A Single Mutation of the Neurokinin-2 (NK2) Receptor Prevents Agonist-induced Desensitization

DIVERGENT CONFORMATIONAL REQUIREMENTS FOR NK2 RECEPTOR SIGNALING AND AGONIST-INDUCED DESENSITIZATION IN XENOPUS OOCYTES

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Receptor activation and agonist-induced desensitization of the human neurokinin-2 (NK2) receptor expressed in Xenopus oocytes have been investigated. When neurokinin A (NKA) was applied repeatedly at 5-min intervals, the second and subsequent applications gave no responses. This desensitization was not observed with the specific agonists (Lys3,Gly8-R-gave no responses. This desensitization was not observed in

In contrast, the protein kinase C inhibitor staurosporine, stimulation with GR64349 or (Nle10)-NKA(4–10). However, in the presence of the protein kinase inhibitor staurosporine, stimulation with GR64349 or (Nle10)-NKA(4–10) induced receptor desensitization. In contrast, the protein kinase C inhibitor Ro-31–8220 was not able to enhance GR64349-mediated desensitization. We created a mutation (F248S) in the third cytoplasmic loop of NK2 that impairs NKA-induced desensitization. In the presence of either staurosporine or Ro-31–8220, the mutant receptor was desensitized in response to NKA application but not to GR64349. Also, truncation mutants Δ62 and Δ87, lacking serine and threonine residues in the cytoplasmic COOH-terminal tail, were functionally active and were partially resistant to desensitization. These observations indicate that 1) there are different conformational requirements for NK2 receptor signaling and agonist-induced desensitization, 2) the third intracellular loop and the cytoplasmic tail of NK2 are functional domains important for agonist-induced desensitization, and 3) some agonists at the NK2 receptor cause much more desensitization than others and suggest that this might result from phosphorylation by receptor-specific kinases and other non-identified protein kinases.

The NK2 receptor is a cell surface receptor mediating the actions of the tachykinin peptide neurokinin A (NKA, substance K) in the central and peripheral nervous system (1). NK2 belongs to the superfamily of receptors coupled to G proteins. Stimulation of G protein-coupled receptors by specific ligands triggers intracellular signaling pathways via activation of G proteins, which regulate several effector enzymes and second messenger production. Prolonged or repeated agonist stimulation induces desensitization of receptor activity. For several G protein-coupled receptors, the mechanism of desensitization has been shown to involve agonist-induced phosphorylation of the receptor by a receptor-specific kinase and uncoupling of the receptor from the effector enzyme (2). Binding of agonist by receptors stabilizes an active conformation that couples to G protein. Likewise, an agonist-induced conformational mechanism is believed to promote interaction and activation of receptor-specific kinase (3).

When expressed in Xenopus oocytes, the NK2 receptor causes release of Ca2+ from intracellular stores as a result of stimulation of phospholipase C and production of inositol trisphosphate. Elevation of intracellular Ca2+ concentration activates Ca2+-dependent chloride channels, which can be electrophysiologically recorded. In this report, we have investigated the signaling and acute desensitization of NK2 receptor expressed in Xenopus oocytes in response to agonists of different chemical structures. We have examined the roles of a point mutation in the third cytoplasmic loop (F248S) and deletions in the cytoplasmic tail (Δ62 and Δ87) in G protein activation and desensitization.

EXPERIMENTAL PROCEDURES

Materials—NKA, neuropeptide K (NPK) and (Nle10)-NKA(4–10) (N(4–10)) were from Bachem (Bubendorf, Switzerland). GR64349 was synthesized as described (4). SP6 and T7 polymerases were from Promega. XhoI and BsmAI endonucleases were from New England Biolabs. Staurorosine, bacitracin, chymostatin, leupeptin, benzamidine, MS222, and collagenase were from Sigma. Ro-31–8220 was from Calbiochem. [3H]SR48698 (28 Ci/mmol) was obtained from Amersham (Buckinghamshire, United Kingdom).

Expression Vectors for NK2 and Mutants—The gene encoding the human ileum NK2 receptor (5) was subcloned, downstream of the SP6 polymerase initiation site, in a transcription vector (pGEMNK2) (6). The pGEMNK2 vector was optimized for efficient transcription, containing a short 5′-non-translated region with low secondary structure forming potential (7) and a Kozak eukaryotic consensus sequence for efficient initiation of translation at AUG (8). NK2 deletions mutants Δ62 and Δ87 were obtained by cutting pGEMNK2 vector at unique sites using restriction endonucleases XhoI or BsmAI, respectively. NK2 site-specific mutants F248S and F248A were constructed by polymerase chain reaction and subcloned into pBlueScript. F248E mutant was constructed by the method of Kunkel (25) using the following primer (sense strand): 5′-GCCAAGAAGGATGTTAGAGG-3′ (mutation underlined). All constructs were confirmed by restriction mapping and DNA sequencing. Capped mRNA transcripts for NK2 and mutants were obtained by run-off transcription of linearized pGEMNK2 or pBlueScript plasmids with SP6 polymerase or T7 polymerase, respectively, in the presence of capping nucleotide using the RNA capping kit from Stratagene. The yield was approximately 10 μg of capped mRNA from 1 μg of plasmid DNA. The mRNA was used directly for injection into oocytes.

Preparation and Microinjection of Oocytes—The female frogs (Xenopus laevis) were anesthetized by immersion in 0.4% MS222 at room temperature for about 15–30 min and killed by decapitation. To remove follicle cells, oocytes were collected and incubated with collagenase 0.2% in 50 ml of OR2 medium without Ca2+ and Mg2+ (OR2 = 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na2HPO4, 15 mM Hepes, 2 mM CaCl2, 1 mM MgCl2),
pH adjusted to 7.6) in tubes under slow agitation for 1 h at room temperature. The oocytes were washed with OR2 medium and incubated again for 1 h with a fresh solution of collagenase (0.2%) in OR2. When inspection indicated that most of the oocytes were free from their follicles, the oocytes were rinsed carefully with OR2 medium and then with ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM sodium pyruvate, 5 mM HEPES, and adjusted to pH 7.5 with NaOH). Oocytes were allowed 1–2 h for recovery after collagenase treatment before selecting stages V–VI for injections. Selected oocytes were incubated in filtered ND96 containing 100 μM penicillin, 100 μM streptomycin, overnight at 18°C before injection. Oocytes were microinjected using an Inject-Matic air pump (Gabay). Injection needles were made from calibrated Drummond capillaries of 0.6 μl volume. mRNA (25 ng in 50 nl) was injected into the cytoplasm. Oocytes were individually transferred to wells of 96-well flat bottom culture plates and incubated in ND96 at 18°C for 24 h before voltage-clamp recording or for 22 h before Ca2+ efflux assays.

Electrophysiology—Electrophysiological recordings were carried out on oocytes superfused with OR2 medium at 18–20°C under voltage-clamped conditions using two microelectrodes (1–2 MΩ, both filled with 3 M KCl). The membrane potential was clamped routinely at −100 mV using a Gene Clamp 500 (Axon) instrument. Each test substance was dissolved in OR2 medium and applied by constant flow superfusion over 6 s. Staurosporine (1 mM) and Ro31–8220 (5 mM) were applied externally by addition to the medium 5 min before testing and to the superfusate.

Preparation of Oocyte Membrane Fraction—The membranes were prepared according to Kobilka (9) with the following modifications. Oocytes were homogenized, 24 h after injection, in ice-cold buffer A (75 mM Tris-HCl, pH 7.4, 12.5 mM MgCl2, 1 mM EDTA, 30% sucrose, 0.15 mg/ml benzamidine, 0.1 mg/ml bacitracin) by passing the mixture through a pipette tip equipped with a 200-μl tip ten times. The homogenate was centrifuged at 3,000 × g for 10 min to remove pigment granules. The supernatant fraction was centrifuged at 100,000 × g for 10 min. The supernatant was then centrifuged at 400,000 × g for 30 min. The pellet, containing the membranes, was resuspended in buffer A in a sonicator bath for 5 min. Protein concentration was determined by the method of Lowry et al. (10) using bovine serum albumin as a standard. Typically, the yield was about 10 μg of total membrane protein per oocyte. Membrane fractions were stored at −80°C.

Binding Assays—[3H]JSR48968 binding assays on membrane fraction from 12 oocytes were performed by incubation for 1 h at ambient temperature in a 0.5-ml total volume of binding assay medium comprising 50 mM Tris- HCl, pH 7.4, 100 mM NaCl, 3 mM MnCl2, 0.2 mg/ml bovine serum albumin, 40 μg/ml bacitracin, 0.8 mg/ml chymotatin, 1.6 mg/ml leupeptin. Separation of protein-bound ligand from free ligand was achieved by rapid filtration on Whatman GF/B filters, using a Brandel cell harvester, followed by washing with three washes with ice-cold 50 mM Tris-HCl, pH 7.4. The filters were then dried and counted in a Beckman LS-5000CE liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH2 (R396). Km and Bmax values were determined by Scatchard analysis. Saturation binding curves and Scatchard plots were calculated using the programs RadLig and Grafit.

Ca2+ Mobilization Assays—45Ca2+ efflux assays were performed as described (11) with the following modifications. Pools of 10 oocytes were incubated in 0.2 ml of ND96 containing 45Ca2+ (75 μCi/ml) for 2 h at 18°C. They were then carefully washed with 0.3 ml of ND96. This medium was changed every 10 min for 50 min. NKA or GR64349 was added at 0.1 μM for 1 min. Oocytes were then washed twice and incubated for 15 min in 0.2 ml of ND96. The medium was removed and added to 3 ml of scintillation fluid and counted.

RESULTS

Receptors and Ligands—The predicted structure of the human NK2 receptor and mutants is shown in Fig. 1. Mutants F248S, F248A, and F248E contain a single amino acid change in the COOH-terminal portion of the third cytoplasmic loop, a region that is known to be important for G protein interaction. Mutants Δ62 and Δ87 are truncated forms of the receptor with 62 and 87 amino acids deleted from the COOH terminus, respectively. Δ62 lacks 15 serine/threonine residues, and Δ87 lacks all of the 18 serine/threonine residues as well as the putative palmitoylation site at cysteines 324–325 of the cytoplasmic COOH-terminal domain.

NK2 ligands used in this study are listed in Table I. The decapeptide NKA and its elongated form NPK are natural ligands acting on NK2 receptor. The modified peptides GR64349 (4) and (Nle10)-NKA(4–10)(N(4–10)) (12) are shorter forms of NKA that have high selectivity and potency at NK2.

Functional Expression of NK2 Receptors in Xenopus Oocytes—Oocytes were injected with 25 ng of cRNA for NK2 or mutants and stimulated by superfusion with NK2 agonist after 24 h, under voltage-clamp at a holding potential of −100 mV. Electrophysiological recordings shown in Fig. 2 are characteristic of inward Ca2+-dependent chloride currents elicited by NK2 agonists at 1 μM. Similar results were obtained at 0.1 μM. The waveforms generated by wild-type NK2 and NK2 mutants (F248S, F248A, F248E, Δ62, or Δ87) were very similar. The onset of response for F248E was delayed with a mean latency of 13 ± 3 s compared to 4 ± 1 s for wild-type NK2 and the other mutants. The current amplitudes varied from oocyte to oocyte, but the average values for NK2 and mutants F248S, F248A, or F248E were not significantly different. Moreover, for a given receptor, the amplitudes were independent of the structure of the ligand used to elicit the signal at 1 μM (Fig. 3). Oocytes not injected with cRNA did not elicit currents in response to NKA. The amplitude of inward current peak was function of the amount of cRNA injected. Typically, for NK2 after 24 h, the responses at 2.5 and 0.25 ng of cRNA were 20–50% and 2–10%, respectively, of the response at 25 ng of cRNA.

Functional activity of NK2 and mutant F248S was further evaluated by Ca2+ efflux assay (11). Activation of either NK2 or mutant F248S by NKA or GR64349, at 0.1–1 μM concentrations, produced identical acceleration of 45Ca2+ efflux (Fig. 4).

To characterize NK2 and mutant receptors expressed in Xenopus oocytes, equilibrium binding studies with the specific NK2 antagonist [3H]JSR48968 were performed on membrane preparations of oocytes in at least two independent experiments. Each receptor preparation showed a single class of saturable binding sites with Kd in the range from 0.5 to 1.0 nM. The Kd values were comparable to the Kd values of 2.7 ± 0.3 nM for NK2 in COS cells (13). The maximum binding capacity Bmax varied from batch to batch of oocytes in the range of 70–160 fmol/mg of total membrane protein.

![Schematic diagram showing the F248S point mutation in the third intracellular loop (black dot) and Δ62 and Δ87 truncation mutations in the cytoplasmic tail.](http://www.jbc.org/)

**FIG. 1.** Putative structure of human NK2 receptor and mutants. Schematic diagram showing the F248S point mutation in the third intracellular loop (black dot) and Δ62 and Δ87 truncation mutations in the cytoplasmic tail. Gray dots represent serine or threonine residues in cytoplasmic loops that are potential sites for phosphorylation by G protein receptor kinases.
Desensitization Mechanisms of the NK2 Receptor

**Fig. 2.** Ca\(^{2+}\)-dependent chloride current evoked by stimulation of NK2 receptor in X. laevis oocytes. Oocytes were injected with 25 ng of cRNA into the cytoplasm and incubated at 18 °C for 24 h. Representative traces of voltage-clamp recordings for wild-type NK2 and NK2 mutant F248S after stimulation with the appropriate ligand (1 μM, 6 s) are shown. The oocytes were washed for 5 min before the second stimulation. In each panel, traces from left to right are recordings after the first and second stimulation, respectively. Typical data obtained from three different oocyte batches are shown.

![Image](image)

**Fig. 3.** Agonist-induced Ca\(^{2+}\)-dependent Cl\(^{-}\) currents elicited by various ligands in oocytes injected with wild-type NK2 or F248S mutant cRNA. Oocytes were stimulated with agonist (1 μM, 6 s) 24 h postinjection (empty bars). Oocytes were then washed for 5 min and stimulated a second time in the same conditions (filled bars). Data are mean ± S.E. of 5 oocytes from the same source. Comparable results were obtained in three to six separate batches of oocytes.

![Image](image)

**Fig. 4.** Effects of NK2 agonists on 45Ca\(^{2+}\) efflux from Xenopus oocytes injected with wild-type NK2 or F248S mutant receptor cRNA. Oocytes were loaded with 45CaCl\(_2\) 22 h after injection and then stimulated with 0.1 μM agonist for 1 min as described in Ref. 11. Each bar represents the mean 45Ca\(^{2+}\) efflux ± S.E. from triplicates of 10 oocytes in a representative experiment.

![Image](image)

Agonist-induced Desensitization of NK2 Receptor—Restimulation of oocytes expressing wild-type NK2 with 1 μM NKA 5 min after the first stimulation failed to elicit chloride channel activity (Figs. 2 and 3). This lack of response lasted for more than 30 min and even up to 1–3 h in some batches. Quite strikingly, this lack of response was observed only when NKA or NPK was used as a ligand at a 0.1 or 1 μM concentration. In contrast, second stimulation by either GR64349 or N(4–10) produced a chloride channel activity comparable to the first stimulation in amplitude but slightly greater in latency of response by an average of 2–3 s. Moreover, up to four repeated stimulations by 1 μM GR64349 at 5-min intervals gave responses, whereas a single stimulation with NKA at a concentration 10-fold lower (0.1 μM) caused desensitization. Identical results were obtained with oocytes expressing the mutant receptor F248A. When oocytes were co-injected with cRNA for NK2 and interleukin 8 receptor (IL8RA), the NKA-induced desensitization of NK2 did not affect the ability of IL8RA to function in response to stimulation with 1 μM interleukin 8 (data not shown) indicating NK2 receptor desensitization rather than depletion of inositol trisphosphate-sensitive intracellular Ca\(^{2+}\) stores. When oocytes expressing NK2 were injected with 1 pmol of heparin 30 min prior to 1 μM NKA stimulation, the desensitization was clearly attenuated in 70% of the oocytes tested with an amplitude for the second response equal to 10–20% of the initial response (Fig. 5). Treatment of the oocytes expressing NK2 with either the protein kinase inhibitor staurosporine (1 μM) or the specific PKC inhibitor Ro-31–8220 (5 μM), before and during ligand application, had no effect on NKA-induced desensitization. Surprisingly, incubation of the oocytes with staurosporine, but not with Ro-31–8220, restored the ability of the NK2 receptor to desensitize in response to either GR64349 or N(4–10) (Figs. 6 and 7).

A Point Mutation in the Third Intracellular Loop Prevents NK2 Desensitization—A phenylalanine to serine mutation at position 248 in the third intracellular loop of NK2 has no noticeable effect on [3H]SR48968 binding affinity (K\(_{d}\) = 0.62 ± 0.18 nM compared to 0.54 ± 0.1 nM for wild-type NK2). Receptor signal transduction by NK2 or NK2 F248S was identical as assayed by activation of chloride currents or Ca\(^{2+}\) efflux (Figs. 3 and 4). Moreover, stimulation of receptors by NKA, NPK, N(4–10), or GR64349 gave responses of the same amplitude. However, the ability of NK2 F248S to desensitize on repeated application of NKA at 5-min intervals was totally impaired. When oocytes expressing the mutant F248S were treated with the protein kinase inhibitor staurosporine (1 μM) or Ro-31–8220 (5 μM) before and during stimulation with agonists, the ability of the receptor to desensitize in response to NKA was restored. However, and in contrast to the wild-type NK2, the F248S mutant receptor produced unaltered responses to repeated exposures to 1 μM GR64349 regardless of the presence or absence of either Ro-31–8220 or staurosporine (Figs. 6 and 7). The mutant F248A in which phenylalanine at position 248 was replaced by alanine was indistinguishable from wild-type NK2 in regard to functional activation or desensitization (data not shown). We created the mutant F248E, which substitutes a
agonists (Table I) on the NK2 receptor expressed in F248S mutant with identical maximum efficacy in Ca$^{2+}$ potent agonists able to stimulate either wild-type NK2 or phosphorylation of NK2 (Figs. 2 and 3). Both NKA and GR64349 are agonists tested elicited chloride currents, these compounds from the COOH-terminal portion minimum for tachykinin receptor activation. In comparison, NKA can be viewed as a long agonist with amino-terminal extension. The amino-terminal moiety in NK2 may make additional contacts with the NK2 receptor, favoring one conformation that is recognized by receptor kinases. In support of this hypothesis, the extended NKA analog NPK behaves as NKA. In contrast, short agonist lacking this extension is unable to confer this conformation. Alternatively, the amino-terminal portion of the long peptides may lock, or modulate, the binding conformation of the COOH-terminal moiety by intramolecular interactions. Our results do not allow us to distinguish between these two possibilities. Thus, the amino-terminal portion of NKA may have a dual role in, on one hand, stabilizing a conformation leading to receptor kinase activation and desensitization and, on the other hand, conferring selectivity for NK2 over NK1 and NK3 receptor subtypes.

The lack of NK2 desensitization in response to either GR64349 or N(4–10) and the reversal of this effect in presence of staurosporine suggests the involvement of some phosphorylation event hindering receptor phosphorylation by G protein receptor kinases. The absence of effect of Ro-31–8220 in enhancing GR64349-mediated desensitization suggests that this phosphorylation is probably mediated by protein kinase A rather than PKC. The protein kinase recognizing the GR64349-activated NK2 conformer remains yet unidentified. Recently, it has been shown that agonists of different structures bind to α2 adrenergic receptors causing different conformational changes and different degrees of Gs and Gi coupling (16). The existence of divergent conformational requirements for angiotensin II receptor internalization and signaling has been recently proposed (17). Taken together with our results, this clearly indicates that G protein-coupled receptors may have multiple active conformers displaying different specificity for agonist binding and distinct functional activities.

The substance P receptor (NK1) was shown to be a substrate of β-adrenergic receptor kinase 1 and 2 in vitro (18), and attenuation of agonist-induced desensitization of NK1 by truncation of the COOH terminus was demonstrated (19). Similar observations were reported for α1β adrenergic receptor (20) or rhodopsin in vivo (21). Desensitization of the NK2 receptor in oocytes was recently reported (22), but the mechanism of desensitization remained unclear. In this work, two lines of evidence suggest a mechanism of desensitization by phosphorylation of the NK2 receptor.

First, truncations in the COOH-terminal part of the receptor (Δ62 and Δ87), removing serine and threonine sites of phosphorylation, produced active receptors displaying attenuated desensitization. Thus, the COOH terminus of NK2 plays a role in acute desensitization (24). This is in contrast to Josiah et al. (22) that indicated no activity for NK2 Δ81 mutant and com-
plete desensitization for NK2 Δ62 mutant. These discrepancies remain unexplained. The difference in loss of desensitization of the Δ62 mutant compared to the Δ87 mutant suggests that the COOH-terminal 62 residues play only a minimal role and that the COOH-terminal region proximal to the seventh transmembrane domain is determinant for NK2 receptor desensitization. We cannot exclude, however, that the increased loss of desensitization for the Δ87 mutant may be reflective of its reduced activity compared to wild type NK2 (Fig. 8).

Second, injection of heparin, a known inhibitor of receptor-specific kinases, caused a partial resistance to desensitization. This effect was small (10–20%) and occurred only at high heparin concentration (≥1 µM), in contrast to the β2-adrenergic receptor where desensitization was completely abolished by 1 µM heparin (23). Therefore, we cannot exclude that the observed impairment of NK2 desensitization by heparin may be mediated by inhibition of PKC or other signaling pathways. In the absence of a specific inhibitor of G protein receptor kinase, it is difficult to probe the exact role of these kinases in regulation of NK2 desensitization by phosphorylation. Thus, we cannot exclude a mechanism involving other protein kinases (24).

A phenylalanine to serine mutation in the COOH-terminal part of the third cytoplasmic loop of the NK2 receptor reveals the existence of a functional domain important for desensitization. This region had already been shown to be an important determinant for G protein interaction. Our data indicate that specific change of functional groups, or conformational changes, in the cytoplasmic region proximal to the sixth transmembrane segment may alter G protein receptor kinase recognition or activation. The F248S mutation creates a consensus site for phosphorylation by protein kinase C (...S<sup>248</sup>VK...). However, in G protein-coupled receptor systems, receptor phosphorylation causes signal attenuation, whereas here we observe the opposite effect. The differences observed between F248A and F248S, or F248E mutations combined with the effect of staurosporine and Ro-31-8220 implies an agonist-induced phosphorylation event. Our findings are consistent with the hypothesis that agonist-induced conformational changes in the third cytoplasmic loop expose Ser<sup>248</sup> or other sites on the cytoplasmic face of the receptor to a kinase, probably PKC. The resulting phosphorylation alters G protein receptor kinase recognition or activation, thus preventing agonist-induced desensitization. In support of this hypothesis, mutant F248E, which substitutes an acidic residue at position 248 and resembles phosphorylated serine both sterically and electronically, was defective in desensitization. Interestingly, in the case of the mutant F248S, this mechanism functions for receptor interaction with NKA but not GR64349, whereas in the case of wild-type NK2 the differential effect on desensitization is observed with GR64349 and not NKA. This further demonstrates the existence of multiple induced active conformations of G protein-coupled receptors and the key role of the position 248 in modulating these conformations.

In summary, our results indicate that agonist-induced conformational changes of NK2 in oocytes leading to G protein activation and desensitization can be dissociated at the molecular level. We have identified one site in the third cytoplasmic loop that is important for NK2 receptor desensitization. Also, we have shown that the COOH-terminal tail is a functional domain for desensitization. Depending on the structure of the ligand, agonist-occupied receptor may adopt distinct conformations that are functionally active with respect to G protein activation but show different susceptibility to kinases involved in desensitization. Because desensitization is a regulatory mechanism controlling cellular activity, the effect of analog may last longer compared to the natural ligand and alter long term cell behavior. Therefore, the results reported here may have important implications in pharmacology.

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