NK1 Receptor Fused to β-Arrestin Displays a Single-Component, High-Affinity Molecular Phenotype

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ABSTRACT

Arrestins are cytosolic proteins that, upon stimulation of seven transmembrane (7TM) receptors, terminate signaling by binding to the receptor, displacing the G protein and targeting the receptor to clathrin-coated pits. Fusion of β-arrestin1 to the C-terminal end of the neurokinin NK1 receptor resulted in a chimeric protein that was expressed to some extent on the cell surface but also accumulated in transferrin-labeled recycling endosomes independently of agonist stimulation. As expected, the fusion protein was almost totally silenced with respect to agonist-induced signaling through the normal Gq/G11 and Gs pathways. The NK1–β-arrestin1 fusion construct bound non-peptide antagonists with increased affinity but surprisingly also bound two types of agonists, substance P and neurokinin A, with high, normal affinity. In the wild-type NK1 receptor, neurokinin A (NKA) competes for binding against substance P and especially against antagonists with up to 1000-fold lower apparent affinity than determined in functional assays and in homologous binding assays. When the NK1 receptor was closely fused to G proteins, this phenomenon was eliminated among agonists, but the agonists still competed with low affinity against antagonists. In contrast, in the NK1–β-arrestin1 fusion protein, all ligands bound with similar affinity independent of the choice of radioligand and with Hill coefficients near unity. We conclude that the NK1 receptor in complex with arrestin is in a high-affinity, stable, agonist-binding form probably best suited to structural analysis and that the receptor can display binding properties that are nearly theoretically ideal when it is forced to complex with only a single intracellular protein partner.

Activation of seven transmembrane segments (7TM) receptors results in signal transduction involving primarily interaction with intracellular, heterotrimeric G proteins that modulate various downstream effector molecules. Signaling through 7TM receptors is in most cases terminated via interaction with additional cellular proteins to prevent uncontrolled cellular stimulation. In general, this is a two-step process, involving receptor-desensitization followed by receptor-internalization (reviewed by Ferguson, 2001). The key player in both of these processes is β-arrestin1 or -2, which is recruited from the cytosol to turn off receptor signaling (Lohse et al., 1990; Attramadal et al., 1992; Pippig et al., 1993; Oakley et al., 2000). Arrestin binds, via its N-terminal domain, with high affinity in a one-to-one ratio (Sohlemann et al., 1995) primarily to the carboxyl-terminal tail of the receptor and thereby displaces the heterotrimeric G protein from the receptor by competition and steric exclusion (Kuhn et al., 1984). Through its C-terminal domain, arrestin is able to bind to both adaptor protein 2 and clathrin and thus functions as a scaffolding protein that targets the receptor to clathrin-coated pits (Goodman et al., 1997; Krupnick et al., 1997; Laporte et al., 2000) where the internalization cascade is initiated resulting in sequestration of the receptor in coated vesicles (Ferguson et al., 1996; Goodman et al., 1996).

In the plasma membrane, 7TM receptors exist in equilibrium between conformations having different affinities for the hormone or neurotransmitter. This equilibrium is controlled by interaction with various accessory proteins (in particular, the G proteins). This is the basis for the classical ternary complex model, where the receptor when associated with the G protein has a higher affinity for agonists than...
when it is on its own. This phenomenon has been studied in various molecular constructs, where a Go subunit is fused to the C-terminal tail of a 7TM receptor (reviewed by Milligan, 2000). Thus, recently we found that the pharmacological profile of the neurokinin NK1 receptor observed in whole cell experiments could be dissected into two distinct molecular phenotypes by fusing the receptor to either of the G proteins through which it normally signals (Holst et al., 2001). Thus, depending on which of the two G proteins with which the NK1 receptor is associated, Goα or Goq, it exists in one of two distinct active conformations binding the two endogenous ligands, substance P (SP) and neurokinin A (NKA) with different affinities.

It is well established that after agonist stimulation, the NK1 receptor undergoes signal-quenching steps involving both β-arrestin (McConologue et al., 1998, 1999), and clathrin (Grady et al., 1995), and that the NK1 receptor rapidly internalizes together with β-arrestin to early endosomes, where it is dephosphorylated and the empty receptor is recycled to the plasma membrane.

In the present study, we have constructed a fusion protein between the NK1 receptor and β-arrestin1 and find in whole-cell studies that, as expected, it is silenced with respect to signaling and has a shifted steady-state distribution from the plasma membrane to recycling endosomes. Moreover, in contrast to wild-type receptor and in contrast to the NK1 receptor fused to α-subunits of G protein, the arrestin-fusion protein displays an almost perfect monocomponent molecular phenotype with a surprisingly high affinity for all agonists as well as antagonists.

**Experimental Procedures**

**Materials.** Pfu polymerase was purchased from Promega (Madison, WI); restriction enzymes, Dulbecco’s modified Eagle’s medium 1885, and fetal bovine serum, were from Invitrogen (Carlsbad, CA). Ampicillin, isobutylmethylxanthine, poly[t-lysine], and holotransferrin was from Sigma Chemical Co. (St. Louis, MO). Culture plates were from Costar (Corning, NY), and bovine serum albumin (BSA) was from ICN Biomedicals Inc. (Aurora, OH). The cDNA for bovine β-arrestin1 was kindly provided by Robert J. Lefkowitz (Duke University, Durham, NC).

**Ligands, Radioactivity and Antibodies.** Substance P and neurokinin A peptides were obtained from Peninsula (St. Helens, Merseyside, UK), CP96,345 was kindly provided by Dr. John A. Lowe III (Pfizer, Groton, CT) and SR140,333 by Drs. Xavier Edmonds-Alt and Eyad Beyarsse, Umeå, Sweden), rabbit antibody against human transferrin was purchased from DAKO (Glostrup, DK), anti-EEA1 was from Transduction Laboratories (BD Bioscience, Erembodegem, Belgium), rabbit antibody against human LAMP1 (Lgp120) was provided by Dr. Sven Carlsson (University of Umeå, Umeå, Sweden), rabbit antibody against rat TGN38 was provided by George Banting (University of Bristol, Bristol, UK). Goat anti-mouse antibody labeled with Alexa Fluor-488 was from Molecular Probes (Eugene, OR), and goat anti-rabbit antibody labeled with rhodamine was from Pierce & Warriner (Chester, UK).

**Generation of the cDNA Constructs.** The coding sequence of the human tachykinin (hNK1) receptor was inserted in the pTEJS eukaryotic expression vector (Johansen et al., 1990). For fusion with the HA-tag (the epitope ‘YPYDVPDYA’ from the influenza virus HA) sequence to the N-terminal end of the receptor, the coding sequence of the human NK1 receptor was amplified with an appropriate primer with Pfu polymerase and inserted into pTEJS. For fusion with β-arrestin, the coding sequences of the human NK1 receptor without its stop codon and of bovine β-arrestin1 were amplified and fused in frame by PCR. The PCR-fragment was digested, and subcloned into the pNSINeo-vector (NeuroSearch, Ballerup, Denmark) using the HindIII/XbaI site. For N-terminal fusion of the HA-tag to the NK1–β-arrestin1, the cDNA encoding HA-NK1 was digested with HindIII and BstEII and inserted into the NK1R–β-arrestin1 construct in pNSINeo. Synthesis of the NK1–GoqΔtail construct has been described previously (Holst et al., 2001). Briefly, the cDNA encoding a 72-amino acid C-terminally truncated human NK1 receptor was fused in frame with the full-length long splice variant of rat Goα by PCR and inserted into the pTEJS expression-vector. All constructs were verified by restriction endonuclease mapping and sequencing on an ABI Prism (Applied Biosystems, Foster City, CA).

**Cell Culture and Transfection.** COS-7 cells, which express relatively low levels of endogenous β-arrestin (Krupnick et al., 1997) were maintained in Dulbecco’s modified Eagle’s medium 1885 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 1000 μg/ml streptomycin, and kept at 37°C in a 10% CO2 atmosphere. For competition binding and functional assays, the cells were transfected using a calcium phosphate-DNA coprecipitation method with the addition of chloroquine (as described by Holst et al., 2001). Approximately 24 h after transfection, the cells were detached by PBS-EDTA and split into appropriate plates. For immunostaining experiments, cells were transfected by nucleofection (Amaxa, Koeln, Germany) and were grown on glass cover slips.

**Competition Binding Assays.** Transfected cells were transferred to 48- or 24-well poly(t-lysine)-treated culture plates at a density aiming at 5 to 10% binding. Two days after transfection, the cells were challenged with variable amounts of agonists or antagonists against a constant concentration of [3H]-Arrestin NK1 Fusion Protein 31
cAMP and 0.1 mM ATP and incubated for 30 min on ice. The supernatant was transferred first onto Dowex 50W-X4 columns and subsequently onto alumina columns as described previously (Holst et al., 2001). Determinations were performed in duplicate.

**Fluorescence Microscopy.** Cells cultured on glass cover slips were used for immunofluorescence stainings 2 days after transfection. For transferrin experiments, cells were first serum-starved for 30 min at 37°C in binding media (RPMI-1640 without bicarbonate containing 0.2% BSA, 10 mM HEPES, adjusted to pH 7.4), and subsequently incubated with transferrin for 15 min in the binding media at 37°C. All cells were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, quenched with NH₄Cl, and stained with appropriate antibodies after permeabilization using 0.05% saponin. Nonspecific binding was blocked by incubating the cells in PBS containing 0.2% gelatin. Subsequently, the cover slips were incubated with the primary antibody at room temperature for 45 min, washed three times, incubated with the secondary antibody for a further 45 min, and washed 5 times. After staining, cells were mounted in Moviol and analyzed using a Nikon Optiphot-2 microscope equipped with an MRC Bio-Rad 1024 confocal laser scanning system. Digital images were transferred to Adobe Photoshop and adjusted so that the intensity values extended over the full measurable range (0–255 gray levels). Single channel and overlay images were printed directly from Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Calculations.** The results from the binding and functional assays were analyzed by nonlinear regression using Prism 3.0 software (GraphPad Software, Inc. (San Diego, CA)). Values for dissociation and inhibition ($K_d$ and $K_i$) were estimated from competition binding experiments using the equations $K_d = IC_{50} - L$ and $K_i = IC_{50}/(1 + L/K_d)$, where $L$ is the concentration of the radioactive labeled ligand.

**Results**

**Subcellular Localization of NK1–β-Arrestin1.** The cellular localizations of the HA-tagged NK1 wild-type receptor and HA-tagged NK1–β-arrestin1 fusion protein was studied in transiently transfected COS-7 cells by immunofluorescence techniques and confocal microscopy. Cells expressing the wild-type NK1 receptor predominantly showed surface staining (Fig. 2A) in accordance with previous studies (McConologue et al., 1998). In contrast, in the absence of any agonist, cells transfected with the fusion protein generally displayed a more punctuated staining pattern widely distributed in the cytoplasm as well as concentrated in a perinuclear region (Fig. 2C). Importantly, in nonpermeabilized cells expressing the NK1–β-arrestin1 fusion protein, additional staining of the cell surface could be demonstrated (Fig. 2B).

**Fig. 1.** Schematic drawing of the NK1–β-arrestin1 and the NK1–Gₛ₆Δtail fusion constructs. β-Arrestin1 was fused to the far C-terminal tail of the NK1 receptor (A); for comparison, fusion of Gₛ₆ to a truncated tail of the NK1 receptor is also displayed (B) (Holst et al., 2001). In the case of the β-arrestin1 fusion construct, only full-length proteins were used, whereas for the Gₛ₆ fusion construct, the C-terminal tail of the NK1 receptor was truncated to contain only 13 instead of 85 amino acids as found in the wild-type receptor. The numbers indicate the amino acid numbering of the respective proteins.

**Fig. 2.** Subcellular localization of HA-NK1 receptor wild-type and HA-NK1–β-arrestin1 in transiently transfected COS-7 cells. COS-7 cells transiently transfected with HA-NK1 receptor wild-type (A) or HA-NK1–β-arrestin1 (B-C) were stained with the HA-11 antibody and imaged by confocal microscopy. Cells in A and C were permeabilized with saponin. Single optical sections are shown. Microscope-settings were approximately the same for all images. Scale bars, 10 μm.

**Fig. 3.** Colocalization of HA-NK1–β-arrestin1 with markers for recycling and sorting endosomes, lysosomes, and trans-Golgi network. COS-7 cells were transiently transfected to express HA-NK1–β-arrestin1 (left, A-D) and were stained with the primary HA-11 antibody and subsequently with an Alexa-488–conjugated secondary antibody. In addition cells were stained with an antibody against transferrin (A), EEA-1 (B), LAMP1 (C), or TGN38 (D) and afterward with a rhodamine-conjugated second antibody (middle). Right, merged pictures; colocalized structures appear in yellow. Cells were imaged with a confocal microscope as described under Experimental Procedures. Single optical sections are shown. Scale bars, 10 μm.
To identify the intracellular compartment in which the fusion protein accumulated, immuno-fluorescence staining of markers for different organelles in the endocytic pathway was performed. The NK1–β-arrestin1 fusion protein and transferrin, the marker for recycling endosomes, showed very similar distribution patterns with a significant overlap of both diffuse cytosolic and perinuclear vesicles (Fig. 3A), although a few NK1–β-arrestin1–containing vesicles that lacked transferrin were also observed. In contrast, the NK1–β-arrestin1 fusion protein did not show marked overlap with sorting endosomes, indicated by EEA1 (Fig. 3B), or lysosomes, indicated by LAMP1 (Fig. 3C). The fusion protein showed some overlap with the marker for trans-Golgi network, TGN38 (Fig. 3D). However, the staining patterns were very different, and the overlap most probably reflects the preferential location of these two organelles in the perinuclear region of the cell. In conclusion, it was found that some of the NK1–β-arrestin1 fusion protein was located on the cell surface, whereas most of the fusion protein seemed to accumulate in recycling endosomes. This suggests that the NK1–β-arrestin chimera may cycle constitutively between the cell surface and recycling endosomes as suggested for some other 7TM receptors (Signoret et al., 2000).

**Signal Transduction Experiments.** In transiently transfected COS-7 cells, the wild-type NK1 receptor couples through both a Gs and a Gq signaling pathway in response to physiological concentrations of substance P as determined by stimulation of cAMP and phosphatidyl inositol production, respectively (Fig. 4). As expected, no response in cAMP accumulation was observed in response to substance P in the NK1–β-arrestin1 fusion construct (Fig. 4A). Similarly, the robust substance P induced response in phosphatidylinositol production was almost eliminated in the NK1–β-arrestin1 fusion construct. However, a very slight activation of the Gq signaling pathway could be observed in the fusion construct, albeit with an $E_{\text{max}}$ value of approximately 4 fmol/10$^5$ cells, corresponding to only 3% of the wild-type response, and with an $E_{50}$ value of 4.8 nM (Fig. 4B, insert). Because the fusion construct does in fact bind agonists well as determined in the whole cell binding experiments (see below), the severe reduction of signaling indicates that the covalently coupled arrestin molecule inactivates the NK1 receptor either in cis conformation (i.e., by binding to the receptor within the fusion molecule) or in trans conformation (i.e., by binding to “neighborable” receptor molecules).

**Binding Experiments.** Whole-cell binding experiments were performed on transiently transfected COS-7 cells using $^{125}$I-BH-substance P or $^{125}$I-NKA as agonist tracers or the nonpeptide compound [3H]LY303,870 as antagonist tracer. As reported previously, in the wild-type NK1 receptor, nonpeptide antagonists display a simple, high-affinity binding independent of the radioligand used (Cascieri et al., 1992; Hastrup and Schwartz, 1996). In the NK1–β-arrestin1 fusion construct, a very similar binding pattern was observed for the nonpeptide antagonist; the affinity for LY303,870 was with all three radioligands found to be higher in the arrestin fusion construct compared with the wild-type receptor (Fig. 5, A–C; Table 1). Slightly higher affinity in the arrestin fusion construct compared with the wild-type NK1 receptor was also found for two other nonpeptide antagonists, CP96,345 (0.23 ± 0.05 nM versus 0.36 ± 0.07 nM, n = 3) and SR140,333 (0.79 ± 0.30 nM versus 1.01 ± 0.23 nM, n = 3), in competition binding against $^{125}$I-BH-SP.

In the wild-type NK1 receptor, the apparent affinity determined for agonists can vary up to 1000-fold depending on the radioligand employed (Hastrup and Schwartz, 1996; Sagan et al., 1997; Hastrup and T. W. Schwartz, unpublished observations). Thus, in the transfected COS-7 cells, substance P competed for binding against the nonpeptide antagonist [3H]LY303,870 with an affinity of 8.8 nM (Fig. 5D), but against itself ($^{125}$I-BH-substance P) it competed with an affinity of 0.23 nM (Fig. 5E) and against $^{125}$I-NKA with an affinity of 0.02 nM (Fig. 5F). Surprisingly, in the NK1–β-arrestin1 fusion, which according to the lack of signaling is uncoupled from the G protein, the agonist substance P bound with high affinity. Importantly, in the arrestin fusion construct, the measured affinity for substance P was independent of the radioligand employed; the competition binding curves for substance P were almost superimposable for all three radioligands, giving $K_i$ values of 0.17, 0.18, and 0.12 nM (Fig. 5, D–F, Table 1). That is, substance P bound to the NK1–β-arrestin1 fusion construct with an affinity very similar to that determined for the peptide against itself in the

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**Fig. 4.** Substance P induced signaling in the wild-type NK1 receptor and the NK1–β-arrestin1 fusion construct. Cyclic AMP accumulation (A) and inositol phosphate turnover (B) in response to varying concentrations of substance P in the wild-type NK1 (●) and in the NK1–β-arrestin1 fusion protein (○) transiently expressed in COS-7 cells. With respect to cAMP production, the wild-type receptor had an $E_{\text{max}}$ value of 1.28 ± 0.21 nM and an $E_{\text{max}}$ value of 86 ± 18 fmol per 10$^5$ cells, whereas the NK1–β-arrestin1 fusion protein was totally nonresponsive to agonist stimulation. With respect to inositol phosphate turnover, the wild-type receptor had an $E_{\text{max}}$ value of 0.38 ± 0.03 nM and an $E_{\text{max}}$ value of 127 ± 10 fmol per 10$^5$ cells. Inset, very small response to substance P stimulation in the NK1–β-arrestin1 fusion protein with an $E_{\text{max}}$ value of 4.8 ± 3.6 nM and an $E_{\text{max}}$ value of 3.7 ± 1.7 fmol per 10$^5$ cells. The data represent mean ± S.E.M. from at least three independent experiments performed in duplicate.
wild-type receptor (0.23 nM). Furthermore, whereas the Hill coefficient for substance P binding in the wild-type receptor clearly was below unity, $-0.75 \pm 0.05$ (Table 1), it was above unity in the NK1\(-\beta\)-arrestin1 fusion construct, $-1.23 \pm 0.06$.

Similarly, in the wild-type NK1 receptor, NKA competed against the nonpeptide antagonist $[\text{3H}]$LY303,870 with an affinity of 722 nM (Fig. 5G), against $125I$-BH-substance P with an affinity of 22 nM (Fig. 5H), and against itself ($125I$-NKA) with an affinity of 0.28 nM (Fig. 5I). As with substance P, this discrepancy in apparent affinity was not observed in the NK1\(-\beta\)-arrestin1 fusion construct where NKA competed for binding with high affinity independent of the radioligand used [i.e., with $K_d/K_i$ values being 1.09 nM against $[\text{3H}]$LY303,870, 0.80 nM against $125I$-BH-substance P, and 0.59 nM against itself (Fig. 5, G–I)]. Thus, as was the case for substance P, in the NK1\(-\beta\)-arrestin1 fusion construct, NKA bound with an affinity very similar to the affinity determined in homologous binding experiments in the wild-type receptor.

### Figure 5
Homologous and heterologous competition binding experiments in the wild-type receptor and in the NK1\(-\beta\)-arrestin fusion construct. Competition binding experiments of wild-type NK1 receptor (A–C) and of the NK1\(-\beta\)-arrestin1 fusion construct (D–I), where unlabeled LY303,870 (A–C), substance P (D–F), and NKA (G–I) were tested against the radioligands $125I$-BH-SP, $125I$-NKA, and $[\text{3H}]$LY303,870. The receptors were expressed transiently in COS-7 cells, and the binding experiments were performed in whole cells in duplicates as described in the text. Data represent mean $\pm$ S.E.M. from 5 to 12 separate experiments (see Table 1). The dotted lines display the IC$_{50}$ values for the NK1\(-\beta\)-arrestin1 fusion construct in the respective binding experiments.

### Table 1
Competition binding experiments

<table>
<thead>
<tr>
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<th>NK1-Wild-Type</th>
<th>NK1-(\beta)-Arrestin1</th>
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<tbody>
<tr>
<td></td>
<td>LY303,870</td>
<td>Substance P</td>
</tr>
<tr>
<td>[$\text{3H}$]LY303,870</td>
<td>$1.27 \pm 0.20$ (10)</td>
<td>$8.81 \pm 2.79$ (5)</td>
</tr>
<tr>
<td>$125I$-BH-SP</td>
<td>$-1.40 \pm 0.12$</td>
<td>$-0.86 \pm 0.10$</td>
</tr>
<tr>
<td>$125I$-NKA</td>
<td>$0.57 \pm 0.12$ (5)</td>
<td>$0.23 \pm 0.06$ (7)</td>
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* $K_d/K_i$ $\pm$ S.E.M. (n).

$^a$ Hill coefficient $\pm$ S.E.M.
(i.e., $K_d$ was 0.59 versus 0.28 nM, independent of the choice of radio-ligand).

Previously, we have observed that the differences between affinities for agonists determined in heterologous versus homologous binding assays in the wild-type NK1 receptor disappeared when the receptor was fused to a Gs subunit. That is, this was observed only when the C-terminal extension of the receptor was shortened to preclude promiscuous interference from other accessory proteins (Holst et al., 2001) (Fig. 6, A and B). However, although the competition curves determined for NKA against itself, $^{125}$I-NKA, and against the other agonist $^{125}$I-BH-substance P were almost superimposable in the NK1–Gs-Δtail fusion construct, NKA still competed against the nonpeptide antagonist $[^3H]$LY303,870 with an apparent 50- to 100-fold lower affinity, as shown in Fig. 6B. This was also the case for the agonist substance P, which in the NK1–Gs-Δtail fusion protein competed against itself with a $K_d$ value of 0.02 nM, but against $[^3H]$LY303,870 with a $K_d$ value of 5.9 nM. Also, in the corresponding NK1-Gq-Δtail construct, NKA and substance P still competed with apparent low affinity against the nonpeptide antagonist (data not shown). However, as shown in Fig. 6C for comparison, when the NK1 receptor was fused to β-arrestin1, the competition curves for NKA (not only against itself and substance P but, importantly, also against the nonpeptide antagonist) were closely similar to each other and to the competition curve for NKA against itself in the wild-type receptor.

In the wild-type NK1 receptor, another interesting phenomenon is that different radioactive ligands do not have access to the same number of receptors; large differences are observed in the calculated $B_{max}$ values for different radioligands (Hastrup and Schwartz, 1996; Sagan et al., 1997). Thus, whereas the $B_{max}$ value for the nonpeptide antagonist $[^3H]$LY303,870 in the wild-type NK1 receptor in the present study was determined to be 458 ± 34 fmol/10⁵ cells (corresponding to 275,000 receptors per cell), $^{125}$I-BH-substance P had a $B_{max}$ value of $167 ± 20$ fmol/10⁵ cells and $^{125}$I-NKA a $B_{max}$ value of $47 ± 4$ fmol/10⁵ cells. That is, apparently substance P bound only approximately 35% and NKA even less (only ~10%) of the number of wild-type NK1 receptors, which were seen by the nonpeptide antagonist on the same cells. The NK1–β-arrestin1 fusion construct was in general not expressed as well as the wild-type NK1 receptor (i.e., with respect to receptors detected on the surface of the cell in the binding experiments). The most striking observation, however, was that in the fusion construct, the $B_{max}$ values for the three different radioligands were of the same order of magnitude. In fact, in the NK1–β-arrestin1 fusion construct, $^{125}$I-BH-substance P displayed the highest $B_{max}$ value, $36 ± 11$ fmol/10⁵ cells compared with $^{125}$I-NKA and $[^3H]$LY303,870, which had $B_{max}$ values of $14 ± 3$ and $22 ± 6$ fmol/10⁵ cells, respectively. Also, in the NK1–Gs-Δtail fusion construct, similar $B_{max}$ values were determined for all three radioligands: $[^3H]$LY303,870, 19 ± 5 fmol/10⁵ cells; $^{125}$I-BH-SP, 13 ± 1 fmol/10⁵ cells; and $^{125}$I-NKA, 19 ± 2 fmol/10⁵ cells.

**Discussion**

In the present study, it was found that fusion of the NK1 receptor to β-arrestin1 creates an unprecedented homogeneous molecular phenotype. In the β-arrestin fusion protein, which is expressed on the cell surface and accumulated in recycling endosomes, the NK1 receptor is inactivated after recycling endosomes, the NK1 receptor is inactivated with respect to signaling through the normal Gq/G₁₁ and Gs pathways. Nevertheless, the receptor binds all tested ligands, including two different types of agonists with high affinity; importantly, the phenomenon of major differences in agonist affinities observed in the wild-type receptor dependent on the radioligand used was not seen in the arrestin fusion construct.
Subcellular Localization of the NK1 Receptor β-Arrestin1 Fusion Construct. The wild-type NK1 receptor is known to be located almost exclusively at the cell surface but to undergo agonist-induced, rapid cointernalization with β-arrestin1 to endosomes, where it colocalizes with transferrin (Grady et al., 1995; McConalogue et al., 1999). In addition, it has been found that although most of the receptors returned to the cell surface when studied several hours after agonist-induced internalization, some NK1 receptors remained colocalized with arrestin in a perinuclear pool (McConalogue et al., 1999). In the present study, we found that when the NK1-receptor is fused to β-arrestin1 it is constitutively located in recycling endosomes with a cellular distribution very similar to that of the wild-type receptor and arrestin after agonist treatment. The presence of chimera at the cell surface and in recycling endosomes suggest that it may recycle between the surface and endosomes. Experiments are in progress that should allow us to determine whether this chimera behaves as an agonist-bound receptor–β-arrestin complex. In summary, the NK1–β-arrestin1 chimera seems to be expressed at the cell surface but to traffic in the endosomal compartments and accumulate in the recycling endosomes.

Arrestin-Fusion versus G Protein Fusions. Fusion constructs between 7TM receptors and G proteins have been used extensively as molecular pharmacology tools (Milligan, 2000). To the best of our knowledge, however, fusion constructs with arrestin have been used in only two cases and merely as a control experiments related to receptor targeting (Shiina et al., 2000) and to receptor internalization (Zhang et al., 1999). In the case of the NK1 receptor, we found recently that fusion to the two different types of G proteins that the receptor normally uses (i.e., Gq and Gs) reveals two distinct molecular phenotypes (Holst et al., 2001). In fact, the pharmacological profile of the wild-type NK1 receptor observed in the cell membrane could basically be considered a combination of the two molecular phenotypes observed in each of the two G protein fusion proteins. Importantly, however, despite the fact that the fusion obviously creates a very high local concentration of a relevant G protein, the long C-terminal tail of the NK1 receptor had to be truncated to ensure that the receptor did not interact “promiscuously” with other G proteins in the whole-cell experiments (Holst et al., 2001). Here, we find that fusion of the NK1 receptor to arrestin is more efficient because it totally eliminates interaction with Gs. However, there is still evidence for an interaction—albeit very limited—with Gq, as reflected in the small but significant phosphatidyl inositol response to substance P (Fig. 4B, inset). It remains unclear whether this signal reflects G protein coupling to the intact fusion protein or to a receptor–population without arrestin resulting from proteolytic cleavage of a small fraction of fusion proteins. Thus, it should be emphasized that a simple fusion construct between a receptor and an accessory protein does not mean that the receptor will use only that protein as a partner in the living cell. The selective occupancy by the fusion partner obviously depends on the relative affinities and the cellular availability of other competing accessory proteins.

It is interesting to note that although the tight fusion to the Gq subunit in the NK1–Gq-Δtail construct did eliminate the difference in agonist affinities determined in competition binding among agonists (Fig. 6, A and B) (Holst et al., 2001), the agonists still competed with apparent low affinity against the antagonist in the G protein fusion (Fig. 6B). That was not the case in the arrestin fusion construct, where all the ligands—agonists as well as antagonists—competed with high affinity against each other as they did against themselves. In other words, the NK1–arrestin fusion construct as opposed to the wild-type receptor displays nearly ideal monocOMPONENT binding properties in these whole-cell experiments.

High Affinity Agonist Binding to Arrestin-Receptor Complexes. One of the basic tenets of 7TM receptors is that high-affinity agonist binding is dependent on G protein interaction. This has usually been demonstrated in membrane preparations or in permeabilized cells using nonhydrolyzable GTP analogs, which will stabilize the Gs subunit in its active form and thereby eliminate its binding to the receptor and consequently shift the usual two-component binding curve for agonists to a monocOMPONENT, low-affinity form (Luber et al., 1990). Intuitively, one would assume that the receptor conformation, which is bound by arrestins, would be a low-affinity state with respect to agonist binding because this is a molecular form on its way through desensitization to internalization. That is, arrestin binding is part of a process of signal termination and consequently has no requirement for high-affinity agonist binding. Nevertheless, we here find that β-arrestin in fact stabilizes a high-affinity agonist binding state of the 7TM receptor in a fusion protein construct studied in a whole-cell system. That the high-affinity agonist binding is in fact induced by arrestin as such is supported by molecular reconstitution studies of Gurevich et al. (1997), who observed that purified β-arrestins induced a marked leftward shift in the dose response curves for agonists in both the β2-adrenergic and the muscarinic M2 receptors. However, they found that β-arrestins induced a shallower binding curve for the β2-adrenergic receptor, whereas it remained unchanged for the M2 muscarinic receptor. In contrast, in our whole-cell experiments with the NK1 receptor, we find that β-arrestin induces a steeper slope in the binding curves. Another difference is that in the NK1–β-arrestin fusion construct, we find that the nonpeptide antagonist clearly binds with higher affinity than in the wild-type receptor, whereas no change in the antagonist binding was observed in the arrestin reconstitutions with the monoamine receptors (Gurevich et al., 1997).

The molecular basis for 7TM receptor activation is still unclear, but different types of biophysical and biochemical studies indicate that an outward movement of the inner part of TM-VI away from the center of the helical bundle is an important part of the conformational change occurring during activation (Farrens et al., 1996; Gether et al., 1997; Ghanouni et al., 2001). In has been envisioned that part of the G protein (e.g., the far C-terminal end) would insert into the GTP analogs, which will stabilize the Gs subunit in its active form and thereby eliminate its binding to the receptor and consequently shift the usual two-component binding curve for agonists to a monocomponent, low-affinity form (Luber et al., 1990).
seems not only to function as a “silencer” of G protein coupling but also can in fact act as a scaffolding protein connecting the receptor to other signaling pathways (e.g., mitogen-activated protein kinases) (DeFea et al., 2000).

**Stable Receptor-Arrestin-Fusions for Structural Studies.** The molecular phenotype described in the present study may not be particularly relevant under physiological conditions (i.e., where arrestin association with the receptor is believed to rapidly result in internalization). However, such arrestin-fusion constructs may be useful for structural studies. Recently, the high resolution X-ray structure of the inactive, dark-state of bovine rhodopsin was published (Palczewski et al., 2000). However, apparently there are major problems in obtaining and analyzing similar structures of the interesting active state(s) even of rhodopsin. This is probably a consequence of the facts that 7TM receptors occur in several different, closely related active states and that the active state(s) seem to be rather unstable (Gether, 2000). Because the 7TM-arrestin fusion protein binds agonists with high affinity, it is likely that the structure may actually rather closely relate to the elusive active state of the 7TM receptor. Moreover, the arrestin fusion protein demonstrates a very homogenous picture with respect to ligand binding (Fig. 5 and Fig. 6C). Thus, it is suggested that arrestin fusions may be a shortcut to get a first look at an “active” conformation of a 7TM receptor.

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**References**


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