Heterodimerization of Substance P and mu-Opioid Receptors regulates Receptor Trafficking and Resensitization*

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Running Title: NK1-MOR1 heterodimerization

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Summary

The μ-opioid receptor (MOR1) and the substance P receptor (NK1) coexist and functionally interact in nociceptive brain regions, however, a molecular basis for this interaction has not been established. Using coimmunoprecipitation and bioluminescence resonance energy transfer (BRET), we show that MOR1 and NK1 can form heterodimers in HEK 293 cells coexpressing the two receptors. Although NK1-MOR1 heterodimerization did not substantially change the ligand binding and signalling properties of these receptors, it dramatically altered their internalization and resensitization profile. Exposure of the NK1-MOR1 heterodimer to the MOR1-selective ligand [D-Ala²,Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) promoted cross-phosphorylation and cointernalization of the NK1 receptor. Conversely, exposure of the NK1-MOR1 heterodimer to the NK1-selective ligand substance P (SP) promoted cross-phosphorylation and cointernalization of the MOR1 receptor. In cells expressing MOR1 alone, β-arrestin directs the receptors to clathrin-coated pits, but does not internalize with the receptor. In cells expressing NK1 alone, β-arrestin internalizes with the receptor into endosomes. Interestingly, in cells coexpressing MOR1 and NK1 both DAMGO and SP induced the recruitment of β-arrestin to the plasma membrane and cointernalization of NK1-MOR1 heterodimers with β-arrestin into the same endosomal compartment. Consequently, resensitization of MOR1-dependent receptor functions was severely delayed in coexpressing cells as compared to cells expressing MOR1 alone. Together, our findings indicate that MOR1 by virtue of its physical interaction with NK1 is sequestered via an endocytotic pathway with delayed recycling and resensitization kinetics.

Key Words: opioid receptor – MOR1 – substance P receptor – NK1 – dimerization – heterodimerization – BRET – internalization – β-arrestin – resensitization
Introduction

The formation of homo- and heterodimeric receptors seems to be a general principle for many, if not all, G protein-coupled receptors (GPCRs), and for some GPCRs, e.g. the GABA\textsubscript{B} and the T1 taste receptor, it appears to be an absolute requirement for functional activity (1-11). We have previously shown that opioid and somatostatin receptors exist as constitutive homodimers when expressed alone and as constitutive heterodimers when coexpressed in human embryonic kidney (HEK) 293 cells (12-14). Heterodimerization of somatostatin receptor subtypes modulates their ligand binding, signalling, and trafficking properties, e.g. the sst\textsubscript{2A}-sst\textsubscript{3} somatostatin receptor heterodimer behaved like the sst\textsubscript{2A} homodimer but it did not reproduce the pharmacological characteristics of the sst\textsubscript{3} homodimer, suggesting that physical interaction of sst\textsubscript{3} with sst\textsubscript{2A} induced a functional inactivation of the sst\textsubscript{3} subtype (13). Unlike that observed for the sst\textsubscript{2A}-sst\textsubscript{3} heterodimer, sst\textsubscript{2A}-\textmu-opioid receptor (MOR1) heterodimerization did not significantly affect the ligand binding or coupling properties but promoted cross-modulation of phosphorylation, internalization, and desensitization of these receptors (14). Given the high level of sequence homology between opioid and somatostatin receptors existing in domains proposed to contribute to the dimerization interface (i.e. transmembrane helices) it was not unexpected that these closely related receptors can form heterodimers. However, so far little is known about dimerization of opioid receptors with distantly related GPCRs.

A major prerequisite for the physiological assembly of heterodimeric GPCRs \textit{in vivo} is their coexpression in the same cell. MOR1 and NK1, the principal receptor for substance P (SP), coexist and functionally interact in pain-processing brain regions (15-22). SP is released from nociceptive primary afferents in the spinal and trigeminal dorsal horn, where it activates
spino-thalamic projection neurons. Recently, NK1 and MOR1 have been shown to coexist in trigeminal dorsal horn neurons, suggesting that MOR and NK1 functionally interact in these neurons during nociceptive neurotransmission. Furthermore, MOR1 and NK1 are highly expressed in brain regions implicated in depression, anxiety and stress, but also in other regions such as the nucleus accumbens which mediate the motivational properties of drugs of abuse including opioids. Interestingly, the rewarding effects of morphine are absent in mice lacking the NK1 receptor (20,21). Despite these observations, a molecular basis for the interactions between opioid and substance P receptors has not been established yet.

Here, we report that the MOR1 receptor forms stable heterodimers with the NK1 receptor. MOR1-NK1 heterodimerization did not substantially change ligand binding and signalling properties of the MOR1 receptor but it dramatically altered its trafficking and resensitization profile.
Experimental Procedures

Materials – SP and [D-Ala²,Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) were from Bachem (Heidelberg, Germany), [³H]SP and [³H]DAMGO were from NEN (Zavenstein, Belgium). Dithiobis-(succinimidylpropionate) (DSP) was purchased from Pierce (Rockford, IL). Mouse monoclonal anti-T7 antibody and mouse monoclonal anti-T7 antibody covalently coupled to Sepharose beads were obtained from Novagen (Madison, WI), rat monoclonal anti-HA antibody was purchased from Roche Diagnostics (Mannheim, Germany), mouse monoclonal anti-HA antibody covalently coupled to Sepharose beads were from Covance (Berkeley, CA) and polyclonal guinea pig anti-NK1 antibody was from Chemicon (Hofheim, Germany). In addition, polyclonal rabbit anti-T7, anti-HA and anti-MOR1 {9998} antibodies were used which have been generated in our laboratory and characterized extensively (13,14,30,31). All polyclonal rabbit antisera were affinity purified against their immunizing peptides using the Sulfo-Link coupling gel according to the instructions of the manufacturer (Pierce).

Cell Culture and Transfections – HEK 293 cells (HEK) obtained from ATCC were transfected with plasmids containing either the MOR1 receptor or the NK1 receptor using the calcium phosphate precipitation method. The wild-type HA epitope-tagged rat μ-opioid receptor MOR1 with puromycin resistance was generated as described previously (12). The full length coding region of the rat NK1 cDNA was isolated by RT-PCR from rat brain total RNA using Pfu Turbo DNA polymerase (Stratagene, Gebouw, CA). The sense primer contained a HindIII and the antisense primer a XbaI restriction site. Purified PCR products were digested and ligated into a pcDNA3.1 expression vector encoding a neomycin resistance and the T7 epitope tag sequence MASMTGGQQMG upstream to the HindIII insertion site.
Double strand sequencing was employed to verify the resulting construct. To generate stable lines coexpressing two differentially epitope-tagged receptors, cells expressing HAMOR1 were subjected to a second round of transfection using Lipofectamine (Invitrogen, Karlsruhe, Germany) and selected in the presence of 500 µg/ml G 418 and 1 µg/ml puromycin (Sigma, Deisenhofen, Germany). 6 clones expressing HAMOR1 alone, 4 clones expressing T7NK1 alone and 6 clones coexpressing T7NK1 and HAMOR1 were generated. Receptor expression was monitored using saturation ligand binding assays as described below. In addition, quantitative Western blot analysis was carried out to ensure that clones coexpressing a ~ 1:1 ratio of NK1 and MOR1 protein were selected. Furthermore, double immunofluorescent staining was performed in order to validate that NK1 and MOR1 were coexpressed within the same cells. The $B_{max}$ and $K_D$ values of cells that were used throughout this study are given in Table I.

**Immunoprecipitation and Western Blot Analysis** – Cells were plated onto 100-mm dishes and grown to 80% confluence. Cells were then incubated in cross-linking buffer (phosphate-buffered saline (PBS) containing 10 mM Hepes (pH 7.4) and 2 mM DSP) for 30 min at room temperature. The reaction was quenched by addition of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin and 10 µg/ml bacitracin). Cells expressing T7NK1 or HAMOR1 alone or coexpressing T7NK1 and HAMOR1 were lysed and further processed as described (12-14,32). Receptor proteins were either immunoprecipitated with 50 µl T7 affinity beads (Novagen) or 50 µl HA affinity beads (Covance). Immunocomplexes were eluted from the beads using SDS-sample buffer for 20 min at 60 °C and equal amounts of
protein were loaded onto regular 8% SDS-polyacrylamide gels. After electroblotting, membranes were incubated with either mouse monoclonal anti-T7 (1:5000), rat monoclonal anti-HA (0.1 µg/ml) or affinity-purified rabbit anti-MOR1 antibody (1 µg/ml) overnight at 4 °C, followed by detection using an enhanced chemiluminescence detection system (Amersham, Braunschweig, Germany).

**BRET Assay** – The NK1 and MOR1 coding sequences were amplified using sense and antisense primers harboring unique PstI and HindIII sites. Purified PCR products were digested and ligated into humanized pGFP-N3 and Rluc-N3 expression vectors (BioSignal Packard Biosciences, Montreal, Canada). For BRET measurements, HEK 293 cells were transiently transfected using lipofectamine. Forty-eight h post-transfection, cells were detached with PBS/EDTA and resuspended in PBS containing 0.1% glucose (w/v) and 2 µg/ml aprotinin. Cells were then transferred to 96-well microplates (white Optiplate from BioSignal Packard Biosciences) at a density of 100,000 cells/well. Deep Blue C (coelenterazine; BioSignal Packard Biosciences) was added at a final concentration of 5 µM, and readings were collected using a Fusion microplate analyzer (BioSignal Packard Biosciences) that allows the sequential integration of the signals detected in the 330-490 nm and 485-545 nm windows using filters with the appropriate band pass. The bioluminescence resonance energy transfer (BRET) signal is determined by calculating the ratio of the light emitted by the receptor-GFP (485-545 nm) over the light emitted by the receptor-Rluc (330-490 nm). The values were corrected by subtracting background signal detected in non-transfected cells.

**Whole Cell Phosphorylation Assays** – Phosphorylation studies were carried out as described (12-14). Briefly, after agonist incubation, [32P]orthophosphate-labeled cells were
scraped into radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin and 10 µg/ml bacitracin) and solubilized.

Immunoprecipitations were carried out using 50 µl T7 affinity beads (Novagen) or 50 µl HA affinity beads (Covance). Immunocomplexes were eluted from the beads using SDS-sample buffer for 20 min at 56 °C. Samples were size separated by 8% SDS gels followed by autoradiography. The extent of phosphorylation of receptor monomers was quantitated using a Fuji PhosphorImaging system and BAS 1000 software.

*Immunocytochemistry* – Cells were grown onto poly-L-lysine-coated coverslips overnight. For single immunofluorescence, cells were preincubated with affinity-purified rabbit anti-T7 or anti-HA antibody at a concentration of 1 µg/ml at 4°C for 2 h and then either not exposed or exposed to various agonists. After fixation, bound primary antibodies were detected with cyanine 3.18 (Cy3)-conjugated anti-rabbit antibodies (1: 200, Jackson ImmunoResearch, West Grove, PA). For double immunofluorescence, cells were preincubated with mouse anti-T7 and rabbit anti-HA antibodies at 4°C for 2 h. After fixation, bound primary antibodies were detected with a mixture of cyanine 3.18 (Cy3)- and cyanine 5.18 (Cy5)-conjugated secondary antibodies. Cells were then dehydrated, cleared in xylol and permanently mounted in DPX (Fluka, Neu-Ulm, Germany). To examine trafficking of β-arrestin, cells stably coexpressing T7NK1 and HAMOR1 were transiently transfected with an expression plasmid containing GFP-tagged β-arrestin2 (kindly provided by Dr. M.G. Caron; Duke University Medical Center, Durham, NC). After 48 h, cells were treated, fixed and then...
permanently mounted in Vectashield (Vector Laboratories, Burlingame, CA). Specimens were examined using a Leica TCS-NT laser scanner confocal microscope.

**Internalization Assays** - Cells were seeded at a density of 2 x 10^5 cells per well onto poly-L-lysine-treated 24-well plates. On the next day, cells were preincubated with 1 µg affinity-purified rabbit anti-T7 or anti-HA antibody for 2 h in OPTIMEM 1 (Invitrogen) at 4 °C. Cells were then treated with 1 µM SP or 1 µM DAMGO in OPTIMEM 1 for 2 h. Subsequently, cells were fixed and incubated with peroxidase-conjugated anti-rabbit antibody (1:1000, Amersham) for 2 h at RT. After washing, plates were developed with 250 µl of ABTS solution (Roche Molecular Biochemicals, Mannheim, Germany). After 10-30 min, 200 µl of the substrate solution from each well was transferred to a 96-well plate and analyzed at 405 nm using a microplate reader (BioRad, Munich, Germany).

**Radioligand Binding Assays** - Saturation binding assays were performed on membrane preparations from stably transfected cells as described (12-14). The dissociation constant (K_D) and number of [3H]DAMGO binding sites (B_max) were calculated by Scatchard analysis using at least six concentrations of [3H]DAMGO in a range from 0.25 to 10 nM. Non-specific binding was determined as radioactivity bound in the presence of 1 µM unlabeled DAMGO. K_D and B_max values of NK1 binding sites were calculated by Scatchard analysis with increasing concentrations of [3H]SP ranging from 0.5 to 10 nM. Nonspecific binding was defined in the presence of 1 µM unlabeled SP. For competition binding assays, aliquots of membrane preparations containing 25 µg protein were incubated with either 2.5 nM [3H]SP or 2.5 nM [3H]DAMGO in the presence or absence of SP or DAMGO in concentrations ranging from 10^{-12} to 10^{-5} M.
were seeded at a density of $1 \times 10^5$ per well onto poly-L-lysine-treated 24-well dishes, grown in DMEM medium containing 0.5 % FCS overnight and then pretreated with OPTIMEM 1 for 2 h. Cells were then incubated with 1 µM SP or 1 µM DAMGO for 0, 1, 2, 4 or 6 h in OPTIMEM 1. For resensitization studies, cells were exposed to 1 µM DAMGO for 4 h followed by an additional incubation period of either 0, 10, 20, 30, 40, 50 or 60 min in the absence of agonist. Cells were then exposed to either SP, DAMGO or lysophosphatidic acid (LPA) for 5 min at 37 °C. Incubation was terminated by removal of the culture medium, and subsequent addition of 150 µl boiling SDS-sample buffer. The samples were assayed as previously described (13,14).

Data Analysis - Data from ligand binding and ERK assays were analyzed by non-linear regression curve fitting using GraphPad Prism 3.0 software. Statistical analysis was carried out using the two-tailed paired $t$ test. $P$-values < 0.05 were considered as statistically significant.

Results

Characterization of NK1-MOR1 Heterodimerization by Coimmunoprecipitation - To examine the dimerization of NK1 and MOR1 receptors, we produced HEK 293 cells stably coexpressing T7-tagged NK1 receptors and HA-tagged MOR1 receptors. Quantitative Western blot analyses revealed that 4 of 6 clones coexpressed similar levels of NK1 and MOR1 receptors (not shown). Saturation binding experiments revealed that NK1-MOR1 double stable clones used for further studies express nearly equivalent numbers of SP and DAMGO binding sites (Table I). While coexpressing cells had a similar high affinity for SP
as cells expressing T7NK1 alone, these cells exhibited an approximately three-fold higher affinity for DAMGO as cells expressing HAMOR1 alone (Table I). Competition binding assays also showed that SP did not compete with $[^3]$H]DAMGO binding, and DAMGO did not compete with $[^3]$H]SP binding in membranes prepared from T7NK1-HAMOR1 cells. When these cells were treated with the cross-linking agent DSP, solubilized and subjected to immunoprecipitation using T7 affinity beads, the anti-T7 antibody detected a monomeric and a dimeric form of the NK1 receptor (Figure 1A). When immunoprecipitation was carried out with HA affinity beads, the anti-HA antibody detected a monomeric and a dimeric form of the MOR1 receptor (Figure 1B). In addition, a band migrating at ~50 kDa was seen which may correspond to the nonglycosylated form of the MOR1 receptor (12). When T7NK1 receptors were immunoprecipitated using T7 affinity beads, the rat HA antibody detected a monomeric and a dimeric form as well as a nonglycosylated form of the HAMOR1 receptor only in extracts prepared from cells coexpressing T7NK1 and HAMOR1 but not in extracts prepared from a mixture of cells expressing these receptors separately (Figure 1C). The coprecipitation of T7NK1 and HAMOR1 suggests that these receptors may form heterodimeric or higher order heterooligomeric complexes in coexpressing cells.

**NK1 and MOR1 Receptors Form Heterodimers in Living Cells – Detergent**

solubilization of cells during coimmunoprecipitation studies could promote artifactual aggregation of hydrophobic proteins such as receptors. We therefore analyzed NK1-MOR1 heterodimerization in living cells using BRET. This technique is a proximity assay based on non radiative transfer of energy between a bioluminescent donor (Rluc) and a fluorescent acceptor (GFP) that allows real time monitoring of protein-protein interaction in living cells (33-37). To assess NK1 and MOR1 homo- and heterodimerization, fusion constructs linking
the receptor carboxyl-terminal tail to either Rluc or GFP were cotransfected in HEK 293 cells, and the transfer of energy between the two partners was assessed following the addition of Deep Blue C. Upon oxidation of the luciferase substrate, the enzyme emits light with a peak at 400 nm that can excite GFP, which, in turn, re-emits fluorescence with a peak at 510 nm but only if the two partners are within the BRET-permissive distance (< 100 Å). The BRET signal is determined by calculating the ratio of the light emitted by the Receptor-GFP over the light emitted by the Receptor-Rluc. As shown in Figure 2, a strong BRET signal was detected in cells expressing MOR1-Rluc/MOR1-GFP and in cells expressing MOR1-Rluc/NK1-GFP indicating that heterodimers between MOR1 and NK1 also form in living cells. No significant BRET was detected when MOR1-Rluc was coexpressed with soluble GFP, confirming the selectivity of the detected signals. No significant changes were detected in the measured MOR1-Rluc/NK1-GFP BRET levels when the cells were stimulated with either DAMGO, SP or a mixture of DAMGO and SP (not shown).

**Endocytotic Trafficking of the NK1-MOR1 Heterodimer** - We next examined the consequences of NK1-MOR1 heterodimerization on agonist-induced receptor endocytosis using a quantitative enzyme-linked immunosorbent assay and immunocytochemistry. As depicted in Figure 3A, quantitative analysis of receptor internalization after treatment with either 1 µM SP or 1 µM DAMGO revealed that NK1 and MOR1 were selectively internalized only in response to their cognate ligands in cells expressing these receptors alone. In cells coexpressing NK1 and MOR1, NK1 was internalized in response to both SP and DAMGO. Conversely, MOR1 internalized after DAMGO as well as after SP (Figure 3C). As shown in Figure 3B and D, both NK1 and MOR1 receptors were predominantly confined to the plasma membrane in untreated cells (*Control*). After 30 min exposure to SP, a loss of NK1 receptors
from the plasma membrane and a robust internalization was observed in cells expressing the
NK1 receptor alone (Figure 3B, SP). In contrast, the distribution of NK1 receptors did not
change when these cells were treated with DAMGO. Incubation with DAMGO but not SP
induced internalization of MOR1 receptors in cells expressing MOR1 alone (Figure 3B,
DAMGO). The confocal images clearly showed that upon DAMGO treatment the MOR1
internalized and clustered in smaller vesicle-like structures then the NK1 after SP incubation
indicating that these receptor have a different endocytotic fate. As depicted in Figure 3D, SP
induced a robust redistribution of both NK1 and MOR1 when these receptors were expressed
in the same cells. Similar, after treatment with DAMGO, MOR1 as well as NK1 were
internalized (Figure 3D, DAMGO). The confocal images of coexpressing cells revealed that
SP and DAMGO induced cointernalization of NK1 and MOR1 into the same endosomal
compartment.

β-Arrestin2 Trafficking in NK1 and MOR1 Coexpressing Cells – MOR1 and NK1
have been reported to profoundly differ in their patterns of β-arrestin trafficking. For MOR1,
β-arrestin directs the receptors to clathrin-coated pits. However, MOR1-β-arrestin complexes
are relatively unstable and dissociate at or near the plasma membrane. Consequently, β-
arrestin is excluded from MOR1-containing vesicles (25,38). In contrast, NK1 and β-arrestin
form stable complexes, remain associated, and internalize together into the same endocytotic
vesicles (25-28). We therefore examined β-arrestin trafficking in cells coexpressing MOR1
and NK1 after transient transfection of GFP-labeled β-arrestin2. Cells were exposed to either
1 μM SP or 1 μM DAMGO for 30 min and the subcellular distribution of the receptor
proteins and β-arrestin2-GFP was analyzed by confocal microscopy. As shown in Figure 4,
NK1 (left panel, Control, red) and MOR1 (right panel, Control, red) were clearly confined to
the plasma membrane whereas β-arrestin2 (Control, green) was distributed throughout the cytoplasm but excluded from the nucleus in untreated cells. Like that seen in cells expressing NK1 alone, the NK1 receptor was internalized together with β-arrestin2-GFP into large clusters of early endosomes after 30 min exposure to SP in cells coexpressing NK1 and MOR1 (Figure 4, left panel, SP). Interestingly, under these conditions the MOR1 receptor was cointernalized together with NK1 into same endosomes which cocontained β-arrestin2-GFP (Figure 4, right panel, SP). Moreover, unlike that seen in cells expressing MOR1 alone, the MOR1 receptor was also internalized together with β-arrestin2-GFP into large clusters of early endosomes after 30 min exposure to DAMGO in cells coexpressing NK1 and MOR1 (Figure 4, left panel, DAMGO). Under identical conditions the NK1 receptor was cointernalized together with MOR1 into same endosomes which cocontained β-arrestin2-GFP (Figure 4, right panel, DAMGO). These results suggest that activation of the NK1-MOR1 heterodimer by both SP and DAMGO induced the formation of stable β-arrestin complexes and cotrafficking of the receptor heterodimer and β-arrestin into the same endosomal compartment thereby promoting a functional switch of MOR1 from a class A to a class B receptor.

*Phosphorylation of the NK1-MOR1 Heterodimer* – Formation of stable complexes between β-arrestin and GPCRs strongly depends on the presence of clusters of phosphate acceptor sites (defined as serine/threonine residues occupying three consecutive positions or three out of four positions) within the carboxyl-terminal tail of the receptor. These clusters are remarkably conserved in their position within the carboxyl-terminal domain and serve as primary sites of agonist-dependent receptor phosphorylation (26,27). While such clusters of phosphate acceptor sites are present in the NK1 receptor, the carboxyl-terminal tail of MOR1
does not contain such a motif. To delineate a mechanistic basis for the observed switch of MOR1 from a class A to a class B receptor, we assessed whole cell receptor phosphorylation in response to either SP or DAMGO in cells coexpressing NK1 and MOR1. As shown in Figure 5, SP induced a rapid and robust phosphorylation of the NK1 receptor monomer (~ 4 fold over basal). Interestingly, SP exposure of the NK1-MOR1 heterodimer also significantly increased phosphorylation of the MOR1 receptor monomer (~ 3 fold over basal). As expected DAMGO produced a rapid and robust phosphorylation of the MOR1 receptor monomer (~ 4.5 fold over basal). DAMGO also significantly increased phosphorylation of the NK1 receptor monomer (~ 2 fold over basal) indicating that activation of the MOR1 subunit of the NK1-MOR1 heterodimer resulted in cross-phosphorylation of the NK1 subunit and *vice versa*. This cross-phosphorylation was not simply due to cross-reactivity of the agonists, because it was not observed in cells expressing either T7NK1 or HAMOR1 alone (not shown). Thus, NK1-MOR1 cross-phosphorylation may provide a plausible explanation for the altered β-arrestin trafficking in response to activation of this heterodimeric receptor.

*De- and Resensitization of the NK1-MOR1 Heterodimer* – NK1 and MOR1 differ in their intracellular signalling in that NK1 stimulates inositol triphosphate formation via $G_\text{q}$ and MOR1 inhibits cAMP formation via $G_\text{i}$. However, both NK1 and MOR1 stimulate a rapid and transient ERK1/2 phosphorylation. Analysis of mitogenic signalling of the NK1-MOR1 heterodimer revealed that SP and DAMGO produced similar dose- and time-dependent responses in cells coexpressing T7NK1 and HAMOR1 as in cells expressing these receptors alone. We then examined the desensitization of ERK signalling of the NK1-MOR1 heterodimer. Cells coexpressing T7NK1 and HAMOR1 were preincubated in the presence or absence of either SP or DAMGO for 4 h. The medium was removed and the ability of SP,
DAMGO or LPA to stimulate ERK1/2 activity was determined. As depicted in Figure 6, preincubation with either SP or DAMGO for 4 h significantly attenuated both NK1- and MOR1-dependent responses. In contrast, mitogenic signalling of the LPA receptor, a third receptor which is endogenously expressed in this system, was unchanged suggesting that the NK1-MOR1 heterodimer underwent homologous cross-desensitization under these conditions. Class A and class B receptors profoundly differ in their resensitization kinetics. Given our observation that NK1-MOR1 heterodimerization promoted a functional switch of MOR1 from a class A to a class B receptor, we compared resensitization of µ-opioid receptor-dependent responses in MOR1-expressing cells and in cells coexpressing NK1 and MOR1. Cells were preincubated in the presence or absence of DAMGO for 4 h. After a DAMGO-free incubation period the medium was removed and the ability of DAMGO to stimulate ERK1/2 activity was determined. The results depicted in Figure 7 show that resensitization of MOR1-mediated mitogenic signalling was severely delayed in cells coexpressing NK1 and MOR1 as compared to cells expressing MOR1 alone.
**Discussion**

The neuropeptide SP and endogenous opioids, as well as their corresponding G protein coupled receptors, are intimately involved in the regulation of nociceptive transmission (15-22). At the level of the spinal cord, many SP-containing terminals target neurons that encode noxious stimuli. These neurons have recently been shown to contain both NK1 and MOR1 receptors (15,16,22). Increased NK1 receptor stimulation in mice lacking noradrenaline leads to reduced opioid efficacy (19). Electrical stimulation of afferent C-fibres is associated with a NK1-dependent increase in the excitability of spinal cord neurons – a phenomenon termed “wind-up”. Wind-up is effectively abolished by opioids (18). Substance P-opioid chimeric peptides produce analgesia without formation of tolerance (17). In addition, MOR1 and NK1 are highly coexpressed in brain regions implicated in depression, anxiety and stress, but also in other regions such as the nucleus accumbens which mediate the motivational properties of drugs of abuse including opioids. Interestingly, the rewarding effects of morphine are absent in mice lacking the NK1 receptor (20,21). Despite these observations, a molecular basis for the interactions between opioid and substance P receptors has not been established yet.

Here, we provide biophysical and biochemical evidence for heterooligomerization of the substance P and the μ-opioid receptor. The coimmunoprecipitation experiments carried out in the present study clearly demonstrate that NK1 and MOR1 receptors exist as constitutive heterooligomeric complexes at the plasma membrane when coexpressed in HEK 293 cells. The immunoprecipitation of T7-tagged NK1 receptors resulted in coprecipitation of HA-tagged MOR1 receptors only from coexpressing cells but not from a mixture of cells expressing these receptors separately, suggesting that NK1-MOR1 heterooligomers preexisted in these cells prior to cell lysis and were not artifactually formed during sample preparation.
When cells were treated with the cross-linker DSP, precipitation of T7-tagged NK1 receptors resulted in coprecipitation of HA-tagged MOR1 receptors with migration properties corresponding to that of the nonglycosylated MOR1 receptor (12). The fact that this lower molecular weight band was not seen in whole cell phosphorylation assays suggests that NK1-MOR1 heterooligomers may have already formed in the cytoplasmic reticulum. By utilizing BRET, we demonstrated that NK1 and MOR1 form constitutive heterooligomeric complexes in living cells. Recent evidence from quantitative BRET analysis of β1- and β2-adrenergic receptor homo- and heterodimerization suggests that most of the receptors expressed in cells exist as constitutive dimers and that, at least in undifferentiated fibroblasts, the proportion of homo- and heterooligomers is determined by the relative level of receptor expression (39). Given the frequent coexpression of MOR1 and NK1 in spinal cord neurons, a direct protein-protein interaction between these receptors is possible. Such a physical interaction could provide a plausible explanation for crosstalk between substance P and µ-opioid receptors during nociceptive transmission. Our analysis of the functional consequences of NK1-MOR1 heterodimerization revealed no substantial changes of the ligand binding and signalling properties of these receptors. In contrast, NK1-MOR1 heterodimerization markedly altered the internalization and desensitization profile of both receptors. Exposure of the NK1-MOR1 heterodimer to the MOR1-selective ligand DAMGO promoted cointernalization and cross-desensitization of the NK1 receptor. Conversely, exposure of the NK1-MOR1 heterodimer to the NK1-selective ligand SP promoted cointernalization and cross-desensitization of the MOR1 receptor.

Based on trafficking patterns and affinity for β-arrestin, GPCRs are categorized into two classes (23-29). Class A receptors include the µ-opioid, β2 and α1B adrenergic, endothelin
A and dopamine D1A receptors. These receptors bind to β-arrestin2 with higher affinity than to β-arrestin1. In addition, their interaction with β-arrestin is transient. β-arrestin is recruited to the receptor at the plasma membrane translocates with it to clathrin-coated pits, however, the receptor-β-arrestin complex dissociates upon internalization of the receptor, such that, as the receptor proceeds into the endosomal pool, the β-arrestin recycles back to the plasma membrane (26,38). Class B receptors, represented by the substance P, angiotensin AT1a, neurotensin 1 and vasopressin 2 receptors bind to β-arrestin1 and β-arrestin2 with equal affinity. These receptors form stable complexes with β-arrestin, such that the receptor-β-arrestin complex internalizes as a unit that is targeted to endosomes (23,26-28). While class A receptors recycle and resensitize rapidly, class B receptors recycle and resensitize slowly (25).

Interestingly, in cells coexpressing NK1 and MOR1, we observed that both DAMGO and SP induced recruitment of β-arrestin to the plasma membrane and cointernalization of NK1-MOR1 heterodimers with β-arrestin into endosomes. Consequently, resensitization of MOR1-dependent receptor functions was severely delayed in coexpressing cells as compared to cells expressing MOR1 alone indicating that MOR1 by virtue of its physical interaction with NK1 is sequestered via a class B receptor-like pathway with delayed recycling and resensitization kinetics.

The structural features that dictate the stability of the receptor-β-arrestin complex reside within specific clusters of putative phosphate acceptor sites (defined as serine/threonine residues occupying three consecutive positions or three out of four positions) in the carboxyl-terminal tail of the receptor (27). Such clusters are remarkably conserved in their position within the carboxyl-terminal tail with respect to the NPXXF motif (marking the end of the seventh transmembrane domain) and the end of the receptor (27). While such clusters of
phosphate acceptor sites are present in the NK1 receptor, the carboxyl-terminal tail of MOR1 does not contain such a motif. In whole cell receptor phosphorylation assays, we demonstrate that SP exposure of the NK1-MOR1 heterodimer significantly increased phosphorylation of the MOR1 receptor monomer. Conversely, DAMGO exposure of the NK1-MOR1 heterodimer significantly increased phosphorylation of the NK1 receptor monomer. Thus, homologous cross-phosphorylation of the NK1-MOR1 heterodimer may provide a mechanistic basis for cotrafficking of NK1-MOR1 heterodimers with β-arrestin into endosomes.

The stability of the receptor-β-arrestin interaction determines not only the recycling and resensitization profile of the receptor but also the mechanisms and functional consequences of downstream signalling (24). Tohgo et al. have recently shown that activated ERK remains associated with stable class B receptor-β-arrestin complexes which, in turn, limits nuclear translocation of ERK and attenuates Elk1-driven transcription (29). In contrast, class A receptors promote nuclear translocation of activated ERK and stimulate Elk1-driven transcription (29). Thus, altered β-arrestin trafficking induced by NK1-MOR1 heterodimerization may lead to cytosolic retention of activated ERK and distinct patterns of ERK-dependent transcription.

In conclusion, we provide biophysical, biochemical and functional evidence for heterodimerization of two distantly related receptors namely the substance P and the µ-opioid receptor. We show that heteromeric assembly of NK1 and MOR1 cross-modulates internalization and desensitization of both receptors. In addition, we demonstrate that MOR1 by virtue of its physical interaction with NK1 is sequestered via a class B receptor-like pathway with delayed recycling and resensitization kinetics. Altered β-arrestin trafficking in
cells coexpressing MOR1 and NK1 could, thus, not only impact on opioid receptor
resensitization but also on the long-term cellular effects of opioids.

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Figure Legends

FIG. 1. Characterization of NK1-MOR1 heterodimers. HEK 293 cells coexpressing T7NK1 and HAMOR1 or a mixture of cells expressing either T7NK1 or HAMOR1 alone were treated with DSP, lysed in detergent buffer and subjected to immunoprecipitation using T7 affinity beads (A, C) or HA affinity beads (B) and immunoblotted using anti-T7 (A) or anti-HA (B, C) antibodies. Note, immunoprecipitation of T7-tagged NK1 receptors resulted in coprecipitation of HA-tagged MOR1 receptors only when T7NK1 and HAMOR1 were coexpressed in the same cell but not when cells expressing T7NK1 or HAMOR1 individually were mixed prior to immunoprecipitation. The position of the NK1-MOR1 heterodimer is indicated on the right. The positions of molecular mass markers are indicated on the left (in kDa). Three additional experiments gave similar results.

FIG 2. Characterization of NK1-MOR1 heterodimers in living cells. HEK 293 cells were transiently cotransfected with MOR1-Rluc in combination with soluble GFP, MOR1-GFP or NK1-GFP. A transfection with Rluc-GFP fusion protein was used as positive control. Cells were harvested 48 h post-transfection, counted, and transferred to 96-well plates. The energy transfer was initiated by addition of 5 μM Deep Blue C coelenterazine to each well, and BRET was assessed in a Fusion microplate reader as described under “Experimental Procedures”. The results represent mean ± S.E. of 3 independent experiments performed in triplicate.
FIG. 3. Agonist-induced endocytosis of NK1-MOR1 heterodimers. A and C, HEK 293 cells expressing either T7NK1 or HAMOR1 (A) or cells coexpressing T7NK1 and HAMOR1 (C) were either left untreated or exposed to 1 µM SP or 1 µM DAMGO. Cell surface receptors were labeled with rabbit anti-T7 or anti-HA antibodies followed by a peroxidase-conjugated secondary antibody. Receptor sequestration, quantified as the percent loss of cell-surface receptors in agonist-treated cells, was measured by ELISA. Data are presented as mean ± S.E. of five independent experiments performed in triplicate. B, HEK 293 cells expressing either T7NK1 (upper panel) or HAMOR1 (lower panel) were either not exposed (Control) or exposed to 1 µM SP or 1 µM DAMGO for 30 min. Cells were subsequently fixed, fluorescently labeled with either anti-T7 or anti-HA antibodies and the subcellular distribution of receptor proteins was examined by confocal microscopy. D, HEK 293 cells coexpressing T7NK1 and HAMOR1 were either not exposed (Control) or exposed to 1 µM SP or 1 µM DAMGO for 30 min. Cells were subsequently fixed, subjected to dual immunofluorescence labeling with a mixture of anti-T7 and anti-HA antibodies and the subcellular distribution of receptor proteins was examined by confocal microscopy. The upper panel shows NK1-Li, and the lower panel shows MOR1-Li. Shown are representative results from one of three independent experiments performed in duplicate. Note, that in untreated cells both NK1 and MOR1 were almost exclusively confined to the plasma membrane revealing extensive colocalization. Exposure of the NK1-MOR1 heterodimer to SP promoted cointernalization of MOR1 together with NK1. Conversely, exposure of the NK1-MOR1 heterodimer to DAMGO induced cointernalization of NK1 together with MOR1. The asterisks indicate a significant difference ($p < 0.05$) between T7NK1-HAMOR1 expressing cells and cells expressing these receptors alone (two-tailed paired $t$ test). Scale bars, 20 µm.
FIG. 4. **Agonist-induced β-arrestin trafficking in cells coexpressing NK1 and MOR1.** *Left panel,* HEK 293 cells coexpressing T7NK1 and HAMOR1 were transiently transfected with β-arrestin2-GFP and either not exposed (*Control*) or exposed to 1µM SP or 1 µM DAMGO for 30 min. Cells were fixed, and T7NK1 receptors were labeled using anti-T7 antibodies. *Right panel,* HEK 293 cells coexpressing T7NK1 and HAMOR1 were transiently transfected with β-arrestin2-GFP and either not exposed (*Control*) or exposed to 1µM SP or 1 µM DAMGO for 30 min. Cells were fixed, and HAMOR1 receptors were labeled using anti-HA antibodies. Note, in untreated cells both NK1 and MOR1 (*shown in Red*) were almost exclusively confined to the plasma membrane whereas β-arrestin2 (*shown in Green*) was distributed throughout the cytoplasm. Exposure of the NK1-MOR1 heterodimer to either SP or DAMGO promoted internalization of both NK1 and MOR1 together with β-arrestin2-GFP (*Yellow in overlay*). Representative images from two independent experiments performed in duplicate are shown. Scale bar, 20 µm.

FIG. 5. **Agonist-induced cross-phosphorylation of the NK1-MOR1 heterodimer.** HEK 293 cells coexpressing T7NK1 and HAMOR1 were exposed to 1 µM SP or 1 µM DAMGO for 20 min, and whole cell receptor phosphorylation was determined. T7NK1 was immunoprecipitated with rabbit anti-T7 antibodies, and HAMOR1 was immunoprecipitated with rabbit anti-HA antibodies. *A,* autoradiographs from representative experiments are shown. *B,* means ± S.E. of three independent experiments quantified by PhosphorImager analysis. The *asterisks* indicate significant agonist-induced phosphorylation compared with basal levels in the absence of agonist (*p* < 0.05; two-tailed paired *t* test). Note, phosphorylation of MOR1 was significantly increased above basal levels in the presence of
the NK1-selective agonist SP. Conversely, phosphorylation of NK1 was significantly increased above basal levels in the presence of the MOR1-selective agonist DAMGO. The data were normalized to basal phosphorylation in the absence of agonist for each receptor monomer. The positions of molecular mass markers are indicated on the left (in kDa).

**FIG. 6.** *Agonist-induced cross-desensitization of coupling to ERK1/2 of NK1-MOR1 heterodimers.* HEK 293 cells coexpressing T7NK1 and HAMOR1 were serum-starved overnight and then incubated in the presence or absence of 1 µM SP or 1 µM DAMGO for 4 h. The cells were washed and then exposed to either 1 µM SP, 1 µM DAMGO or 1 µM lysophosphatidic acid (*LPA*) for 5 min. Cells were lysed, equal amounts of protein were resolved by SDS-PAGE and levels of total ERK2 and phosphorylated ERK1/2 were determined by immunoblotting. A, results were quantified by densitometric analysis. Data were normalized to total ERK2 and expressed as the fold ERK1/2 phosphorylation over basal in untreated cells. Values represent means ± S.E. of three independent experiments performed in duplicate. The *asterisks* indicate a significant difference (*p* < 0.05) between cells preincubated with either SP or DAMGO and cells that had not been preincubated (two-tailed paired *t* test). *B*, representative immunoblots for phospho-ERK1/2 (*upper panel*) and total ERK2 (*lower panel*), respectively. The positions of phospho-ERK1/2 (*pERK1/2*) and total ERK2 (*ERK2*) are indicated on the right. The positions of molecular mass markers are indicated on the left (in kDa).
FIG. 7. Differential Resensitization of µ-Opioid Receptor Responses in MOR1- and NK1-MOR1-expressing cells. HEK 293 cells expressing HAMOR1 or coexpressing T7NK1 and HAMOR1 were serum-starved overnight and incubated in the presence or absence of 1 µM DAMGO for 4 h. Cells were washed followed by a DAMGO-free interval of either 0, 10, 20, 30, 40, 50 or 60 min. Cells were then exposed to DAMGO for 5 min and subsequently lysed. Equal amounts of protein were resolved by SDS-PAGE, and levels of phosphorylated ERK1/2 were determined by immunoblotting. A, results were quantified by densitometric analysis. Data were normalized to total ERK2 and expressed as the fold ERK1/2 phosphorylation over the basal value in untreated cells. The maximum agonist-induced activation of ERK1/2 without agonist preincubation was defined as 100%. Values represent means ± S.E. of three independent experiments performed in duplicate. The asterisks indicate a significant difference ($p < 0.05$) between MOR1-expressing cells and cells coexpressing NK1 and MOR1 (two-tailed paired t test). B, representative immunoblots for MOR1-expressing cells (upper panel) and cells coexpressing NK1 and MOR1 (lower panel), respectively. The position of phospho-ERK1/2 ($pERK1/2$) is indicated on the right. The positions of molecular mass markers are indicated on the left (in kDa).
FOOTNOTES

The abbreviations used are: BRET, bioluminescence resonance energy transfer; DAMGO, [D-\text{Ala}^{2},\text{Me-Phe}^{4},\text{Gly}^{5}-\text{ol}]\text{enkephalin}; DMEM, Dulbecco’s modified Eagle medium; ERK, extracellular signal-regulated kinases; FCS, fetal calf serum; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; LPA, lysophosphatidic acid; MOP, \(\mu\)-opioid-peptide-receptor; MOR1, \(\mu\)-opioid receptor; NK1, neurokinin 1 receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate; SP, substance P; sst, somatostatin receptor; TPBS, Tris/phosphate-buffered saline.
TABLE I

*Ligand binding properties of NK1, MOR1 and NK1-MOR1 receptors*

Saturation binding assays were performed on membranes prepared from stably transfected cells. The dissociation constant \((K_D)\) and number of \([^3]H\)DAMGO binding sites \((B_{max})\) were calculated by Scatchard analysis as described under “Experimental Procedures”. Data are presented as mean ± S.E. of three or four independent experiments performed in triplicate.

|                      | \[^3\]HSP binding                      | \[^3\]DAMGO binding                   |
|----------------------|----------------------------------------|---------------------------------------|
|                      | \(K_D\) (nM)                           | \(B_{max}\) (fmol/mg protein)         |
| T7NK1-HAMOR1         | 5.27 ± 0.35                            | 1,752 ± 315                           |
| T7NK1                | 5.79 ± 0.78                            | 1,178 ± 135                           |
|                      |                                        |                                       |
| T7NK1-HAMOR1         | 4.38 ± 0.43                            | 1,137 ± 199                           |
| HAMOR1               | 1.28 ± 0.03                            | 3,991 ± 242                           |
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Figure 1
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Figure 2
Pfeiffer et al.
Figure 3
Pfeiffer et al.

Figure 4

T7NK1-HAMOR1 coexpressing cells + β-Arrestin2-GFP

Control

SP

DAMGO
Pfeiffer et al.

Figure 6
Pfeiffer et al.
Figure 7
Heterodimerization of substance P and mu-opioid receptors regulates receptor trafficking and resensitization

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