Disulfide Bridge Engineering in the Tachykinin NK_1 Receptor†

Christian E. Elling,* Ute Raffetseder, Søren Møller Nielsen, and Thue W. Schwartz

Laboratory for Molecular Pharmacology, Department of Pharmacology, Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark

Received July 30, 1999; Revised Manuscript Received November 8, 1999

ABSTRACT: As in most other seven-transmembrane receptors, the central disulfide bridge from the extracellular end of TM-III to the middle of the second extracellular loop was essential for ligand binding in the NK_1 receptor. However, introduction of "extra", single Cys residues in the second extracellular loop, at positions where disease-associated Cys substitutions impair receptor function in the vasopressin V_2 receptor and in rhodopsin, did not cause mispairing with the Cys residues involved in this central disulfide bridge. Cys residues were introduced in the N-terminal extension and in the third extracellular loop, respectively, in such a way that disulfide bridge formation could be monitored by loss of substance P binding and breakage of the bridge could be monitored by gain of ligand binding. This disulfide bridge formed spontaneously in the whole population of receptors and could be titrated with low concentrations of reducing agent, dithiothreitol. Another putative disulfide bridge "switch" was constructed at the extracellular ends of TM-V and -VI, i.e., at positions where a high-affinity zinc site previously had been constructed with His substitutions. Disulfide bridge formation at this position, monitored by loss of binding of the nonpeptide antagonist [^{3}H]LY303.870, occurred spontaneously only in a small fraction of the receptors. It is concluded that disulfide bridges form readily between Cys residues introduced appropriately in the N-terminal extension and the third extracellular loop, whereas they form with more difficulty between Cys residues placed at the extracellular ends of the transmembrane segments even at positions where high-affinity metal ion sites can be constructed with His residues.

Cysteine residues are found quite frequently in G protein-coupled or seven-transmembrane segment receptors (7TM receptors).[1] Depending on the receptor domain in which they occur, they obviously play different structural and functional roles. In the transmembrane segments, Cys residues are usually just used as small hydrophobic residues mostly facing the lipid bilayer. In the C-terminal intracellular extension, one or several Cys residues are frequently located 15−20 residues after TM-VII and are here involved in a dynamic, reversible palmitoylation process which is regulated by receptor usage (*1*). In the extracellular domains, Cys residues are believed most often to be involved in intramolecular disulfide bridge formation. Recently, it has also been demonstrated that intermolecular disulfide bridges can be formed as part of dimerization events, for example, in some of the metabotropic glutamate receptors and the Ca^{2+}-sensing receptor (*2*−*4*). However, many receptors appear to form dimers and oligomers without intermolecular disulfide bridges (*5*). In contrast, one of the most well-conserved structural features of 7TM receptors is a putative disulfide bridge from the extracellular end of TM-III to the middle of extracellular loop 2. This disulfide bridge transforms extracellular loop 2 into two short loops connecting the extracellular end of TM-III to TM-IV and -V, respectively. TM-III is located very much in the center of the seven-helix bundle (*6*−*11*), and the fact that it is connected to the rest of the helical bundle by four small loops and not only by the two protein backbone loops underlines the crucial structural and possible functional role of this helix. Mutational analysis of the Cys residues involved in this presumed central disulfide bridge in, for example, rhodopsin, the β_2-adrenergic receptor, and the angiotensin AT_1 receptor has demonstrated the importance of this bridge for the structural and functional integrity of the receptors (*12*−*16*).

In a number of 7TM receptors, two additional Cys residues are found in the N-terminal extension and in the third extracellular loop. Although they are located far from each other in the primary structure, these residues are likely to be in spatial proximity in the folded seven-helix bundle (*7*, *11*), and hence, they are likely to form a second structurally constraining disulfide bridge. Such a bridge would create an extra loop between TM-I and -VII and thereby "close up" the circle of the seven-helix bundle. However, whether such a bridge is in fact formed is difficult to probe. Substitutions of these Cys residues in some cases such as the chemokine CXCR-1 and CCR-2 receptors seriously impair agonist binding (*17*, *18*), whereas in other cases, such as the bradykinin B_2 receptor (*19*) and the NPY Y_1 receptor,

† This study was supported by grants from the Biotechnology Center for Molecular Recognition through the Danish MRC as well as grants from the Carlsberg and Lundbeck foundations.

* To whom correspondence should be addressed: Laboratory for Molecular Pharmacology, Department of Pharmacology, Panum Institute, University of Copenhagen, Blegdamsvej 3C, DK-2200 Copenhagen, Denmark. Phone: +45 3532 7602. Fax: +45 3535 2995. E-mail: elling@molpharm.dk.

[1] Abbreviations: 7TM receptors, G protein-coupled or seven-transmembrane segment receptor; NPY, neuropeptide Y; DTT, dithiothreitol; [^{125}I]BH-SP, [^{125}I]Bolton-Hunter-labeled substance P; ΔCysIV−VI−NK_1, [C152V/C199V/C255V/C260S]NK_1; ΔCys-NK_1, [C152V/C199V/C255V/C260S/C306A/C307F/C386S]NK_1.

10.1021/bi991777b CCC: $19.00
Published on Web 01/07/2000

it has a negligible effect on ligand binding (unpublished observations).

Certain diseases associated with receptor malfunction are caused by the introduction of a single, extra Cys residue in extracellular loop 2. Hereditary, nephrogenic diabetes insipidus in which the kidneys are insensitive to the antidiuretic hormone vasopressin is associated with a number of different mutations in the vasopressin V_2 receptor (*20*−*27*). Interestingly, a disproportionate number of these mutations, which are located in extracellular loop 2, are Cys substitutions (*27*). Similarly, one of the rhodopsin mutations causing retinitis pigmentosa is an introduction of a single Cys in extracellular loop 2 (*28*−*31*). Thus, apparently an extra, single Cys residue is not allowed in these receptors in extracellular loop 2.

In the study presented here, we have performed a number of substitutions and introductions of Cys residues in the tachykinin NK_1 receptor. The aim was to probe the importance of the presumed central disulfide bridge, and the effect of introduction of single "free" Cys residues in extracellular loop 2 to test if a malfunctioning receptor phenotype could be induced and characterized, and to try to introduce functional disulfide bridge "switches". Rather extensive mutational analysis has previously been performed on this receptor with respect to characterizing especially ligand−receptor interactions (*32*−*42*) and metal ion site engineering (*43*−*46*). Residues that are important for the binding of the peptide agonist, substance P, have been identified in the N-terminal extension and at the exterior ends of TM-III and -VII (*32*, *40*). Interestingly, affinity cross-linking studies using chemically modified substance P analogues have identified an additional major interaction site as a methionine residue, Met^{181}, apparently located in the middle of extracellular loop 2 but, importantly, close to the conserved Cys residue in this loop (*47*). Thus, because this Cys residue makes a disulfide bridge to the Cys residue at the extracellular end of TM-III in the folded receptor structure, Met^{181} is in fact not found far out in an extracellular loop but more likely just one helical turn above a His residue in TM-III, His^{108} (HisIII:04), which through mutational analysis has been implicated in peptide binding (*32*). Thus, the agonist binding site is composed of many residues scattered in the primary structure even far out in the extracellular loop, but in fact making a binding pocket around the extracellular ends of TM-III, -VI, and -VII. Mutations which affect the binding of nonpeptide antagonists have been located more deeply in the receptor between the helices, especially among TM-III−VI (*34*−*39*, *48*, *49*). These differences in binding sites for peptide agonists and nonpeptide antagonists are exploited in the study presented here, where, for example, the Cys substitutions and disulfide bridge engineering affect peptide binding, but where a radioactive nonpeptide antagonist still binds normally and therefore can serve as a control ligand.

EXPERIMENTAL PROCEDURES

Peptide and Reagents. The nonpeptide NK_1 receptor antagonist LY303.870 [(*R*)-1-[*N*-(2-methoxybenzyl)acetylamino]-3-(1*H*-indol-3-yl)-2-[*N*-(2-(4-(piperidin-1-yl)piperidin-1-yl)acetyl)amino]propane] (*50*) was generously provided by P. A. Hipskind (Lilly Research Laboratories, Indianapolis, IN). Substance P was obtained from Peninsula. Dithiothreitol (DTT) was from Boehringer Mannheim. Restriction endonucleases were from New England Biolabs and Life Technologies. T4 DNA polymerase was from Life Technologies. *Pfu* polymerase was from Stratagene. [^{125}I]Bolton-Hunter reagent was from Amersham.

Construction of Receptor Mutants. The cDNA encoding the human NK_1 receptor, generously provided by N. P. Gerard (Boston, MA), was cloned into the pTEJ-8 expression vector (*51*). Mutations were introduced either by combined extension of mutated internal primers by T4 DNA polymerase followed by selective amplification of the mutated DNA strand by the polymerase chain reaction (PCR) from added-on external primers according to methods described in ref *52* or by recombinant PCR (*53*, *54*) on a single-stranded M13 bacteriophage template of the NK_1 receptor (*35*). All PCRs were performed using *Pfu* polymerase according to the manufacturer's instructions. The PCR fragments were digested with restriction endonucleases, purified by agarose gel electrophoresis, and ligated into the pTEJ-8-NK_1 receptor. All mutations were verified by restriction endonuclease mapping and by DNA sequence analysis (ALFexpress DNA sequencer, Amersham-Pharmacia Biotech).

Transfections and Tissue Culture. Cloned receptors were transiently expressed in COS-7 cells transfected by calcium phosphate precipitation according to previously reported methods (*55*). COS-7 cells were grown in Dulbecco's Modified Eagle's Medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/mL gentamicin in 10% CO_2 and 90% air.

Binding Experiments. Monoiodinated [^{125}I]Bolton-Hunter-labeled substance P ([^{125}I]BH-SP) was prepared and purified by high-performance liquid chromatography (*55*). [^{3}H]-LY303.870 was generously provided by P. A. Hipskind. The transfected COS-7 cells were transferred to culture plates 1 day after transfection and assayed 2 days after transfection. Competition binding and titration experiments were performed on whole cells at 4 °C for 3 h using 40 pM [^{125}I]-BH-SP with variable amounts of unlabeled peptide or DTT in 0.5 mL of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM $MnCl_2$, 0.1% (w/v) BSA (Sigma), and 100 μg/mL bacitracin. All determinations were carried out in triplicate, and the level of nonspecific binding was determined as the level of binding in the presence of 1 μM substance P. Competition binding data were analyzed and IC_{50} values determined by nonlinear regression analysis using Prizm (GraphPad Software, San Diego, CA). Values of the dissociation constants (K_d) and the number of binding sites (B_{max}) were estimated from competition binding data using the equations $K_d = IC_{50} - L$ and $B_{max} = (B_0 IC_{50})/L$, where L is the concentration of radioligand and B_0 is the amount of specific bound radioligand.

Cu^{2+}(phenanthroline)$_3$ Disulfide Cross-Linking. Cells were washed in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 5 mM $MnCl_2$ and incubated with 1 mM Cu^{2+}-(phenanthroline)$_3$ in 10 mM sodium phosphate buffer containing 3.3% (v/v) glycerol (pH 7.0) for 30 min at 37 °C. Cells were washed, and binding was performed as described above.

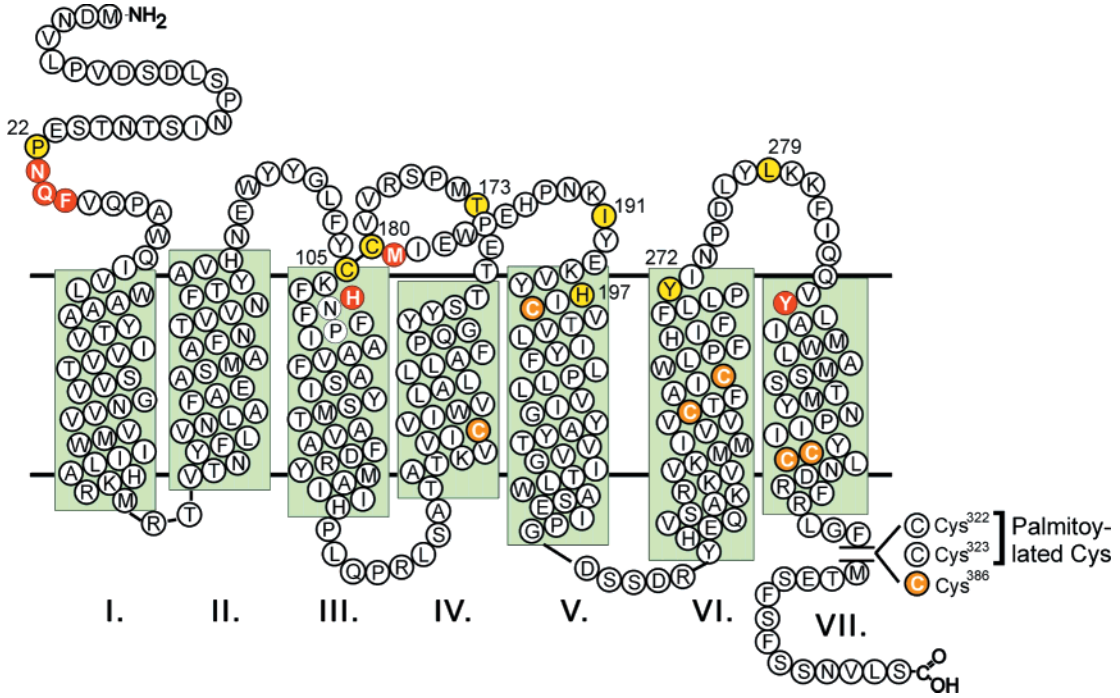

FIGURE 1: Serpentine diagram of the human NK_1 receptor. Black letters on yellow circles represent residues substituted with cysteine or serine probing existing and engineering of new disulfide bridges. White letters on orange circles represent endogenous cysteines substituted withr Val, Ser, Ala, or Phe aimed at constructing a ΔCys-NK_1 receptor devoid of free cysteines. White letters on red circles represent residues previously implicated in binding or cross-linking of substance P to the NK_1 receptor. The putative disulfide bridge between the extracellular end of TM-III and the second extracellular loop is indicated. Helices have been extended on the intracellular side according to Hubbell and co-workers (*70*, *71*).

Table 1: Binding of Substance P to Wild-Type and Cysteine Mutants of the NK_1 Receptor

	substance P		B_{max} (fmol/ 100000 cells)
	$K_d \pm$ SEM (nM)	*n*	
substituted cysteines			
NK_1	0.22 ± 0.04 (0.90 ± 0.05)[c]	7	86 ± 11
[C105S]	NB	2	–
[C180S]	NB	2	–
[C105S/C180S]	NB	2	–
introduced cysteines			
[P22C]	0.12 ± 0.02 (1.04 ± 0.06)	7	6.0 ± 0.8
[L279C]	0.19 ± 0.04 (0.95 ± 0.07)	6	59 ± 16
[P22C/L279C] state I[a]	NB[b]	5	–
[P22C/L279C] state II[a]	0.13 ± 0.02 (0.98 ± 0.09)	5	3.0 ± 1.2
[I191C]	0.14 ± 0.05 (1.06 ± 0.04)	3	8.0 ± 2.9
[T173C/I191C]	0.11 ± 0.02 (1.10 ± 0.08)	7	16 ± 5
[I191C/L279C]	0.08 ± 0.01 (0.89 ± 0.02)	3	17 ± 3

[a] Ses Figure 5 for the definition of states I and II. [b] NB, no detectable radioligand binding. [c] Hill coefficients.

RESULTS

Probing the Obligatory Disulfide Bridge. The two cysteine residues presumed to be linked in a disulfide bridge between the extracellular end of TM-III, Cys^{105}, and the middle of the second extracellular loop, Cys^{180}, were substituted for serine residues (Figure 1). Substitution of either Cys^{105} or Cys^{180} separately in both instances eliminated detectable binding of the radiolabeled substance P (Table 1). To test whether it was the lack of the putative disulfide bridge or the presence of the free thiol group of the remaining Cys residue that impaired substance P binding, the double mutant Cys^{105}Ser/Cys^{180}Ser was also constructed. Again, this double mutant did not exhibit any detectable radioligand binding, suggesting that the putative disulfide bond is essential either for receptor binding of the endogenous ligand substance P or for the general expression of the mutant receptors or both in accordance with previous studies (*12*−*15*, *19*) (Table 1).

Introduction of Cys Residues in Extracellular Loop 2. Two residues in the NK_1 receptor, Thr^{173} and Ile^{191} located just outside TM-IV and -V, respectively, were selected for Cys substitutions since they correspond to positions in the vasopressin V_2 receptor and rhodopsin where Cys substitution is associated with impaired receptor function in the hereditary diseases nephrogenic diabetes insipidus (vasopressin V_2 receptor) and retinitis pigmentosa (rhodopsin) (*20*−*31*). Introduction of a single cysteine at position Ile^{191} did not impair substance P binding with an affinity of 0.14 nM, virtually identical to the value of 0.22 nM for the wild-type (Table 1). Introduction of cysteines at both positions Thr^{173} and Ile^{191} again did not decrease but in fact increased the affinity for substance P to 0.11 nM (Table 1). Similarly, combining cysteines at position Ile^{191} in extracellular loop 2 and position Leu^{279} in extracellular loop 3 (see below) did not impair binding of substance P (Table 1). Hence, the introduction of novel cysteines in extracellular loop 2 is tolerated in the NK_1 receptor at least at these positions in contrast to the vasopressin V_2 receptor and rhodopsin.

Attempt To Establish a Disulfide Bridge Switch between the N-Terminal Extension and Extracellular Loop 3. A disulfide bridge is presumed to be present in certain receptors on the basis of the concomitant occurrence of single Cys residues both in the N-terminal extension close to TM-I and in the middle of the third extracellular loop. However, it has as yet only been possible to address the presence of such a disulfide bridge by conventional mutational analysis using loss of function as a monitor (*16*−*18*, *56*, *57*) with all the well-known uncertainties that implies. Instead, we here try to construct a disulfide bridge, which in its oxidized,

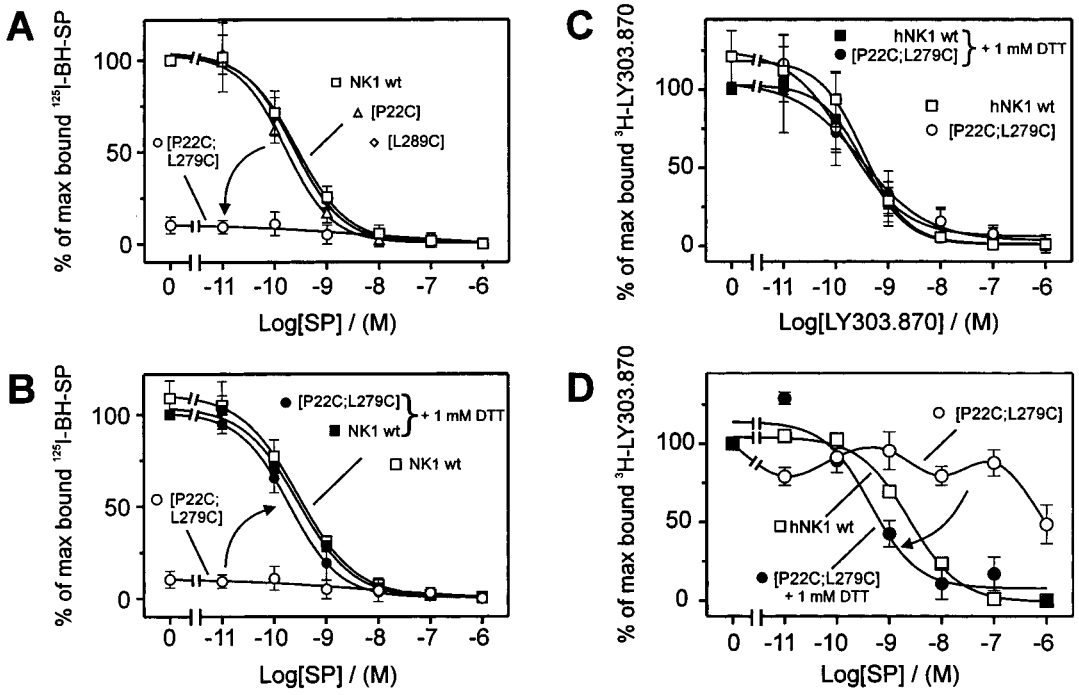

FIGURE 2: Engineering of a disulfide bridge switch between the N-terminal extension and extracellular loop 3. (A) Cysteine substitution of either Pro^{22} (△) or Leu^{279} (◇) did not impair agonist binding; however, combination of these in the double mutant [P22C/L27C] (○) eliminated agonist binding. (□) Wild-type NK_1 receptor. (B) Selective reduction of the engineered disulfide bridge between Cys^{22} and Cys^{279} with 1 mM DTT (black symbols) salvages the binding of the agonist. White symbols depict data for the treatment with buffer only: (□ and ■) wild-type NK_1 receptor and (○ and ●) [P22C/L27C]. (C) Binding of the nonpeptide antagonist LY303.870 is unaffected by the engineered disulfide bond. K_d values were as follows: [P22C/L279C], 0.37 ± 0.12 nM (with 1 mM DTT) and 0.19 ± 0.06 nM (with buffer); wild-type NK_1 receptor, 0.34 ± 0.17 nM (with 1 mM DTT) and 0.33 ± 0.05 nM (with buffer). Symbols as in panel B. (D) High-affinity substance P competition for [^{3}H]LY303.870 binding only occurs with the [P22C/L279C]NK_1 receptor construct in the reduced form. Symbols as in panel B.

connected form should constrain the conformation of the extracellular extension and loops of the receptor in a way which should prevent the binding of the peptide agonist; however, in its reduced, disconnected form, it should behave like the wild-type receptor. Pro^{22}, located approximately 10 residues before the start of TM-I, was selected for Cys substitution in the N-terminal extension because the neighboring residues (Asn^{23}-Gln^{24}-Phe^{25}) by Ala substitution have been strongly implicated in substance P binding (*32*). Leu^{279}, located in the middle of the third extracellular loop, was selected for Cys substitution as a possible disulfide bridge partner for Cys^{22} on the basis of the fact that a Cys residue is found at the corresponding position in extracellular loop 3 of the homologous human NK_2 receptor and is suspected to be involved in a disulfide bridge to the N-terminal extension in that receptor. Introduction of Cys residues corresponding to position Pro^{22} or Leu^{279} in the NK_1 receptor did not impair substance P binding when performed individually with an affinity for the peptide of 0.12 and 0.19 nM, respectively (Figure 2A and Table 1), indicating that cysteines at these positions are allowed and do not induce, for example, mispairing with any of the conserved Cys^{105} or Cys^{180} residues. However, when the Cys substitutions of Pro^{22} and Leu^{279} were combined in a double mutant, the binding of the radioactive substance P tracer was eliminated, suggesting that a constraining disulfide bridge had been formed between Cys^{22} and Cys^{279} (Figure 2A). In contrast, the nonpeptide antagonist LY303.870 bound to the [P22C/L279C]NK_1 receptor construct with an affinity similar to that observed in the wild-type receptor (Figure 2C), indicating that the loss of substance P binding was not due to the lack of expression of the receptor mutant.

Titration with the reducing agent DTT was performed to probe whether the artificially constructed and conceivably more solvent-exposed disulfide bridge located several residues removed from the extracellular end of the transmembrane segments could be opened without breaking the structurally important, central disulfide from the extracellular end of TM-III. In the wild-type NK_1 receptor, the binding of substance P was impaired by DTT only at high concentrations, i.e., above 10 mM (Figure 3A). In contrast, in the double mutant [P22C/L279C]NK_1, which could not bind radioactive substance P under normal conditions, treatment with low DTT concentrations, i.e., 0.03−0.5 mM, restored substance P binding (Figure 3B). This indicates that the lack of binding of radioactive substance P to the [P22C/L279C]-NK_1 receptor is caused by the spontaneous[2] formation of a disulfide bond between the two Cys residues and not by the combined introduction of these residues. Like in the wild-type receptor, concentrations of reducing agent greater than 10 mM impaired substance P binding also in the [P22C/L279C]NK_1 receptor construct (Figure 3B). In both receptors, this loss of substance P binding at high DTT concentrations is presumably caused by reduction of the central disulfide bond between the extracellular end of TM-III and the second extracellular loop, which according to the [C105S/C180S]-NK_1 receptor construct is essential for receptor integrity. To

[2] The word spontaneous is here used to describe the fact that the disulfide bridge is formed without addition of any additional oxidizing agent or catalyst besides oxygen in the air.

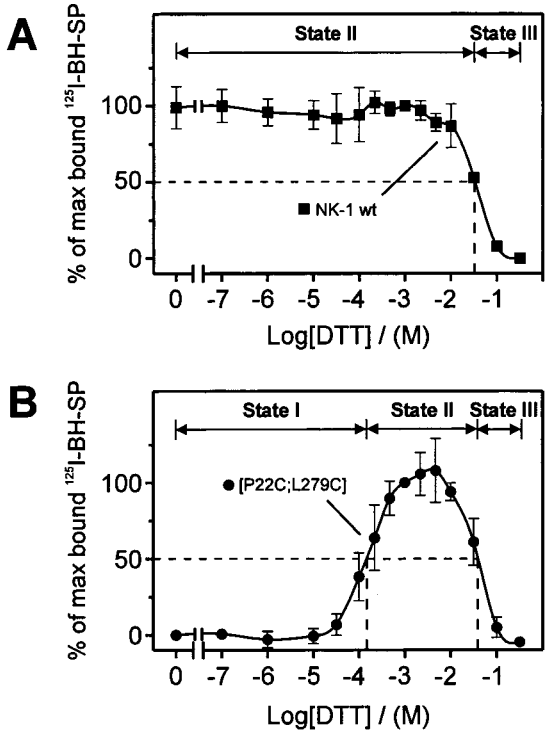

FIGURE 3: Titration of the engineered disulfide bridge between Cys^{22} and Cys^{279} in the extracellular domain of the NK_1 receptor using the reducing agent dithiothreitol. (A) Titration of the wild-type NK_1 receptor (■) with the reducing agent DTT inhibited the binding of substance P at high DTT concentrations, presumably due to reduction of the obligatory disulfide bridge between the extracellular end of TM-III and extracellular loop 2. (B) Titration of the double mutant [P22C/L279C] (●) with DTT salvaged the binding of the agonist with half-maximal binding of substance P at DTT concentrations of 0.15 mM (reduction of the [P22C/L279C] disulfide bridge) and 39 mM (presumably reduction of the obligatory disulfide bridge between the extracellular end of TM-III and extracellular loop 2). States I−III refer to the various reduction states of the receptors as depicted in Figure 5.

verify that the reduced form of the [P22C/L279C]NK_1 receptor had a phenotype comparable to those of the [P22C]-NK_1 and [L279C]NK_1 receptor constructs, competition binding experiments were performed in the presence of 1 mM DTT (Figure 2B). Under these conditions, the [P22C/L279C]NK_1 receptor construct exhibited an affinity for substance P of 0.13 nM, i.e., virtually similar to those of the receptor constructs with single Cys substitutions and to that of the wild-type receptor. The competition binding curve of the wild-type receptor was unchanged under these conditions (Figure 2B). Also, the binding of LY303.870 was virtually identical with and without reduction by DTT, i.e., 0.37 ± 0.12 and 0.19 ± 0.06 nM, respectively, and importantly similar to the affinity observed in the wild-type NK_1 receptor, 0.34 ± 0.17 and 0.33 ± 0.05 nM with and without DTT, respectively (Figure 2C), whereas high-affinity substance P competition for [^{3}H]LY303.870 binding only occurred with the [P22C/L279C]NK_1 receptor construct in the reduced form (Figure 2D).

Attempt To Build a Disulfide Bridge Switch between TM-V and -VI. Previously, we have by introduction of His residues constructed a high-affinity metal ion site between TM-V and -VI (*43*). At this location, we here try to build a disulfide bridge switch, which should affect especially the binding of nonpeptide antagonists (*34*, *36*). This site is located at the very ends of the transmembrane helices as such and could therefore be expected to be more conformationally constrained than the site used for the other artificial disulfide bridge described above, which was located relatively far out in the extracellular loops (Figure 1).

To work with a cleaner system, we initially engineered a series of constructs where free Cys residues were substituted in groups in the NK_1 receptor, which also would make the receptor more suitable for future biophysical studies using Cys labeling, for example, with spin-labels and flourescent labels. Four Cys residues were not substituted, viz., the two involved in the naturally occurring central disulfide bridge from TM-III to extracellular loop 2 and the two residues, Cys^{322} and Cys^{323}, in the C-terminal intracellular extension and presumably involved in palmitoylation events. The remaining Cys residues were substituted in groups with either Val, Ser, Ala, or Phe to preserve high-affinity ligand binding and the high expression level as shown in Table 2. Initially, a construct in which Cys residues in transmembrane helices IV−VI were substituted (dubbed ΔCysIV−VI−NK_1, which corresponds to [C152V/C199V/C255V/C260S]NK_1) was created. This construct bound LY303.870 with an affinity identical to that of wild-type NK_1 (Table 2). Finally, a construct suited for biophysical studies called ΔCys-NK_1, which corresponds to [C152V/C199V/C255V/C260S/C306A/C307F/C386S]NK_1, was created. This construct bound substance P with an affinity of 0.27 nM, compared to 0.13 nM in the wild-type NK_1 receptor, and bound the nonpeptide antagonist LY303.870 with an affinity of 0.16 nM, compared to 0.11 nM in wild-type NK_1 (Table 2).

Of the residues constituting the original tridentate metal ion site, residues His^{197} in TM-V and His^{272} (originally Tyr^{272}) in TM-VI were considered to be most optimally located for disulfide bridge construction between these two helices (*43*). Whereas the disulfide bridge formed spontaneously in the [P22C/L279C]NK_1 receptor construct and totally eliminated ligand binding, in that case substance P binding, substitution of both Tyr^{272} and His^{197} with Cys only decreased the affinity of the nonpeptide antagonist LY303.870 from 0.11 to 1.34 nM. Importantly, the Hill coefficient was decreased from just above unity in the ΔCysIV−VI−NK_1 construct to 0.76 in the [H197C/Y272C]ΔCysIV−VI−NK_1 construct (Table 2 and Figure 4B). Two-component analysis of the binding curve revealed that 75% of the construct was in a high-affinity state (0.98 nM) corresponding to the nonsubstituted ΔCysIV−VI−NK_1 construct and 25% in a low-affinity state (39 nM). It was assumed that the low-affinity state could correspond to a state in which the intended disulfide bridge had formed spontaneously between the two introduced Cys residues. This notion was supported by the observation that treatment of the [H197C/Y272C]ΔCysIV−VI−NK_1 with the reducing agent DDT (10 mM) resulted in a monocomponent binding curve for LY303.870 with a Hill coefficient of 0.95 and with an affinity of 0.66 nM, more closely resembling that observed for the ΔCysIV−VI−NK_1 construct (Table 2 and Figure 4B). In contrast, treatment of the [H197C/Y272C]ΔCysIV−VI−NK_1 construct with the redox catalyst Cu^{2+}(phenanthroline)$_3$ (1 mM) (*58*) strongly impaired ligand binding (Figure 4B), an effect which could at least partly be reversed by subsequent treatment with 10 mM DTT (Figure 4B). Importantly, neither treatment with 1 mM Cu^{2+}(phenanthroline)$_3$ nor treatment with 10 mM DTT

Table 2: Binding of the Nonpeptide Antagonist LY303.870 to Wild-Type and Cysteine Mutants of the NK1 Receptor

		LY303.870			B_{max}
	helical segment	$K_d \pm$ SEM (nM)	n	F_{mut}	(fmol/100000 cells)
hNK_1		0.11 ± 0.038 (0.97 ± 0.05)[b]	3	1.0	49.7 ± 4.93
hNK_1[a]		0.13 ± 0.06 (0.9 ± 0.1)	2	1.0	27.8 ± 7.7
[Y272C]ΔCysIV−VI	TM-IV−VI	0.22 ± 0.040 (1.07 ± 0.06)	6	2.0	10.2 ± 3.80
[H197C/Y272C]ΔCysIV−VI	TM-IV−VI	1.34 ± 0.140 (0.76 ± 0.04)	13	12.2	7.2 ± 2.00
[C152V/C199V/C255V/C260S]ΔCysIV−VI	TM-IV−VI	0.10 ± 0.040 (1.05 ± 0.09)	4	0.9	23.1 ± 4.20
[C152V/C199V/C255V/C260S/C306S/C307S]ΔCysIV−VI	TM-IV−VII	0.01 ± 0.005 (1.16 ± 0.13)	3	0.1	4.2 ± 0.80
[C152V/C199V/C255V/C260S/C306A/C307F]ΔCysIV−VI	TM-IV−VII	0.04 ± 0.010 (1.24 ± 0.04)	3	0.4	4.0 ± 0.80
[C152V/C199V/C255V/C260S/C306A]ΔCysIV−VI	TM-IV−VII	0.06 ± 0.007 (0.96 ± 0.06)	2	0.6	11.8 ± 1.60
[C152V/C199V/C255V/C260S/C307F]ΔCysIV−VI	TM-IV−VII	0.09 ± 0.020 (1.36 ± 0.11)	3	0.8	12.4 ± 0.78
[C152V/C199V/C255V/C260S/C306A/C307F/C386S]ΔCys	TM-IV−VII and C-terminus	0.16 ± 0.030 (1.11 ± 0.05)	7	1.5	12.9 ± 1.60
[C152V/C199V/C255V/C260S/C306A/C307F/C386S]ΔCys[a]	TM-IV−VII and C-terminus	0.27 ± 0.04 (1.06 ± 0.2)	4	1.5	34.0 ± 3.8

[a] Binding performed using substance P and [^{125}I]Bolton-Hunter-labeled substance P. [b] Hill coefficients.

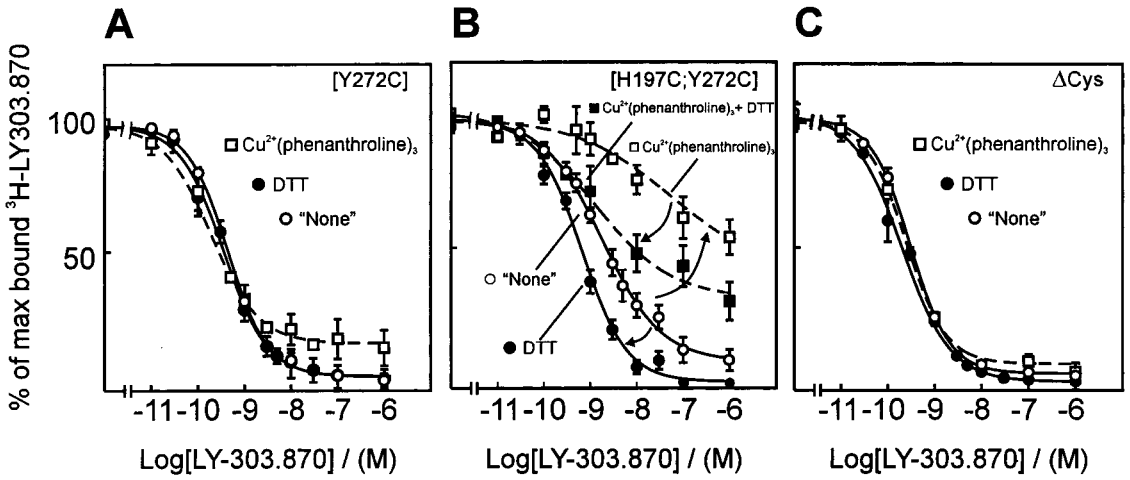

FIGURE 4: Engineering of a disulfide bridge switch between TM-V and -VI of the NK_1 receptor. Binding of LY303.870 to (A) [Y272C]ΔCysIV−VI−NK_1, (B) [H197C/Y272C]ΔCysIV−VI−NK_1, and (C) ΔCys-NK_1. (□) Oxidation of receptor constructs by Cu^{2+}-(phenanthroline)$_3$ followed by reduction (■) with 10 mM DTT. (●) Reduction using 10 mM DTT *without* prior treatment with oxidizing agent. (○) Addition of buffer alone.

affected ligand binding in the [Y272C]ΔCysIV−VI−NK_1 construct or in the ΔCys-NK_1 construct (Figure 4A,C). Thus, a putative disulfide switch has been constructed between Cys^{197} in TM-V and Cys^{272} in TM-VI. However, this bridge only forms spontaneously in a fraction of the receptor molecules conceivably due to the fact that these Cys residues are more constrained due to their location in the transmembrane helices as such.

DISCUSSION

Conserved, Central Disulfide Bridge from TM-III to Extracellular Loop 2. The Cys residue found in the middle of the second extracellular loop is the only highly conserved residue in rhodopsin-like 7TM receptors located outside the transmembrane segments. This issue is usually not picked up by conventional sequence alignment programs. In fact, a Cys residue located at the start of the third hydrophobic segment and another located somewhere in the connecting "loop" between the fourth and fifth hydrophobic segments are often the only "sequence identity" between distantly related families of 7TM receptors. For example, this pair of Cys residues is found in the receptors of apical neurons in the mammalian vomeronasal organ (*59*) and in the smoothened receptor in *Drosophila* (*60*).

As expected on the basis of this high degree of structural conservation, we here find that this disulfide bridge from TM-III to extracellular loop 2 is of major functional importance in the substance P NK_1 receptor as has previously been shown for rhodopsin and subsequently many other 7TM receptors (*12*−*17*, *19*, *56*, *61*, *62*).

Originally, we suspected that the reason introduction of Cys residues in the second extracellular loop was not tolerated in 7TM receptors was that these "extra" Cys residues were involved in mispairing with the cysteines involved in the central, well-conserved disulfide bridge from TM-III (*63*). However, surprisingly, we found that extra, free Cys residues were in fact well tolerated in extracellular loop 2 in the NK_1 receptor. Accordingly, there must be some other reason these substitutions are not tolerated, particularly in the vasopressin V_2 receptor and in rhodopsin. Interestingly, in both of these receptors, there are in fact not one but two Cys residues in extracellular loop 2, and no obvious disulfide bridge partner can be found for this naturally occurring extra Cys residue in the wild-type receptors (*20*, *64*). Thus, in the nonfunctional mutant version of both the V_2 receptor and of rhodopsin, there are actually three Cys residues in extracellular loop 2. One of these is expected to form the important disulfide bridge to the extracellular end of TM-III. This

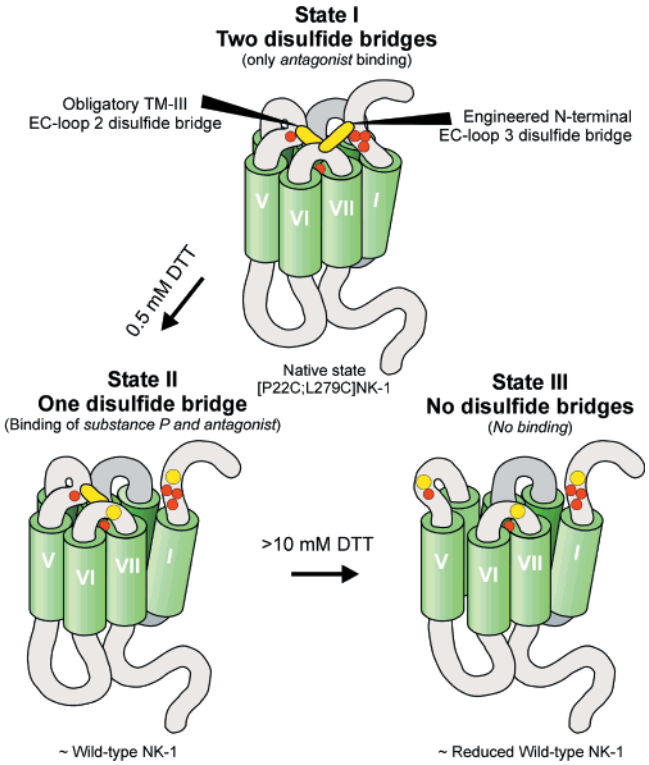

FIGURE 5: Schematic drawing of the different "redox states" of the [P22C/L279C] cysteine-engineered NK_1 receptor. Color coding as in Figure 1. In state I, two disulfide bridges are present in the native [P22C/L279C]NK_1 receptor construct: one linking the extracellular end of TM-III with the second extracellular loop ("the obligatory bridge") as also found in the wild-type receptor and the engineered bridge between the N-terminal extension and the third extracellular loop. This form of the receptor is *unable* to bind the agonist substance P but can bind the nonpeptide antagonist LY303.870 with wild-type affinity. In state II, by selective reduction of the engineered bridge, the receptor is able to adopt a conformation comparable to the wild-type receptor that is able to bind both the agonist and antagonist. In state III, further reduction of the obligatory disulfide bridge eliminates the binding of the agonist [compare with that of the [C105S/C180S]NK_1 receptor mutant (Table 1)].

leaves two Cys residues in extracellular loop 2, which could form a disulfide bridge within the loop. Although such an intraloop disulfide bridge may be functionally disabling in some receptors (the V_2 receptor and rhodopsin), it may be well tolerated in other receptors, for example, the β_2-adrenergic receptor in which three cysteines are located in the second extracellular loop (*15*).

Formation of a Disulfide Bridge between the N-Terminal Segment and the Third Extracellular Loop. On the basis of the knowledge of the binding site for substance P in the NK_1 receptor (*32*, *40*, *47*, *65*), a receptor could here be constructed where we by loss of function could monitor the formation and by gain of function (substance P binding) could monitor the breakage of a disulfide bridge from the extracellular N-terminal extension and the third extracellular loop. Thus, in the [P22C/L279C]NK_1 receptor construct, a disulfide bridge is formed between the N-terminal extension and third extracellular loop which prevents the binding of substance P but does not affect nonpeptide antagonist binding (Figure 5, state I). Upon *selective* reduction of the engineered disulfide bridge in the [P22C/L279C]NK_1 receptor construct with low concentrations of DTT, the mutant receptor is apparently allowed to adopt a conformation that is able to bind substance P in a manner similar to that of the wild-type receptor (Figure 5, state II). It has previously been demonstrated that residues Asn^{23}, Gln^{24}, and Phe^{25} in the N-terminal extension of the NK_1 receptor are crucial for binding of the endogenous agonist substance P and the related tachykinin peptides, whereas these residues do not seem to be involved in binding nonpeptide antagonists (*32*). The engineered disulfide bridge involves Cys^{22}, located immediately preceding these residues. Hence, one model for the lack of binding of substance P in the oxidized form of the [P22C/L279C]NK_1 receptor construct could be that the disulfide bridge prevents the proper interaction of these residues with the peptide agonist. More extensive reduction in response to high concentrations of DTT conceivably leads to reduction also of the central, conserved disulfide bridge between the extracellular end of TM-III and the second extracellular loop, eliminating binding of substance P in both the [P22C/L279C]NK_1 receptor construct and in the wild-type receptor (Figure 5, state III). Interestingly, despite the fact that the location of the introduced Cys residues in both the N-terminal extension and in the third extracellular loop was chosen rather arbitrarily, the disulfide bond formed spontaneously, i.e., without the aid of added oxidative agent and apparently in the whole population of receptors. In this context, it may be noted that disulfide bridges can form between α-helices in soluble, globular proteins between Cys residues introduced even up to 20 Å apart due to thermal motions (*66*, *67*). However, the extent of disulfide formation seems most strongly correlated with the spatial separation of the cysteines with the formation rate dropping sharply beyond a separation of approximately 8 Å (*66*, *67*). On the basis of the observations in this study, we conclude that in 7TM receptors where a Cys residue is located in the N-terminal extension close to TM-I and another Cys residue is located in extracellular loop 3, they most likely do form a disulfide bridge which closes the seven-helix bundle connecting TM-I and -VII. This is found, for example, in chemokine receptors and in some neuropeptide and peptide hormone receptors.

Disulfide Bridge Engineering between the Extracellular Ends of TM-V and -VI. As discussed above, the disulfide bridge constructed between the N-terminal extension and extracellular loop 3 formed spontaneously in basically the whole population of receptors. In contrast, the putative bridge between the Cys residues introduced in the most extracellular turns of transmembrane helices V and VI formed spontaneously only in a small fraction of the receptors. This disulfide bridge was like the other one designed to function as a switch, i.e., in such a way that the formation and breakage of the bridge could be monitored as loss of function and gain of function, respectively, in this case with respect to binding of the nonpeptide antagonist [^{3}H]LY303.870. In principle, the disulfide bridge switch between TM-V and -VI also worked; i.e., oxidation with Cu^{2+}(phenanthroline)$_3$ impaired LY303.870 binding conceivably through formation of the disulfide bridge between the two introduced Cys residues, and subsequent reduction with DTT improved ligand binding conceivably through breakage of the disulfide bridge. However, the signal was not as easy to monitor as in the disulfide bridge switch constructed between the N-terminus and extracellular loop 3, where a total loss of ligand binding (in that case substance P) was observed upon

bridge formation and a complete reversion to full, high-affinity binding could be obtained upon breakage of the bridge. Previously, Oprian and co-workers have used the corresponding positions in rhodopsin for construction of an interhelical disulfide bridge also based upon the previously constructed high-affinity zinc binding site (*68*, *69*). As in the NK_1 receptor, spontaneous disulfide formation was only observed in a relatively small fraction of the rhodopsin molecules and treatment with Cu^{2+}(phenanthroline)$_3$ was required to produce quantitative disulfide formation (*68*). The fact that two His residues are able to form a high-affinity zinc site but two Cys residues only to a limited degree are able to spontaneously form a disulfide bridge indicates that a surprising degree of conformational constraint is present in this part of the receptor structure, conceivably due to the relatively rigid helical structure. The binding site of LY303.870 appears to be rather elusive in contrast to a number of other nonpeptide NK_1 receptor antagonists that seem to bind in the transmembrane domain between TM-III, -V, -VI, and -VII (*49*). Presumably, the binding site for LY303.870 is also located in the transmembrane domain, and the inhibition of binding of LY303.870 could speculatively be attributed to, for example, fixation of the helical structure of the receptor which thereby restricts the binding of the nonpeptide antagonist.

Establishing a ΔCys-NK$_1$ Receptor for Biophysical Studies. The ΔCysIV−VI−NK_1 receptor was here used as a clean "background" to construct and monitor the disulfide bridge switch between TM-V and -VI. However, the ΔCys-NK_1 receptor was mainly constructed with future biophysical, structural studies in mind. Several of these techniques rely on the possibility of incorporating cysteine reactive probes into the molecule of interest at selective positions by genetic engineering. A prerequisite for such a technique is often that the site of probe incorporation be known and that selective incorporation at one site be realized. The free cysteines in the NK_1 receptor were all located in the transmembrane segments of the receptor (Cys^{152}, Cys^{199}, Cys^{255}, Cys^{260}, Cys^{306}, and Cys^{307}) except for a single cysteine (Cys^{386}) located in the C-terminus of the cytoplasmic tail. The four remaining cysteines in the ΔCys-NK_1 receptor construct are conceivably all subject to post-translational modifications and hence unavailable for labeling. This ΔCys-NK_1 receptor had an affinity for substance P and LY303.870 which was similar to that observed in the wild-type receptor. An important issue when performing biophysical studies on any membrane protein is the level of expression as the amounts needed are substantial and require robust expression levels of the protein of interest. The ΔCys-NK_1 receptor had an expression level of 25% of that of the wild type with respect to LY303.870 antagonist binding, whereas its B_{max} for substance P agonist binding was slightly higher compared to that of the wild type. Thus, the ΔCys-NK_1 receptor construct appears to be suited for further studies using, for example, bacculovirus expression.

REFERENCES

1. Morello, J. P., and Bouvier, M. (1996) *Biochem. Cell. Biol. 74*, 449−457.
2. Romano, C., Yang, W.-L., and O'Malley, K. L. (1996) *J. Biol. Chem. 271*, 28612−28616.
3. Bai, M., Trivedi, S., and Brown, E. M. (1998) *J. Biol. Chem. 273*, 23605−23610.
4. Pace, A. J., Gama, L., and Breitwieser, G. E. (1999) *J. Biol. Chem. 274*, 11629−11634.
5. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., and Marshall, F. H. (1998) *Nature 396*, 679−682.
6. Schertler, G. F. X., Villa, C., and Henderson, R. (1993) *Nature 362*, 770−772.
7. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. (1997) *Nature 389*, 203−206.
8. Krebs, A., Villa, C., Edwards, P. C., and Schertler, G. F. X. (1998) *J. Mol. Biol. 282*, 991−1003.
9. Schertler, G. F. X. (1998) *Eye 12*, 504−510.
10. Baldwin, J. M. (1993) *EMBO J. 12*, 1693−1703.
11. Baldwin, J. M., Schertler, G. F., and Unger, V. M. (1997) *J. Mol. Biol. 272*, 144−164.
12. Karnik, S. S., Sakmar, T. P., Chen, H. B., and Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8459−8463.
13. Karnik, S. S., and Khorana, H. G. (1990) *J. Biol. Chem. 265*, 17520−17524.
14. Dohlman, H. G., Caron, M. G., DeBlasi, A., Frielle, T., and Lefkowitz, R. J. (1990) *Biochemistry 29*, 2335−2342.
15. Noda, K., Saad, Y., Graham, R. M., and Karnik, S. S. (1994) *J. Biol. Chem. 269*, 6743−6752.
16. Yamano, Y., Ohyama, K., Chaki, S., Guo, D.-F., and Inagami, T. (1992) *Biochem. Biophys. Res. Commun. 187*, 1426−1431.
17. Leong, S. R., Kabakoff, R. C., and Hébert, C. A. (1994) *J. Biol. Chem. 269*, 19343−19348.
18. Monteclaro, F. S., and Charo, I. F. (1997) *J. Biol. Chem. 272*, 23186−23190.
19. Herzig, M. C. S., Nash, N. R., Conolly, M., Kyle, D. J., and Leeb-Lundberg, L. M. F. (1996) *J. Biol. Chem. 271*, 29746−29751.
20. Birnbaumer, M., Seibold, A., Gilbert, S., Ishido, M., Barberis, C., Antaramian, A., Brabet, P., and Rosenthal, W. (1992) *Nature 357*, 333−335.
21. Lolait, S. J., O'Carroll, A.-M., McBride, O. W., Konig, M., Morel, A., and Brownstein, M. J. (1992) *Nature 357*, 336−339.
22. Sharif, M., and Hanley, M. R. (1992) *Nature 357*, 279−280.
23. Rosenthal, W., Seibold, A., Antaramian, A., Lonergan, M., Arthus, M.-F., Hendy, G. N., Birnbaumer, M., and Bichet, D. G. (1992) *Nature 359*, 233−235.
24. Pan, Y., Metzenberg, A., Das, S., Jing, B., and Gitschier, J. (1992) *Nat. Genet. 2*, 103−106.
25. Rosenthal, W., Antaramian, A., Gilbert, S., and Birnbaumer, M. (1993) *J. Biol. Chem. 268*, 13030−13033.
26. Pan, Y., Wilson, P., and Gitschier, J. (1994) *J. Biol. Chem. 269*, 31933−31937.
27. Pan, Y., Metzenberg, A., Das, S., Jing, B., and Gitschier, J. (1992) *Nat. Genet. 2*, 99−102.
28. Sung, C.-H., Davenport, C. M., Hennessey, J. C., Maumenee, I. H., Jacobson, S. G., Heckenlively, J. R., Nowakowski, R., Fishman, G., Gouras, P., and Nathans, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6481−6485.
29. Sung, C.-H., Schneider, B. G., Agarwal, N., Papermaster, D. S., and Nathans, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8840−8844.
30. Applebury, M. L. (1992) *Curr. Biol. 2*, 113−115.
31. Berson, E. L. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4526−4528.
32. Fong, T. M., Huang, R.-R. C., and Strader, C. D. (1992) *J. Biol. Chem. 267*, 25664−25667.
33. Fong, T. M., Yu, H., Huang, R.-R. C., and Stader, C. D. (1992) *Biochemistry 31*, 11806−11811.
34. Gether, U., Johansen, T. E., Snider, R. M., Lowe, J. A., III, Nakanishi, S., and Schwartz, T. W. (1993) *Nature 362*, 345−348.
35. Gether, U., Johansen, T. E., and Schwartz, T. W. (1993) *J. Biol. Chem. 268*, 7893−7898.
36. Fong, T. M., Cascieri, M. A., Yu, H., Bansal, A., Swain, C., and Strader, C. D. (1993) *Nature 362*, 350−353.
37. Gether, U., Yokota, Y., Emonds-Alt, X., Breliere, J.-C., Lowe, J. A., III, Snider, R. M., Nakanishi, S., and Schwartz, T. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6194−6198.

38. Gether, U., Emonds-Alt, X., Breliére, J.-C., Fujii, T., Hagiwara, D., Pradier, L., Garret, C., Johansen, T. E., and Schwartz, T. W. (1994) *Mol. Pharmacol. 45*, 500−508.
39. Gether, U., Nilsson, L., Lowe, J. A., and Schwartz, T. W. (1994) *J. Biol. Chem. 269*, 23959−23964.
40. Huang, R.-R. C., Yu, H., Strader, C. D., and Fong, T. M. (1994) *Biochemistry 33*, 3007−3013.
41. Maggi, C. A., and Schwartz, T. W. (1997) *Trends Pharmacol. Sci. 18*, 351−355.
42. Regoli, D., Boudon, A., and Fauchere, J. L. (1994) *The American Society for Pharmacology and Experimental Therapeutics 46*, 551−599.
43. Elling, C. E., Nielsen, S. M., and Schwartz, T. W. (1995) *Nature 374*, 74−77.
44. Elling, C. E., and Schwartz, T. W. (1996) *EMBO J. 15*, 6213−6219.
45. Thirstrup, K., Elling, C. E., Hjorth, S. A., and Schwartz, T. W. (1996) *J. Biol. Chem. 271*, 7875−7878.
46. Elling, C. E., Thirstrup, K., Nielsen, S. M., Hjorth, S. A., and Schwartz, T. W. (1997) *Folding Des. 2*, S76−S80.
47. Kage, R., Leeman, S. E., Krause, J. E., Costello, C. E., and Boyd, N. D. (1996) *J. Biol. Chem. 271*, 25797−25800.
48. Gether, U., Johansen, T. E., Snider, R. M., Lowe, J. A., III, Emonds-Alt, X., Yokota, Y., Nakanishi, S., and Schwartz, T. W. (1993) *Regul. Pept. 46*, 49−58.
49. Holst, B., Zoffmann, S., Elling, C. E., Hjorth, S. A., and Schwartz, T. W. (1998) *Mol. Pharmacol. 53*, 166−175.
50. Gitter, B. D., Bruns, R. F., Howbert, J., Waters, D. C., Threlkeld, P. G., Cox, L. M., Nixon, J. A., Lobb, K. L., Mason, N. R., Stengel, P. W., Cockerham, S. L., Silbaugh, S. A., Gehlert, D. R., Schober, D. A., Iyengar, S., Calligaro, D. O., Regoli, D., and Hipskind, P. A. (1995) *J. Pharmacol. Exp. Ther. 275*, 737−744.
51. Johansen, T. E., Sch¢ller, M. S., Tolstroy, S., and Schwartz, T. W. (1990) *FEBS Lett. 267*, 289−294.
52. Stappert, J., Wirsching, J., and Kemler, R. (1992) *Nucleic Acids Res. 20*, 624−625.
53. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
54. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 61−68.
55. Gether, U., Marray, T., Schwartz, T. W., and Johansen, T. E. (1992) *FEBS Lett. 296*, 241−244.
56. Blanpain, C., Lee, B., Vakili, J., Ranz, B. J., Govaerts, C., Migeotte, I., Sharron, M., Dupriez, V., Vassart, G., Doms, R. W., and Parmentier, M. (1999) *J. Biol. Chem. 274*, 18902−18908.
57. Tournamille, C., Kim, C. L. V., Gane, P., Blanchard, D., Proudfoot, A. E., Cartron, J. P., and Colin, Y. (1997) *J. Biol. Chem. 272*, 16274−16280.
58. Kobashi, K. (1968) *Biochim. Biophys. Acta 158*, 239−245.
59. Dulac, C., and Axel, R. (1995) *Cell 83*, 195−206.
60. Alcedo, J., Ayzenzon, M., Von-Ohlen, T., Noll, M., and Hooper, J. E. (1996) *Cell 86*, 221−232.
61. Savarese, T. M., Wang, C.-D., and Fraser, C. M. (1992) *J. Biol. Chem. 267*, 11439−11448.
62. D'Angelo, D. D., Eubank, J. J., Davis, M. G., and Dorn, G. W., II (1996) *J. Biol. Chem. 271*, 6233−6240.
63. Kono, M., Yu, H., and Oprian, D. D. (1998) *Biochemistry 37*, 1302−1305.
64. Nathans, J., and Hogness, D. S. (1983) *Cell 34*, 807−814.
65. Boyd, N. D., Kage, R., Dumas, J. J., Krause, J. E., and Leeman, S. E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 433−437.
66. Careaga, C. L., and Falke, J. J. (1992) *J. Mol. Biol. 226*, 1219−1235.
67. Careaga, C. L., and Falke, J. J. (1992) *Biophys. J. 62*, 209−219.
68. Yu, H., Kono, M., McKee, T. D., and Oprian, D. D. (1995) *Biochemistry 34*, 14963−14969.
69. Struthers, M., Yu, H., Kono, M., and Oprian, D. D. (1999) *Biochemistry 38*, 6597−6603.
70. Altenbach, C., Klein-Seetharaman, J., Hwa, J., Khorana, H. G., and Hubbell, W. L. (1999) *Biochemistry 38*, 7945−7949.
71. Altenbach, C., Cai, K., Khorana, H. G., and Hubbell, W. L. (1999) *Biochemistry 38*, 7931−7937.

BI991777B