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Residue 78 in the Second Transmembrane Domain of the Neurokinin-1 Receptor Is Important in Coupling High Affinity Agonist Binding to Multiple Second Messenger Responses

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SUMMARY

The neurokinin-1 tachykinin receptor is a member of the G protein-coupled receptor superfamily. An unusual feature of the neurokinin-1 receptor is the presence of glutamic acid (residue 78) in the second putative transmembrane domain, at the location of a highly conserved aspartate residue in the G protein-coupled receptor superfamily. The rat neurokinin-1 receptor cDNA was mutated to lysine, aspartate, and glutamine at this site and functionally expressed in Chinese hamster ovary cells, and clonal cell lines were isolated and characterized. Radioligand binding demonstrated that the Asp^{78} and Lys^{78} receptors have substance P binding affinities indistinguishable from those of the wild-type receptor and are expressed at roughly the same number of receptors per cell. The Gln^{78} receptor variant, on the other hand, exhibited no detectable agonist binding. Although wild-type and Asp^{78} receptors have essentially the same ability to stimulate inositol phospholipid turnover, cAMP production, and arachidonic acid release, the Lys^{78} variant is markedly attenuated in its ability to activate any of these pathways. These data indicate that residue 78 plays a role in the coupling of the rat neurokinin-1 receptor to cellular effectors. In addition, both Asp^{78} and Lys^{78} receptors show a greater percentage of high affinity binding that is resistant to guanosine-5′-*O*-(3-thio)triphosphate than does the wild-type receptor, indicating a potential difference in G protein coupling between wild-type and mutated receptors.

The SP-activated NK-1 tachykinin receptor is involved in diverse biological functions, including pain transmission, smooth muscle contraction, and neuronal excitation (1–3), and has been linked to several disease states, such as asthma, arthritis, inflammatory bowel disease, and migraine (4, 5). The NK-1 receptor activates intracellular effector proteins typically via coupling to pertussis toxin-insensitive G proteins. Primary sequence analysis has revealed that the GPCR family shares a high degree of structural similarity, with the areas of greatest homology occurring in the seven putative TMs (see Ref. 6 for review). It therefore seems reasonable that the TM residues conserved in virtually all members of this pharmacologically diverse group of receptors may dictate the tertiary structure required for receptor folding, structural integrity, and/or signal transduction.

Of particular interest in recent years, with regard to receptor structure-function considerations, has been an aspartate residue located in TMII, which is 98% conserved within the GPCR family. Family members not sharing this high degree of aspartate residue conservation (see Ref. 6 for review) are the NK-1, odorant F6, and odorant I7 receptors, which contain a glutamate at this position, the cytomegalovirus US33 and GnRH receptors, which have asparagine, the *Dictyostelium* cAMP receptor, which has phenylalanine, and the blue opsin receptor, which has glycine. Many GPCRs have been utilized to elucidate the structure-activity relationship of the highly conserved TMII aspartate. These receptors and the primary sequences of their putative TMII regions are illustrated in Table 1. The TMII aspartate was mutated to an asparagine residue in the AT_{1a} receptor (7), LH/CG receptor (8), α_2-AR (9–11), β_2-AR (12), muscarinic cholinergic subtype 1 receptor (13), and 5-HT_2 receptor (14), which were then functionally expressed. When ligand binding affinities of mutant receptors and their wild-type counterparts were compared, the mutant LH/CG receptor and

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ABBREVIATIONS: SP, substance P; NK, neurokinin; GPCR, G protein-coupled receptor; TM, transmembrane domain; GnRH, gonadotropin-releasing hormone; AT_{1a}, angiotensin II subtype 1a; LH, luteinizing hormone; CG, chorionic gonadotropin; AR, adrenergic receptor; 5-HT, 5-hydroxytryptamine; IP, inositol phosphate; CHO, Chinese hamster ovary; Y^{-1}-SP, Tyr^{-1}-substance P; Y^{-1}-SPFA, Tyr^{-1}-substance P free acid; Y^{-1}-SPOMe, Tyr^{-1}-substance P methyl ester; Y^{-1}-SPOEt, Tyr^{-1}-substance P ethyl ester; HPLC, high performance liquid chromatography; Gpp(NH)p, guanyl-5′-(β,γ-imido)diphosphate; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTP-γS, guanosine-5′-*O*-(3-thio)triphosphate; PCR, polymerase chain reaction.

TABLE 1

Sequence comparison of TMII within various GPCRs

The predicted TMII region of the rat NK-1 receptor is compared with corresponding sequences of other members of the GPCR family (35). The highlighted residue represents the position at which aspartic acid is highly conserved in this receptor superfamily with notable exceptions shown.

Receptor	Predicted TMII Sequence
Rat NK-1	F L V N L A F A E A C M A A F N T V V N F
Human NK-1	F L V N L A F A E A S M A A F N T V V N F
Human NK-2	F I V N L A L A D L C M A A F N A A F N F
Human NK-3	F L V N L A F S D A S M A A F N T L V N F
Human GnRH	L L K H L T L A N L L E T L I V M P L D G
Rat AT_1	F L L N L A L A D L C F L L T L P L W A V
Rat LH/CG	L M C N L S F A D F C M G L Y L L L I A S
Human α_{2A}-AR	F L V S L A S A D I L V A T L V I P F S L
Human β_{2A}-AR	F I T S L A C A D L V M G L A V V P F G A
Rat M_1[a]	F L L S L A C A D L I I G T F S M N L Y T
Rat 5-HT_2	F L M S L A I A D M L L G F L V M P V S M

[a] M_1, muscarinic cholinergic type 1.

β_2-AR exhibited a significant decrease in binding affinity for their respective agonists, whereas all other mutant receptors had comparable binding affinities, with only subtle variations being noted when multiple agonists and antagonists were tested. These studies indicate that variations in ligand binding affinity produced by the TMII aspartate to asparagine mutation are likely due to an indirect conformational change, rather than an alteration at the ligand binding site itself. With respect to signal transduction, all of the aforementioned mutant receptors, with the exception of the α_2-AR, lacked the ability to respond along the tested signal pathway(s). The Asn^{79} α_2-AR mutant exhibited coupling to both inhibition of cAMP production and suppression of calcium currents via a pertussis toxin-sensitive pathway but could not increase inwardly rectifying potassium currents. The receptor systems in which the mutants were tested for the ability of GTP to cause a rightward shift in binding affinity (β_2-AR and 5-HT_2 receptor) illustrated that the aspartate to asparagine mutants lost the GTP-induced rightward affinity shift seen with their wild-type counterparts. A previous study on the AT_{1a} receptor mutated the conserved aspartate not only to asparagine but also to glutamate (7). Although the resulting mutant AT_{1a} receptor exhibited normal binding, compared with the wild-type receptor, it demonstrated a lack of both phosphoinositide hydrolysis and calcium influx, a result identical to that for the aspartate to asparagine mutant. A similar structure-activity relationship study was conducted with the NK-1 receptor (15). As noted above, the wild-type NK-1 receptor has a glutamate instead of an aspartate in TMII (specifically, residue 78 in the NK-1 receptor). In this study, Glu^{78} was mutated to both glutamine and alanine, and both mutant receptors were transiently expressed in COS cells. Although wild-type and mutant receptors exhibited comparable binding of the nonpeptide antagonist L-703,606, the binding affinity of SP was reduced 28- and 379-fold with the Ala^{78} and Gln^{78} mutants, respectively. With respect to phosphoinositide hydrolysis, the NK-1 receptor TMII mutants yielded essentially no response, similarly to TMII mutagenesis studies performed with other GPCR family members. Of interest is whether all second messenger pathways activated by a single receptor (16) are affected by this mutation or whether transduction is altered only for certain pathways, as has been shown for the α_2-AR (11).

Glu^{78} in the NK-1 receptor is conserved among all currently cloned NK-1 receptors (17), including a recently characterized bullfrog NK-1 receptor,[1] whereas the NK-2 and NK-3 tachykinin receptors contain an aspartate. This striking conservation may be important for NK-1 receptor structure-function relationships; consequently, we replaced the wild-type glutamate residue with an aspartate, lysine, or glutamine. Stable transfection into CHO cells was performed and clonally selected cell lines were subjected to saturation and displacement binding analysis. Signaling studies were performed for total IP accumulation, cAMP production, and arachidonic acid release. Also, the effects of nonhydrolyzable GTP analogs on agonist binding to wild-type and residue 78 mutant NK-1 receptors were studied.

Experimental Procedures

Materials. *myo*-[^{3}H]Inositol (specific activity, 20 Ci/mmol) and [^{3}H]arachidonic acid (specific activity, 200 Ci/mmol) were from American Radiolabeled Chemicals, and the RIANEN ^{125}I-cAMP radioimmunoassay kit was from DuPont. Oligonucleotides, Y^{-1}-SP, SP, and Y^{-1}-SPFA were synthesized by the Protein and Nucleic Acid Chemistry Laboratory, Washington University School of Medicine, and peptides were purified by HPLC as described previously (18). Briefly, HPLC was performed on a Vydac C_4 column using an elution gradient of 15% to 40% acetonitrile, over 50 min, in 0.2 M sodium phosphate, pH 2.5. The retention time for Y^{-1}-SPFA was ~31 min. Other materials were the highest purity available from Sigma Chemical Co. or Fisher Scientific and have been described previously (19, 20).

Y^{-1}-SP ester derivatives. Y^{-1}-SPOMe and Y^{-1}-SPOEt were synthesized from 2.1 mg of Y^{-1}-SPFA by esterification with the appropriate alcohol (21). The products were purified by HPLC to ≥95% purity, as described above for Y^{-1}-SPFA. The retention times for Y^{-1}-SPOMe and Y^{-1}-SPOEt were ~36 min and ~39 min, respectively. For Y^{-1}-SPOMe, the yield was 1.1 mg (52%) of purified peptide. For Y^{-1}-SPOEt, the yield was 1.4 mg (67%).

Radioligand preparation, binding assays, and data analysis. Radioiodination of Y^{-1}-SP was performed using the chloramine T oxidative iodination procedure, and ^{125}I-Y^{-1}-SP was isolated by reverse phase HPLC, with >98% purity (specific activity, 2175 Ci/mmol). Ligand binding was performed as described previously (22). Briefly, for displacement binding, incubation of stably transfected CHO cells with 0.07 nM ^{125}I-Y^{-1}-SP was performed at 4° for 2 hr (to equilibrium), in the presence of 1 mg/ml BSA, 20 μg/ml chymostatin, 20 μg/ml leupeptin, 0.2 mg/ml bacitracin, and increasing amounts of unlabeled competitor. Total binding was determined by rapid filtration over no. 32 glass filters (Schleicher and Scheull). Nonspecific binding was defined as the counts bound in the presence of 1 μM SP and was <10% of total binding. IC_{50} values were determined by the equation $\log [B_I/(B_o - B_I)] = n_H \cdot \log [I] + n_H \cdot \log IC_{50}$, where B_I is the amount (cpm) of ^{125}I-Y^{-1}-SP specifically bound in the presence of unlabeled ligand, B_o is the amount specifically bound in the absence of unlabeled ligand, [I] is the the concentration of unlabeled ligand, and n_H is the indirect Hill coefficient. For saturation binding, stably transfected CHO cells were incubated for 2 hr at 4°. Nonspecific binding was determined for each concentration of ^{125}I-Y^{-1}-SP in the presence of 1 μM SP. Nonlinear regression analysis of the data was performed using the program LIGAND (23). Displacement binding assays on membrane preparations (24) were performed essentially as described above, using 20 μg of total protein/assay tube in 50 mM Tris·HCl, pH 7.4, 3.0 mM $MnCl_2$. The effect of GTPγS or Gpp(NH)p on ligand binding was assessed by preincubation of membranes for 30 min at 37° in buffer containing increasing amounts of GTPγS/

[1] M. A. Simmons and J. E. Krause, unpublished observations.

Gpp(NH)p. After preincubation, membrane homogenates were incubated for 2 hr at 4° with 0.07 nM ^{125}I-Y^{-1}-SP plus proteinase inhibitors (described above), followed by rapid filtration as described previously. Displacement binding studies assessing the effect of Na^+ were performed as described above but in an isotonic buffer (25) of the following composition: 121 mM NaCl or 121 mM *N*-methyl-D-glucamine chloride, 0.82 mM $CaCl_2$, 1.18 mM $MgCl_2$, 0.81 mM $MgSO_4$, 5.2 mM KCl, 11 mM glucose, 1 mg/ml BSA, 20 μg/ml chymostatin, 20 μg/ml leupeptin, and 0.2 mg/ml bacitracin.

Construction and expression of point-mutated NK-1 receptors. Glu[78] in the rat NK-1 receptor was mutated to aspartate, lysine, or glutamine using PCR. Two sequential reactions were performed. In the first PCR, two "half" receptors were generated, which contained the desired mutation in each half. The following oligonucleotides were used: for 5′ receptor halves, 5′ NK-1 (5′-CCAAGCTTGCGCCACCATGGATAACGTCCTTCC-3′), D[78] (5′-GCAGGCGTCAGCGAAGGCCA-3′), K[78] (5′-GCAGGCCTTAGCGAAGGCCA-3′), and Q[78] (5′-GCAGGCCTGAGCGAAGGCCA-3′) (the latter three were used for mutation to aspartate, lysine, and glutamine, respectively); for 3′ receptor halves, 3′ NK-1 (5′-CGCGGATCCTAGGCCAGCATGTTAGAGT-3′) and the complementary oligonucleotides for D[78], K[78], and Q[78]. Twelve cycles were performed for amplification. For the second reaction, the two halves of each mutant were combined by strand separation and reannealing of the halves in the reaction, followed by extension and 15 cycles of amplification. The full length cDNAs for each mutant were isolated from low-melting point agarose after electrophoresis and were subcloned into pBLUESCRIPT using *Hind*III and *Bam*HI. The nucleotide sequence of the cDNAs was verified (20), and the cDNAs were subcloned into the expression vector pM[2]. CHO cells, grown in minimal essential medium-α containing 10% fetal bovine serum, were stably transfected with the appropriate pM[2] construct using a calcium phosphate procedure described previously (22). After 6–10 days of selective growth in the presence of 0.8 mg/ml active G418, cell clones were isolated, propagated, and screened by ligand binding.

IP, cAMP, and arachidonic acid release assays. The accumulation of total [^{3}H]IPs was measured in the presence of LiCl, as described previously (20). Briefly, cells that had been prelabeled with *myo*-[^{3}H]inositol (10 μCi/ml) for 16–20 hr were washed with phosphate-buffered saline, harvested by scraping, and resuspended in phosphate-buffered saline containing 0.9 mM $CaCl_2$, 11 mM glucose, 20 mM LiCl, and 20 mM HEPES, pH 7.4, at a density of 4.0×10^5 cells/ml. After preincubation for 15 min at 37°, the cell aliquots were stimulated for 45 min at 37° with SP. Total IPs were purified and quantified from cell lysates using ion exchange methods.

cAMP assays were performed as described previously (20). Cells were washed, scraped, and resuspended in serum-free minimal essential medium-α plus 1.0 mM 3-isobutyl-1-methylxanthine, at a density of 1.0×10^6 cells/ml. After a 20-min preincubation at 37°, the cells were incubated for 15 min at 37° with SP plus 1.0 mg/ml BSA, 20 μg/ml chymostatin, 20 μg/ml leupeptin, and 0.2 mg/ml bacitracin. The reaction was terminated with the addition of 0.5 volume of 3.0 M trichloroacetic acid. After centrifugation and six extractions with diethyl ether, the cell lysates were subjected to a standardized RIANEN ^{125}I-cAMP radioimmunoassay.

Arachidonic acid release assays were performed using similar procedures previously established for muscarinic cholinergic subtype 1 receptor-expressing cells (26). Subconfluent cells plated in a 12-well format were labeled for 16–20 hr with [^{3}H]arachidonic acid (1 μCi/ml). The labeled cells were washed in the plate four times, for 10 min at 37°, with minimal essential medium-α plus 20 mM HEPES, pH 7.4, and 0.2% fatty acid-free BSA, to remove unincorporated [^{3}H]arachidonic acid. The cells were stimulated with SP in minimal essential medium-α plus 20 mM HEPES, pH 7.4, 0.2% fatty acid-free BSA, 20 μg/ml chymostatin, 20 μg/ml leupeptin, and 0.2 mg/ml bacitracin. At the indicated time the medium, which contained released [^{3}H]arachidonic acid that comigrated with authentic arachidonic acid in thin layer chromatography, was collected, centrifuged to remove cell debris, and counted in a scintillation counter.

Results

Radioligand Binding

Saturation binding. To determine the effect that rat NK-1 receptor mutations at residue 78 have on ligand binding, wild-type (i.e., Glu[78]) (R-NK1), Asp[78] (R-E78D), Lys[78] (R-E78K), and Gln[78] (R-E78Q) receptors were analyzed by saturation binding. ^{125}I-Y^{-1}-SP was used to determine the dissociation constants (K_d) and numbers of receptors/cell (B_{max}) for the wild-type and mutant receptors. The R-NK1, R-E78D, and R-E78K receptors bound ^{125}I-Y^{-1}-SP with similar affinities (Table 2). The B_{max} values for R-NK1, R-E78D, and R-E78K indicate that, in spite of subtle variations in the numbers of sites/cell (Table 2), the two mutant receptors are processed and appropriately expressed at the cell surface. On the other hand, although the R-E78Q mutant transcribed a comparable amount of mRNA (as determined by solution hybridization) and translated a protein with an appropriate apparent molecular weight in an *in vitro* translation assay, binding of ^{125}I-Y^{-1}-SP at concentrations as high as 0.7 nM was not detected (data not shown).

Displacement binding. To assess the ligand binding characteristics of the residue 78 mutant NK-1 receptors, displacement binding studies were performed. Displacement of ^{125}I-Y^{-1}-SP by SP, Y^{-1}-SPFA, Y^{-1}-SPOMe, Y^{-1}-SPOEt, and the nonpeptide antagonist CP-96,345 was performed. The IC_{50} values derived from the whole-cell binding assays indicate that the binding affinities of the mutant NK-1 receptors are either indistinguishable or minimally different from those of the wild-type receptor (Table 3). To demonstrate agonist binding to the R-E78Q mutant receptor, a displacement binding study was performed on membrane homogenates prepared from wild-type and mutant receptors, including R-E78Q. Displacement of 0.07 nM ^{125}I-Y^{-1}-SP with SP yielded IC_{50} values comparable to those determined in whole-cell binding studies, with the exception of R-E78Q, which demonstrated no detectable binding under these conditions (data not shown).

Regulation of ligand binding affinity by monovalent cations has been observed with members of the GPCR family such as the α_2-AR, dopamine D_2 receptor, and muscarinic acetylcholine receptor. It has been suggested that the carboxylate ion of the TMII aspartate residue pairs with Na^+ and allosterically modulates binding affinity. Displacement of ^{125}I-Y^{-1}-SP by SP was performed with both wild-type (Glu[78]) NK-1 receptors and residue 78 mutants, with particular attention to the effect of Na^+ versus the Na^+ substitute *N*-

TABLE 2

Saturation binding of wild-type and residue 78 mutant NK-1 receptors

All experiments were performed in duplicate on whole cells, and data are expressed as the average ± standard error of eight independent experiments.

Receptor	K_d	B_{max}
	nM	*10^3 sites/cell*
R-NK1	0.211 ± 0.043	240.2 ± 45.7
R-E78D	0.354 ± 0.064	318.3 ± 66.4
R-E78K	0.257 ± 0.024	205.6 ± 24.4
R-E78Q	ND[a]	ND

[a] ND, binding not detected.

TABLE 3

Displacement of ^{125}I-Y^{-1}-SP binding to wild-type and residue 78 mutant NK-1 receptors

Results were derived from displacement studies utilizing the indicated agonist or antagonist. Displacement studies were performed in duplicate on whole cells, as described in Experimental Procedures, and data are expressed as the average ± standard error. All experiments yielded a Hill coefficient of ~1. The numbers in parentheses represent the numbers of independent experiments performed.

Receptor	IC_{50}				
	SP	Y^{-1}-SPOMe	Y^{-1}-SPOEt	Y^{-1}-SPFA	CP-96,345
			nM		
R-NK1	1.1 ± 0.2 (3)	51 ± 2 (3)	32 ± 4 (4)	400 ± 47 (2)	27 ± 4 (3)
R-E78D	0.6 ± 0.08 (3)	13 ± 1 (2)	32 ± 1 (2)	390 ± 10 (2)	25 ± 7 (3)
R-E78K	0.6 ± 0.13 (3)	19 ± 2 (3)	59 ± 14 (4)	340 ± 20 (2)	84 ± 17 (3)

methyl-D-glucamine chloride (as described in Experimental Procedures). IC_{50} values derived for R-NK1, R-E78D, and R-E78K with and without Na^+ (0.43/0.40 nM, 1.18/1.19 nM, and 0.55/0.42 nM, respectively) suggest that no such effect is observed with the NK-1 receptor regardless of the residue 78 identity. A similar result was reported for the AT_{1a} receptor (7), where Na^+ had no major effect on K_d values for either wild-type (Asp^{74}) or mutated (Asn^{74} and Glu^{74}) receptors. In addition, saturation binding experiments were performed to study the effect of Na^+ on the binding of [^{3}H]SP to rat submaxillary-sublingual membranes (24). These data also showed no Na^+ effect on agonist binding. The ability of Na^+ to allosterically regulate ligand binding to GPCRs varies regardless of the identity of the conserved TMII residue (7). It is likely that additional unidentified receptor regions play a role in the allosteric regulation of agonist binding by Na^+. The displacement binding data coupled with the saturation binding data demonstrate that agonist binding to the residue 78 mutant NK-1 receptors R-E78D and R-E78K is virtually identical to that to the wild-type rat NK-1 receptor (Tables 2 and 3).

Second Messenger Response Characteristics

IP accumulation. The effects of the residue 78 NK-1 receptor mutations on SP-induced IP accumulation were examined as described in Experimental Procedures. Fig. 1A shows that, whereas the wild-type and R-E78D receptors yielded nearly identical dose-response curves, the R-E78K mutant produced no detectable IPs in paired experiments. Further analysis illustrated that even at SP concentrations as high as 10 μM the R-E78K mutant was unresponsive (data not shown). Dose-response studies performed with wild-type and R-E78D receptors yielded comparable EC_{50} values (0.69 nM and 0.30 nM, respectively) (Fig. 1B), suggesting that these two receptors possess similar abilities to activate phospholipase C. The levels of IP accumulation (4.4-fold above basal levels) and EC_{50} values are comparable to our previous data on wild-type rat NK-1 receptors (20) and provide sufficient levels of signal for us to conclude that the Lys^{78} mutation markedly attenuates SP-induced phosphoinositide hydrolysis. Additional assays were performed with particular attention to the SP-induced accumulation of IPs from the R-E78Q mutant. At SP concentrations as high as 10 μM, no [^{3}H]IPs were detected with the R-E78Q mutant (data not shown).

cAMP production. We and others previously demonstrated that wild-type rat NK-1 receptors stably expressed in CHO cells are able to mediate SP stimulation of adenylate cyclase, leading to measurable cAMP production (20, 22, 27). SP-stimulated cAMP production assays were performed with

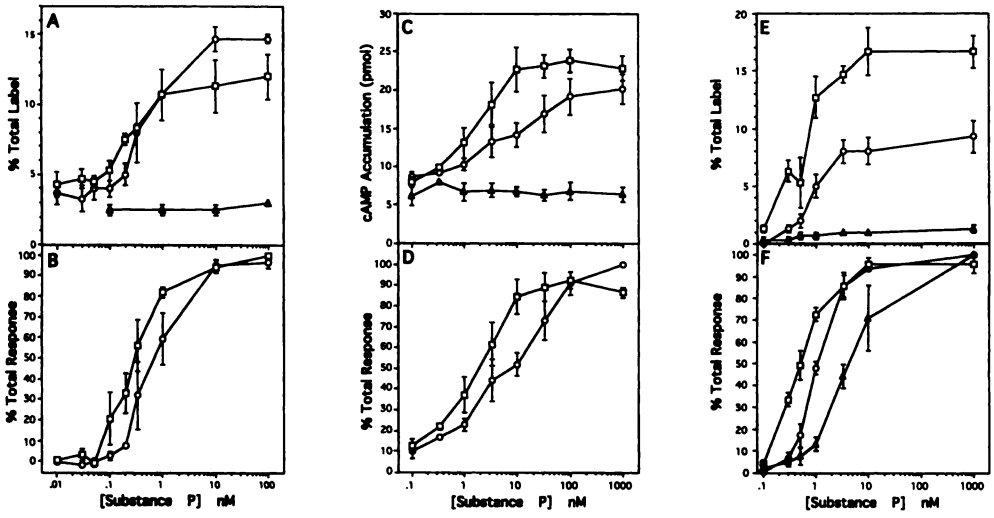

Fig. 1. SP stimulation of wild-type and residue 78 mutant NK-1 receptors. CHO cells stably transfected with the wild-type rat NK-1 receptor (○) or the residue 78 receptor mutant R-E78D (□) or R-E78K (△) were stimulated with increasing amounts of SP and assayed with respect to total IP accumulation (A and B), cAMP production (C and D), and arachidonic acid release (E and F), as described in Experimental Procedures. Upper panels A and E show % total label, which is defined as the amount of [^{3}H] IPs or [^{3}H] arachidonic acid produced or released, divided by the total cellular radioactivity incorporated. Upper panel C represents the total amount of cAMP produced. Lower panels B, D, and F represent the extent of response at the indicated agonist concentration divided by the maximum response. All experiments were performed in duplicate, with all three receptor forms being assayed concurrently, and each data point represents the average ± standard error of three independent experiments.

wild-type rat NK-1, R-E78D, and R-E78K receptors as described in Experimental Procedures. As illustrated in Fig. 1C, both wild-type and R-E78D receptors yielded a dose-dependent cAMP response, resulting in comparable levels of cAMP production. The R-E78K mutant produced no detectable cAMP increase at SP concentrations as high as 1 μM. Whereas the wild-type receptor yielded an EC_{50} for cAMP production of 6.4 nM, comparable to our previous results (20), the R-E78D mutant had a somewhat reduced EC_{50} of 1.8 nM (Fig. 1D). Using the cAMP production assay system described above, we detected a maximal production above basal levels of 11.4, 14.8, and 0.3 pmol of cAMP for wild-type, R-E78D, and R-E78K receptors, respectively. These data demonstrate a marked reduction in the ability of the R-E78K mutant receptor to activate adenylate cyclase.

Arachidonic acid release. Phospholipase A_2 activity leads to the direct release of arachidonic acid from a variety of phospholipids that, along with products of further oxidative metabolism, are involved in a variety of physiological responses. Agonist-stimulated arachidonic acid release has recently been demonstrated for the NK-2 receptor expressed in CHO cells (28). To determine whether the R-E78K mutant can stimulate the release of arachidonic acid, wild-type and residue 78 mutant NK-1 receptors were subjected to an [^{3}H]arachidonic acid labeling procedure and examined for agonist-stimulated arachidonic acid release as described in Experimental Procedures. As illustrated in Fig. 1, E and F, both wild-type and R-E78D mutant NK-1 receptors exhibited a dose-dependent and saturable arachidonic acid release response to SP stimulation (Fig. 1E). The R-E78K mutant remained largely unresponsive to stimulation by SP. The arachidonate release assay provided 29- and 40-fold stimulation over basal levels for R-NK1 and R-E78D, respectively. This sensitive assay illustrated two points not seen in the previous response assays, i.e., 1) the basal and maximal responses from the R-E78D mutant were reproducibly greater than those from the wild-type receptor and 2) the R-E78K mutant demonstrated a limited but detectable response, culminating in a maximal stimulation of 3.3-fold over basal levels. No detectable arachidonic acid release was observed in untransfected CHO cells stimulated with up to 1.0 μM SP, demonstrating that the detectable arachidonic acid release observed in the R-E78K cell line was not due to endogenous NK-1 receptor expression. The R-E78D mutant possessed a somewhat lower EC_{50}, compared with that of wild-type NK-1 receptors (0.5 nM and 1.2 nM, respectively). The enhanced sensitivity of the assay resulted in determination of an EC_{50} for R-E78K of 4.3 nM.

Regulation of ligand binding by G proteins. With respect to GPCRs, GTP and nonhydrolyzable analogs cause a shift in the receptor from a higher to a lower affinity state. This shift is generally considered to indicate an effective interaction with one or more G proteins. Because the ability of the R-E78K receptor to transduce signals to various effector proteins was severely attenuated (Fig. 1), an experiment designed to assess coupling to G proteins was performed. Membrane preparations derived from CHO cells stably transfected with wild-type, R-E78D, or R-E78K receptors were preincubated with increasing amounts of the nonhydrolyzable GTP analog GTPγS before a 2-hr incubation with 0.07 nM ^{125}I-Y^{-1}-SP (see Experimental Procedures). The amount of bound ligand was determined and expressed as a percentage of the maximal binding in the absence of GTPγS (Fig. 2). The results demonstrated a 68% decrease in high affinity binding with the wild-type receptor, whereas the R-E78D and R-E78K mutant receptors showed 43% and 34% decreases, respectively. Comparable data were obtained when Gpp(NH)p was used as the GTP analog (data not shown).

Discussion

The results presented in this study further document the importance of the highly conserved acidic residue in TMII of GPCRs. The NK-1 receptor is unique, in that it possesses a glutamate at position 78, compared with the aspartate in most other GPCRs, as described in the introduction. In contrast to the AT_{1a} receptor, in which glutamate was unable to replace aspartate at this position with regard to maintenance of intracellular signaling, the R-E78D receptor, stably transfected into CHO cells, responds similarly to the wild-type receptor with respect to agonist activation of IP turnover, cAMP accumulation, and arachidonic acid release. This receptor mutant, as well as the R-E78K mutant, shows normal agonist and antagonist binding, in contrast to the β_2-AR (12) and LH/CG receptor (8), where mutation of the conserved aspartate residue leads to dramatic binding affinity changes. Although alteration of the charge characteristics of this residue with a glutamate to lysine conversion results in a NK-1 receptor that is folded and assembled on the cell surface, the receptor is unable to activate IP and cAMP responses and only weakly activates arachidonic acid release. Recently, Huang *et al.* (15) mutated residue 78 in the human NK-1 receptor to alanine and glutamine; the former mutant dis-

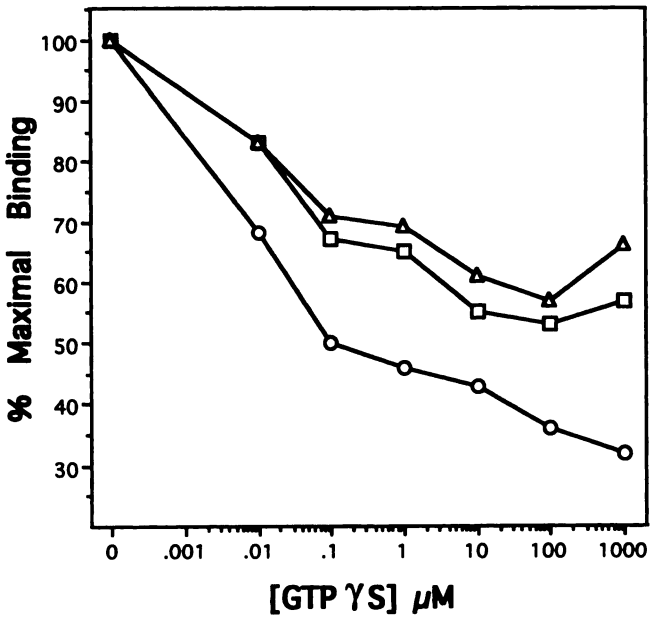

Fig. 2. Effect of GTPγS on SP binding to wild-type and residue 78 mutant NK-1 receptors. CHO cells stably transfected with the wild-type rat NK-1 receptor (○) or the residue 78 receptor mutant R-E78D (□) or R-E78K (△) were harvested and used to prepare membrane preparations. Membrane preparations were preincubated with increasing amounts of GTPγS, incubated with 0.07 nM ^{125}I-Y^{-1}-SP, and subjected to rapid filtration as described in Experimental Procedures. Data are plotted as the percentage of maximal binding that occurred in the absence of GTPγS. Each data point represents the average of two independent experiments performed in duplicate, with the standard error being <12% of the mean value reported.

played a 28-fold decrease in agonist displacement of antagonist binding, while lacking IP responsiveness, and the latter displayed a 379-fold decrease in agonist displacement of antagonist binding, with IP responsiveness being reduced by 80%. Because these binding studies were performed on membrane preparations, it was not clear that the mutant receptors were properly assembled on the cell surface. Taken together, these results document the importance of the TMII acidic residue in receptor-effector coupling processes and highlight the differences in the effects of mutations at this site among several members of the GPCR superfamily.

The NK-1 receptor has been shown previously to directly activate IP metabolism and cAMP production in transfected cell systems (20, 27). As cited in these references, IP metabolism is a typical NK-1 receptor-mediated response, whereas cAMP production may be relevant in only a limited number of cellular situations. Activation of arachidonic acid release has not been an extensively studied property of the NK-1 receptor, although astrocytes and some other cells have been shown to release arachidonic acid or its metabolic products in response to SP stimulation (29, 30). NKA activation of arachidonic acid release has recently been reported in an NK-2 receptor-transfected CHO cell line (28), and it has been concluded that this effector system requires the activation of a G protein complex distinct from $G_{\alpha q/11}$, in conjunction with activation of protein kinase C and stimulation of extracellular Ca^{2+} entry. The EC_{50} for SP stimulation of arachidonic acid release in rat NK-1 receptor-transfected CHO cells is similar to that for the stimulation of IP metabolism (1.2 nM and 0.7 nM, respectively). In our studies, the mechanism by which SP activates arachidonic acid release is not completely understood and is currently under investigation. Based on our finding that the arachidonic acid release assay is more sensitive than the IP or cAMP assays, the detection of an arachidonate release response with the R-E78K mutant may be due to this increased assay sensitivity. Consequently, this NK-1 receptor mutant, although severely compromised, is not completely dysfunctional. To date, the clearest example of a TMII aspartate mutation that results in a separation of effector system activity is that of the α_2-AR, in which an aspartate to Asn^{79} mutation results in blockade of K^+ current coupling but retention of Ca^{2+} current and adenylate cyclase coupling (11). Our mutational studies with the rat NK-1 receptor residue 78 mutants have not clearly demonstrated a separation of multiple effector system activities.

Although the agonist and antagonist binding pockets of R-NK1, R-E78D, and R-E78K appear to be similar, the R-E78K mutant receptor displays a marked attenuation of its coupling to phosphoinositide turnover, cAMP production, and arachidonic acid release, indicating that linkage to effector systems requires more than high affinity binding of agonist. Structure-activity relationship studies with both GnRH and AT_{1a} receptors (31, 32) suggest that a hydrogen-bonding network involving the highly conserved TMII residue (Asn^{87} and Asp^{74} in the GnRH and AT_{1a} receptors, respectively) and a conserved residue in TMVII may be important for this linkage. Similar interhelical interactions may take place in the NK-1 receptor.

In the presence of high concentrations of GTPγS, $^{125}I\text{-}Y^{-1}$-SP binding to the wild-type NK-1 receptor is reduced to 32% of its original value (Fig. 2), a reduction somewhat less than, but consistent with, results from other studies of the NK-1 receptor in tissue preparations (22, 33). Because the concentration of $^{125}I\text{-}Y^{-1}$-SP used in this study is such that it binds only to the high affinity state of the receptor, the data show that ~30% of high affinity binding is GTPγS insensitive. This may be the result of a population of receptors in a state that exhibits high affinity for the receptor but is not coupled to G proteins. A receptor in such a state has been proposed to exist based on mutational analysis of ARs and is represented as R* in the extended or allosteric ternary complex model proposed by Samama *et al.* (34).

The mutant receptors R-E78D and R-D78K show a greater percentage of binding that is resistant to GTPγS than does the wild-type receptor (Fig. 2). This suggests that for both mutant receptors, compared with wild-type receptor, a greater percentage of high affinity binding interactions may occur with uncoupled receptors (as HR*), rather than with coupled receptors (as HR*G). This may be the result of decreased affinity of the G protein complex for the receptor and/or decreased ability of SP to stabilize the R*G interaction [which corresponds to a decrease in the constants M and/or α, as defined by Samama *et al.* (34)]. There are several possible explanations as to why binding to R-E78K displays some GTPγS sensitivity but exhibits little or no second messenger response. One potential mechanism involves the association of G proteins with R-E78K receptors that do not activate effector pathways or activate second messenger pathways that have not yet been identified. An alternative explanation is that, although G proteins can associate with R-E78K, the interaction is altered from the native situation in such a way as to impair the release and activation of the G proteins. Although regulation of binding by guanine nucleotides may be a gross measure of receptor-G protein coupling, this study demonstrates that this is not necessarily predictive of efficient second messenger production.

In summary, whereas the identity of the highly conserved TMII residue in NK-1 receptors (Glu^{78}) appears to play a minimal role in high affinity agonist binding, its role in signal transduction is critical. Although it is not mechanistically clear how this residue is involved, it may play a role in maintaining receptor conformation, as a result of its charge or hydrogen-bonding properties, in concert with other conserved TM residues. To probe further the functional nature of these conserved regions, studies designed to assess the possible involvement of hydrogen bonding in the signal transduction process of the NK-1 receptor are currently underway.

References

1. Pernow, B. Substance P. *Pharmacol. Rev.* **35**:85–141 (1983).
2. Maggio, J. E. Tachykinins. *Annu. Rev. Neurosci.* **11**:13–28 (1988).
3. Helke, C. J., J. E. Krause, P. W. Mantyh, R. Couture, and M. J. Bannon. Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors and regulatory mechanisms. *FASEB. J.* **4**:1606–1615 (1990).
4. Payan, D. G. Neuropeptides and inflamation: the role of substance P. *Annu. Rev. Med.* **40**:341–352 (1989).
5. Maggi, C. A., R. Patacchini, P. Rovero, and A. Giachetti. Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.* **13**: 23–93 (1993).
6. Probst, W. C., L. A. Snyder, D. I. Schuster, J. Brosius, and S. C. Sealfon. Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol.* **11**:1–20 (1992).
7. Bihoreau, C., C. Monnot, E. Davies, B. Teutsch, K. E. Bernstein, P. Corvol, and E. Clauser. Mutation of Asp^{74} of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling. *Proc. Natl. Acad. Sci. USA* **90**:5133–5137 (1993).
8. Ji, I., and T. H. Ji. Asp^{383} in the second transmembrane domain of the lutropin receptor is important for high affinity hormone binding and cAMP production. *J. Biol. Chem.* **266**:14953–14957 (1991).

9. Horstman, D. A., S. Brandon, A. L. Wilson, C. A. Guyer, E. J. J. Cragoe, and L. E. Limbird. An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* **265**:21590–21595 (1990).
10. Wang, C.-D., M. A. Buck, and C. M. Fraser. Site-directed mutagenesis of α_{2A}-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation of agonists. *Mol. Pharmacol.* **40**:168–179 (1991).
11. Surprenant, A., D. A. Horstman, H. Akbarali, and L. E. Limbird. A point mutation of the α_2-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science (Washington D. C.)* **257**:977–980 (1992).
12. Chung, F.-Z., C.-D. Wang, P. C. Potter, J. C. Venter, and C. M. Fraser. Site-directed mutagenesis and continuous expression of human β-adrenergic receptors. *J. Biol. Chem.* **263**:4052–4055 (1988).
13. Fraser, C. M., C.-D. Wang, D. A. Robinson, J. D. Gocayne, and J. C. Venter. Site-directed mutagenesis of m_1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**:840–847 (1989).
14. Wang, C.-D., T. K. Gallaher, and J. C. Shih. Site-directed mutagenesis of the serotonin 5-hydroxytrypamine$_2$ receptor: identification of amino acids necessary for ligand binding and receptor activation. *Mol. Pharmacol.* **43**:931–940 (1993).
15. Huang, R. C., H. Yu, C. D. Strader, and T. M. Fong. Interaction of substance P with the second and seventh transmembrane domains of the neurokinin-1 receptor. *Biochemistry* **33**:3007–3013 (1994).
16. Milligan, G. Mechanisms of multifunctional signalling by G protein-linked receptors. *Trends Pharmacol. Sci.* **14**:239–244 (1993).
17. Krause, J. E., P. Blount, and B. S. Sachais. Molecular biology of receptors, in *The Tachykinin Receptors* (S. H. Buck, ed.). Humana Press, Totowa, NJ, 165–218 (1994).
18. MacDonald, M. R., J. Takeda, C. M. Rice, and J. E. Krause. Multiple tachykinins are produced and secreted upon post-translational processing of the three substance P precursor proteins, α, β, and γ preprotachykinin. *J. Biol. Chem.* **264**:15578–15592 (1989).
19. Hershey, A. D., and J. E. Krause. Molecular characterization of a functional cDNA encoding the rat substance P receptor. *Science (Washington D. C.)* **247**:958–962 (1990).
20. Blount, P., and J. E. Krause. Functional nonequivalence of structurally homologous domains of neurokinin-1 and neurokinin-2 type tachykinin receptors. *J. Biol. Chem.* **269**:16388–16395 (1993).
21. Watson, S. P., B. E. B. Sandberg, M. R. Hanley, and L. L. Iversen. Tissue selectivity of substance P alkyl esters: suggestive of multiple receptors. *Eur. J. Pharmacol.* **87**:77–84 (1983).
22. Takeda, Y., P. Blount, B. S. Sachais, A. D. Hershey, R. Raddatz, and J. E. Krause. Ligand binding kinetics of substance P and neurokinin A receptors stably expressed in Chinese hamster ovary cells and evidence for differential stimulation of inositol 1,4,5-trisphosphate and cyclic AMP second messenger responses. *J. Neurochem.* **59**:740–745 (1992).
23. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
24. Lee, C.-M., J. A. Javitch, and S. H. Snyder. ^{3}H-Substance P binding to salivary gland membranes: regulation by guanyl nucleotides and divalent cations. *Mol. Pharmacol.* **23**:563–569 (1983).
25. Buettner, K., and M. Ascoli. Na^+ modulates the affinity of the lutropin/choriogonadotropin receptor. *J. Biol. Chem.* **259**:15078–15084 (1984).
26. Felder, C. C., R. Y. Kanterman, A. L. Ma, and J. Axelrod. A transfected m1 muscarinic acetylcholine receptor stimulates adenylate cyclase via phosphatidylinositol hydrolysis. *J. Biol. Chem.* **264**:20356–20362 (1989).
27. Nakajima, Y., K. Tsuchida, M. Negishi, S. Ito, and S. Nakanishi. Direct linkage of three tachykinin receptors to stimulation of both phosphotidylinositol hydrolysis and cyclic AMP cascades in transfected Chinese hamster ovary cells. *J. Biol. Chem.* **267**:2437–2442 (1992).
28. Arkinstall, S., I. Emergy, D. Church, A. Chollet, and E. Kawashima. Calcium influx and protein kinase Cα activation mediate arachidonic acid mobilization by the human NK-2 receptor expressed in Chinese hamster ovary cells. *FEBS Lett.* **338**:75–80 (1994).
29. Marriot, D. R., G. P. Wilkin, and J. N. Wood. Substance P-induced release of prostaglandins from astrocytes: regional specialization and correlation with phosphoinositol metabolism. *J. Neurochem.* **56**:259–265 (1991).
30. Devillier, P., G. M. Acker, C. Advenier, J. Marsac, D. Regoli, and N. Frossard. Activation of an epithelial neurokinin NK-1 receptor induces relaxation of rat trachea through release of prostaglandin E_2. *J. Pharmacol. Exp. Ther.* **263**:767–772 (1992).
31. Zhou, W., C. Flanagan, J. A. Ballesteros, K. Konvicka, J. S. Davidson, H. Weinstein, R. P. Millar, and S. C. Sealfon. A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor. *Mol. Pharmacol.* **45**:165–170 (1994).
32. Marie, J., B. Maigret, M.-P. Joseph, R. Larguier, S. Nouet, C. Lombard, and J.-C. Bonnafous. Tyr^{292} in the seventh transmembrane domain of the AT_{1a} angiotensin II receptor is essential for its coupling to phospholipase C. *J. Biol. Chem.* **269**:20815–20818 (1994).
33. Cascieri, M. A., E. Ber, T. M. Fong, S. Sadowski, A. Bansal, C. J. Swain, E. Seward, B. Frances, D. Burns, and C. D. Strader. Characterization of the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor. *Mol. Pharmacol.* **42**:458–463 (1992).
34. Samama, P., S. Cotecchia, T. Costa, and R. J. Lefkowitz. A mutation-induced activated state of the β_2-adrenergic receptor: extending the ternary complex model. *J. Biol. Chem.* **268**:4625–4636 (1993).
35. Peroutka, S. J., ed. *CRC Handbook of Receptors and Channels*. CRC Press, Boca Raton, Florida (1994).

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