by CHO cells occurred at comparable levels for the wild-type and the mutant receptor. Therefore, the observed impairment in the binding and functional properties of certain agonists (NKB, SP) and antagonists (SR 48968) can be reasonably assumed to be dependent upon discrete alterations in receptor structure and function involving the −OH function of Tyr 289.

A considerable quantitative discrepancy exists between the binding affinity of NKA and its potency in stimulating IP_3 formation for both the wild-type and Tyr289Phe mutant human NK_2 receptor. This discrepancy can be partly attributed to a low, non-physiological level of GTP in the membrane preparation as compared to that present in intact cells. In fact, exogenously added GTP decreased the binding affinity constant of NKA; despite this modification, the estimated binding affinity of NKA was still significantly higher than its agonist potency in producing IP_3 accumulation. On the other hand, no significant difference was found between the estimated binding affinities and antagonist potencies of MEN 11420 at the wild-type and Tyr289Phe mutant receptor. We hypothesize that the GTP-independent difference between K_d and EC_{50} values observed with NKA largely arises from kinetic factors. The EC_{50} values for IP_3 formation were estimated

under non-equilibrium conditions, since IP_3 formation is a rapid and transient event. In fact, when measuring the time–course of IP_3 formation in response to NKA, the maximal response was produced at 15 sec from the addition of the peptide. On the other hand, the binding affinity and antagonist potency of MEN 11420 in preventing IP_3 accumulation were both measured at equilibrium. Moreover, when measuring another end point as index of cellular activation by NKA, the production of prostaglandin E_2, we found that the EC_{50} of NKA (and other agonists as well) closely matches the estimated binding affinities [20]. Notably, the production of prostaglandin E_2 by NKA, which involves a different and independent signaling pathway as compared to IP_3 formation (activation of phospholipase A_2 and C, respectively), reaches equilibrium at 30 min from the addition of the agonist [20].

The ability of the Tyr289Phe mutation to affect the agonist efficacy of SP and NKB without affecting that of NKA and of selective NK_2 receptor agonists is the main new finding of this study. Huang *et al.* [8] previously reported that Tyr289Phe replacement does not affect the NKA binding affinity and agonist efficacy, but they did not investigate the behavior of other natural tachykinins at the mutant receptor.

The binding affinities of NKB and SP were not markedly altered by the Tyr289Phe mutation, indicating that the removal of the −OH function of Tyr289 affected the signaling capacity of the receptor but not its ability to interact with NKB and SP. This conclusion is further supported by the notion that the EC_{50} of NKB does not significantly differ between the wild-type and Tyr289Phe mutant receptor, although the agonist efficacy of NKB was reduced by about 50% at the latter receptor. On this basis, we conclude that the −OH function of Tyr289 is not directly involved in the binding of NKB and SP to the human NK_2 receptor but is, rather, essential for transducing the signal in response to receptor occupancy by these agonists. Interestingly, a nearby residue (Leu292) had been implicated in the binding of SP and NKB but not NKA to the human NK_2 receptor [6]. The results of PBZ alkylation experiments indicate that a large receptor reserve exists in CHO cells transfected with either the wild-type or Tyr289Phe mutant NK_2 receptor. In fact, a concentration of PBZ (10 nM) which did not significantly reduce the ability of NKA to induce IP_3 formation produced about 80% reduction in B_{max}. Our data indicate that the wild-type and Tyr289Phe mutant NK_2 receptor possess a substantially similar sensitivity to inactivation by PBZ with regard to their coupling efficiency in generating IP_3 formation in response to NKA. On the other hand, a minor difference between the mutant and the wild-type receptor exists with regard to sensitivity to PBZ pretreatment, especially at 0.3–1 μM concentration (Fig. 5). Therefore, it is not possible to totally exclude the possibility that the reduction in efficacy for certain agonists (NKB and SP) at the mutant receptor may involve, at least in part, a difference in receptor reserve. Our data demonstrate that SP is a partial agonist in producing IP_3 formation at the wild-type NK_2 receptor, with IP_3 formation kinetics very similar to that of NKA. SP still binds with sizeable affinity at the mutant NK_2 receptor, but it is evidently incapable of inducing an agonist response at this receptor. As discussed above, it is possible that a difference in receptor reserve between the wild-type and the mutant receptor may account for this result. On the other hand, the ability of SP to act as an NKA antagonist at the mutant receptor raises the possibility that SP (and NKB) select a different conformer of the NK_2 receptor from the one "selected" by NKA and that the −OH group of Tyr289 is more important for the former than for the latter interaction. The question was raised as to whether the "apparent antagonism" exerted by SP at the Tyr289Phe mutant NK_2 receptor may involve receptor internalization. To assess this point, we performed saturation experiments at 4° up to equilibrium (120 min) on intact cells after a preincubation for 30 min at 37° with 10 μM SP, and measured about 40% reduction in B_{max} with respect to non-treated cells. Considering the results of PBZ inactivation experiments, this effect cannot explain the failure of SP to induce IP_3 formation at the mutant NK_2 receptor, since a very large receptor reserve exists for producing this effect. The existence of different agonist-dependent conformers of the tachykinin NK_1 receptor has been recently proposed [21]; our results are consistent with the hypothesis that a similar phenomenon occurs with the NK_2 receptor, in particular that the agonist-bound conformer of the NK_2 receptor protein operated by SP differs from that operated by NKA. This hypothesis needs further experimental evaluation. The replacement of Tyr289 by Phe in the human NK_2 receptor has been reported to dramatically affect the binding affinity of the non-peptide NK_2 receptor antagonist, SR 48968, without affecting the binding affinity of NKA [8]. The exact role of the −OH group of Tyr289 in determining the full binding affinity of SR 48968 for the human NK_2 receptor has not been elucidated; given the small extent of the structural variation introduced, it may be speculated that the −OH group of Tyr289 is a direct point of interaction between the NK_2 receptor protein and a suitable moiety in the structure of SR 48968 [8]. An alternative explanation is that the receptor conformer with high affinity for SR 48968 is thermodynamically unaccessible if the −OH function of Tyr289 is lacking. The present findings indicate that the affinity of the peptide-based NK_2 receptor antagonist MEN 11420 is unchanged by Tyr289Phe mutation and establish a clear difference between peptide and non-peptide NK_2 receptor antagonists in their mode of interaction with the tachykinin NK_2 receptor, as suggested by earlier functional studies [22].

The indication that the −OH function of Tyr289 is involved, directly or indirectly, in the signaling capability of NK_2 receptors may have a bearing on the unsurmountable type of antagonism displayed by the non-peptide NK_2 receptor antagonist, SR 48968 in certain bioassays and species [9, 21, 23, 24], despite its competitive mode of

interaction in binding assays [9, 25]. The −OH group of Tyr289 seems crucial for the binding affinity of SR 48968 [8] and, being also involved in signal transduction, it may represent a site of interaction through which SR 48968 exerts a non-competitive type of antagonism. Since the non-competitive antagonism of SR 48968 has been observed when using NKA or [βAla^8]NKA(4–10) as agonists in certain bioassays [9, 21, 23, 24], the above interpretation implies that the NK_2 receptor conformer requiring the −OH function of Tyr 289 for a full signaling capability is physiologically relevant in intact tissues of some species.

In conclusion, the present findings demonstrate that the −OH group of Tyr289 is involved in IP_3 generation in response to natural tachykinins (NKB and SP) but not in response to NKA or synthetic NK_2 receptor agonists. These data suggest the existence of distinct agonist-bound conformers of the tachykinin NK_2 receptor.

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