

Interaction of the μ -Opioid Receptor with Synaptophysin Influences Receptor Trafficking and Signaling

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Received May 2, 2006; accepted September 27, 2006

ABSTRACT

There is increasing evidence that the signal transduction of opioid receptors is modulated by receptor-associated proteins. In the search for proteins regulating μ -opioid receptor (MOPr) endocytosis, synaptophysin was found to bind to the rat μ -opioid receptor in yeast two-hybrid assay. Coimmunoprecipitation experiments and bioluminescence resonance energy transfer assays confirmed that the μ -opioid receptor constitutively interacts with synaptophysin in human embryonic kidney 293 cells overexpressing MOPr and synaptophysin. In this study, we show that overexpression of synaptophysin enhances the μ -opioid receptor endocytosis. One explanation for the ob-

served effects is that synaptophysin recruits dynamin to the plasma membrane, facilitating fission of clathrin-coated vesicles. This suggestion is supported by our finding that overexpression of a synaptophysin truncation mutant, which breaks the interaction between synaptophysin and dynamin, prevents agonist-mediated μ -opioid receptor endocytosis. In addition, the synaptophysin-augmented μ -opioid receptor trafficking leads to attenuated agonist-induced receptor desensitization and faster receptor resensitization. Taken together, our findings strongly suggest that synaptophysin plays an important role in the regulation of μ -opioid receptor trafficking and signaling.

The action of most clinically important opiate drugs (e.g., morphine, as well as drugs of abuse, such as heroin) is mediated by the μ -opioid receptor, which belongs to the G-protein-coupled receptor family (Matthes et al., 1996; Kieffer and Gaveriaux-Ruff, 2002). Similar to other G-protein-coupled receptors, agonist binding to opioid receptors is followed by receptor coupling to G-proteins and subsequent phosphorylation. The binding of β -arrestins to phosphorylated receptors leads to receptor uncoupling from G-proteins, which in turn causes receptor desensitization. In addition, β -arrestins bind to both clathrin and adaptor protein complex AP-2. The coordinated interaction of both clathrin and AP-2 with β -arrestins is necessary for heptahelical receptor internalization via clathrin-coated vesicles. Once the plasma membrane is invaginated, GTPase dynamin wraps around and constricts the necks upon GTP hydrolysis; thus, vesiculation occurs. These vesicles soon shed their clathrin coats and become

early endosomes. The ligands and receptors are separated in an acidified perinuclear compartment. Dissociation of β -arrestins also occurs. Cytosolic phosphatases may dephosphorylate the receptors. The ligands are degraded, whereas the receptors are either recycled to the plasma membrane or degraded in lysosomes (for reviews, see Claing et al., 2002; Mousavi et al., 2004).

The trafficking and signaling of G-protein-coupled receptors are affected by the interaction with various proteins (for review, see Bockaert et al., 2004). For example, the interaction of rat μ -opioid receptors with phospholipase D2 accelerates receptor internalization (Koch et al., 2003). Periplakin, a cytolinker protein, interacts with the human μ -opioid receptor and reduces the coupling of G proteins (Feng et al., 2003). Using the yeast two-hybrid system, we identified another μ -opioid receptor interacting protein, synaptophysin. Synaptophysin is a major integral membrane glycoprotein of synaptic vesicles and is also found in a subset of coated vesicles in brain, presumably as a result of vesicle trafficking (Sudhof et al., 1987). The exact role of synaptophysin remains controversial. In this study, we showed for the first time that synaptophysin was constitutively associated with the μ -opioid receptor and involved in receptor trafficking. Moreover,

This work was supported by grants HO 1027/10-1 and SFB 426/A2 from the Deutsche Forschungsgemeinschaft (to V.H.) and Bundesministerium für Bildung und Forschung grant 01ZZ0407 (to T.K.).

Y.-J.L. and D.-F.W. contributed equally to this work.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.026062.

ABBREVIATIONS: PCR, polymerase chain reaction; Syp, synaptophysin; MOPr, the μ -opioid receptor; CMV, cytomegalovirus; HEK, human embryonic kidney; HA, influenza hemagglutinin-HA-epitope (YPYDVPDYA); HA-MOPr, HA epitope-tagged MOPr; Myc, epitope tag (MASMQKLI-SEEDL); GST, glutathione transferase; BRET, bioluminescence resonance energy transfer; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; DAMGO, [D-Ala², Me-Phe⁴, Glyol⁵]enkephalin; IP, immunoprecipitation; AP, adaptor protein; 3L μ , third intracellular loop of the μ -opioid receptor; ANOVA, analysis of variance.

we provide evidence that the synaptophysin-augmented μ -opioid receptor trafficking plays an important role in agonist-induced receptor desensitization and resensitization.

Materials and Methods

Yeast Two-Hybrid Screen. The Yeast Two-Hybrid System 3 (Clontech, Heidelberg, Germany) was used to investigate interacting proteins of rat μ -opioid receptor. The cDNA fragments encoding the full-length rat μ -opioid receptor, a truncation (amino acids 258–398, from the 3rd intracellular loop to the carboxyl tail), and the carboxyl tail alone (amino acids 340–398) of rat μ -opioid receptor (GI: 6981309) were amplified by PCR and subcloned into vector pGBKT7, containing the Gal4 DNA binding domain. These fusion genes were used as baits to screen a rat brain cDNA library (Clontech), constructed in the vector pACT2 containing the Gal4 DNA activation domain. The bait and library plasmids were sequentially transformed into yeast strain AH109, using the lithium acetate method. The yeast two-hybrid screen was carried out according to the manufacturer's instructions.

Plasmid Construction. For cloning of the full-length cDNA of rat synaptophysin (Syp; GI: 6981621), PCR primers were designed based on the sequence of Syp cDNA segment from the rat cDNA library obtained from yeast two-hybrid assay. The sequence of forward primer introducing a SalI restriction site is 5'-TAG CTT GTC GAC TAT GGA CGT GGT GAA TCA GCT GGT GGC TGG GGG TCA GTT CCG GGT GGT CAA GGA GCC CCT TGG CTT CGT GAA G-3', and sequence of reverse primer introducing a KpnI restriction site is 5'-ACT TCA GGT ACC AGA TTA CAT CTG ATT GGA-3'. Using the pACT2-Syp plasmid containing a partial length of Syp (nucleotides 49–924) as template, the full-length coding region of rat Syp was amplified and subcloned into the SalI/KpnI sites of pCMV-Myc expression vector (Clontech) to produce amino-terminally Myc-tagged Syp. Likewise, the Syp truncations (nSyp, amino acids 1–223; cSyp, amino acids 219–307 of Syp) were amplified by PCR, and subcloned into pCMV-Myc. For transient transfection, the coding region of MOPr was amplified using PCR and then subcloned into pCMV-HA expression vector, which generates amino-terminally HA-tagged MOPr.

Immunoprecipitation and Western Blot Analysis. In a 100-mm dish, HEK293 cells (American Type Culture Collection, Manassas, VA) or HEK293 cells stably expressing HA-MOPr, as described below under *Generation of Stable Cell Lines*, were transiently transfected with pCMV-Myc-Syp plasmid using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were completely lysed in a radioimmunoprecipitation assay 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, and proteinase inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 μ g/ml aprotinin, and 10 μ g/ml bacitracin), and the resulting lysate was subjected to Western blot analysis or immunoprecipitation as described previously (Pfeiffer et al., 2003). In brief, receptor proteins were immunoprecipitated with 100 μ l of protein A agarose beads preloaded with 10 μ g of anti-HA antibody. After SDS-polyacrylamide gel electrophoresis and electroblotting, membranes were incubated with 0.5 μ g/ml anti-HA or anti-Myc antibody (Clontech) followed by detection using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

GST-Affinity Chromatography. cDNA fragment encoding the third intracellular loop of MOPr (3IL μ , amino acids 253–283 of MOPr) was subcloned into bacterial expression vector pGEX-2KT (GE Healthcare) to generate the glutathione transferase (GST) fusion proteins by PCR using the following primers. The forward primer 5'-ACT GTG GGA TCC GGC CTG ATG ATC TTA-3' introduced a BamHI restriction site, and reverse primer 5'-AGC CAC

GAA TTC CAG CAC CAT CCG GGT GAT-3' introduced an EcoRI restriction site. Before produce GST and GST-3IL μ affinity beads, glutathione agarose beads (GE Healthcare) were preincubated with a buffer (20 mM HEPES, pH 7.6, 100 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, and proteinase inhibitors). After overnight incubation (4°C), the beads were incubated with XL1 bacterial lysate containing GST or GST fusion proteins for an additional 3 h at 4°C and followed complete wash. HEK cells transiently transfected with pCMV-Myc-Syp using Lipofectamine 2000 were completely lysed in the radioimmunoprecipitation assay buffer. Debris was removed by pelleting and expressed Myc-Syp proteins were purified using 100 μ l of protein A agarose beads (GE Healthcare) preloaded with 10 μ g of anti-Myc antibody. Eluted Myc-Syp proteins from the beads were incubated with GST-3IL μ or GST beads at 4°C for 3 h. After complete wash, bound proteins were eluted and run on SDS-polyacrylamide gel electrophoresis, and immunoblots were visualized as described above.

Bioluminescence Resonance Energy Transfer Assay. The coding sequence without a stop codon of MOPr was amplified by PCR and ligated into humanized pRluc-N₃ expression vector (BioSignal Packard, Montreal, QC, Canada). The full-length Syp and its truncations (nSyp and cSyp) were amplified by PCR and subcloned into humanized pGFP-C₃ expression vectors (BioSignal Packard). For bioluminescence resonance energy transfer (BRET) measurements, HEK293 cells were transiently cotransfected with Rluc fusion and GFP fusion plasmids using Lipofectamine 2000. Forty-five hours after transfection, cells were detached with PBS/EDTA and resuspended in PBS buffer containing 0.1% (w/v) glucose and 2 μ g/ml aprotinin. Approximately 1×10^5 cells per well were transferred into a white-bottomed 96-well microplate (BioSignal Packard). Deep Blue C (a modified form of coelenterazine; BioSignal Packard) was added at a final concentration of 5 μ M, and readings were collected using a Fusion Universal Microplate Analyzer (BioSignal Packard) 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 BRET signal is determined by calculating the ratio of the light emitted by the GFP (485–545 nm) over the light emitted by the Rluc (330–490 nm) after subtracting background signal detected in nontransfected cells. Using the Fusion Universal Microplate Analyzer, the expression level of GFP-Syp, GFP-cSyp, and GFP-nSyp can be examined by fluorescence assay (light source, halogen CW; excitation, 485 nm; emission, 530 nm), and the expression level of MOPr-Rluc or Rluc can be evaluated (emission, 410 nm) after the addition of Deep Blue C.

Confocal Microscopy. Cells were grown onto poly(L-lysine)-coated coverslip overnight. After treatment with or without monensin for 1 h, cell fixation, permeabilization, and antibody incubation with anti-HA and/or anti-Myc antibodies were carried out as previously described (Koch et al., 2003). Bound primary antibodies were detected with cyanine 2.18- and/or cyanine 3.18-conjugated secondary antibodies (1:400; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cells were then permanently mounted in DPX (Fluka, Neu-Ulm, Germany) and examined using a TCS-NT confocal microscope (Leica, Wetzlar, Germany).

Primary Neuronal Cell Culture, Transfection, and Immunostaining. Neuronal cultures were prepared from the rat cortex of embryonic day 17 embryos. All animal procedures were approved by the Otto-von-Guericke-University Magdeburg. In 12-well plates with poly(D-lysine)-coated coverslips, primary cortical cells were seeded at 4×10^5 cells/well in 1.5 ml of Neurobasal medium (Invitrogen) supplemented with 2% B-27, 0.5 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were cultured at 37°C and 5% CO₂ in a humidified incubator. Half of the volume was removed on day 3 after seeding and replaced with fresh culture medium. Primary neuronal cultures on day 4 were transfected with pCMV-HA-MOPr and pCMV-Myc-Syp plasmids using Lipofectamine 2000. Approximately 45 h after transfection, cells were treated and subsequently

fixed. The distribution of expressed proteins of interest was detected by confocal microscopy as described above.

Generation of Stable Cell Lines. MOPr or Syp was tagged at the amino terminus with the HA or Myc epitope tag using PCR and subcloned into the pEAK10 (Edge Bio Systems, Gaithersburg, MD) or pcDNA3.1 expression vector (Invitrogen), respectively. HEK293 cells were first transfected with pEAK10-HA-MOPr plasmid using Lipofectamine 2000. Stable transfectants were selected in the presence of 1.25 $\mu\text{g/ml}$ puromycin (Sigma, Deisenhofen, Germany). To generate cell lines coexpressing HA-MOPr and Myc-Syp, GFP-nSyp (amino acids 1–223 of Syp), or GFP-cSyp (amino acids 219–307 of Syp), the cells were subjected to a second round of transfection with pcDNA3.1-Myc-Syp, pGFP-C₃-nSyp, or pGFP-C₃-cSyp plasmid, as described above, and selected in the presence of 1.25 $\mu\text{g/ml}$ puromycin plus 1000 $\mu\text{g/ml}$ G418 or 100 $\mu\text{g/ml}$ Zeocin (Invitrogen), respectively. Multiclones were used for further study. Receptor and/or synaptophysin expression were monitored using Western blot and/or confocal microscopy.

Radioligand Binding Assay. Binding studies were performed on membranes prepared from stably transfected cells. The dissociation constant (K_D) and number of [³H]DAMGO binding sites (B_{max}) were calculated by Scatchard analysis using at least seven concentrations of [³H]DAMGO in a range from 0.25 to 10 nM as described previously (Koch et al., 2006).

Quantitative Analysis of Receptor Trafficking. Cells were seeded at a density of 4.0×10^4 per well and grown onto poly(L-lysine)-treated 96-well plates overnight. To estimate endocytosis, cells were specifically surface-labeled with anti-HA antibody (1 $\mu\text{g/ml}$) in UltraMEM (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium) at 4°C for 1.5 h. After the treatment with or without agonist, cells were fixed and incubated with peroxidase-conjugated antibody (1:1000; GE Healthcare) for 1.5 h at room temperature. Subsequently, plates were developed with 50 μl of 2,2'-Azino-di-[3-ethylbenzthiazoline-sulfonate (6)] solution (Roche, Mannheim, Germany). The reaction was analyzed at 405 nm using an Expert Plus Microplate Reader (ASYS, Eugendorf, Austria) for 10 to 20 min in real time. Receptor endocytosis was evaluated as the loss of surface-labeled receptors. To measure recycling, cells were first exposed to 10 μM etorphine (Gampian Pharmaceutical Limited, Dundee, UK) for 2 h to drive agonist-induced endocytosis to a steady-state level. After wash, cells were incubated at 37°C for 30 and 60 min in the presence of 10 μM naloxone to block residual agonist-stimulated internalization of MOPr. Then cells were chilled with 4°C PBS to stop receptor trafficking. Afterward, receptors were surface-labeled at 4°C for 1.5 h. After wash of unbound antibody, cells were fixed, and surface receptors were detected as described in this section. The recycling rate was estimated as a percentage of recovered surface receptors to endocytosed receptors.

Determination of Receptor Desensitization and Resensitization by Measurement of cAMP Accumulation. Approximately 1.0×10^5 cells per well were plated onto poly(L-lysine)-coated 24-well plates overnight. For desensitization assay, cells were exposed to 5 μM DAMGO (Bachem, Heidelberg, Germany) with and without 50 μM monensin (Sigma) for either 0, 1, 2, 4, or 6 h. For resensitization assay, cells were washed after 5-h exposure of 5 μM DAMGO plus 50 μM monensin followed by an additional incubation period of either 0, 5, 10, 30, or 50 min in the absence of agonist. For measurement of cAMP accumulation, cells were washed once with 0.5 ml of serum-free RPMI medium (Cambrex Bio Science Verviers S.p.r.l.). Medium was removed immediately and replaced with 0.25 ml of serum-free RPMI medium containing 25 μM forskolin (Biotrend, Köln, Germany) in the presence or absence of 5 μM DAMGO. The cells were incubated at 37°C for 15 min. After one wash with ice-cold PBS, the intracellular cAMP was extracted immediately with 0.5 ml of ice-cold HCl/ethanol (1 volume of 1 N HCl/100 volumes of ethanol, stored at –20°C). The supernatant was transferred into a 1.5-ml tube and evaporated by vacuum at 30°C. The extracted cAMP content was

determined using a commercially available cAMP (³H) assay system (GE Healthcare).

Results

Identification of Synaptophysin as a μ -Opioid Receptor Interacting Protein by Yeast Two-Hybrid Screen. A yeast two-hybrid screening of a rat cDNA library using both full-length μ -opioid receptor and its truncation mutant (amino acids 258–398, from the 3rd intracellular loop to the carboxyl tail) as baits led to the finding of two cDNA fragments encoding amino acids 17 to 307 and 81 to 307 of synaptophysin (GI: 6981621), respectively. The interaction was verified in yeast mating and β -galactosidase assay. Synaptophysin interacts strongly with full-length μ -opioid receptor and its truncation mutant (amino acids 258–398), but not with the carboxyl cytoplasmic tail (amino acids 340–398 of MOPr) alone, suggesting that the third intracellular loop of the receptor is the potential site for the interaction. Empty Gal4-BD with Gal4-AD-Syp and empty Gal4-AD with Gal4-BD-MOPr, as well as a fusion of Gal4-BD with human lamin C, which neither forms complexes nor interacts with most other proteins, were used as negative controls.

Coimmunoprecipitation of the μ -Opioid Receptor and Synaptophysin. To confirm the interaction between MOPr and synaptophysin, we first carried out coimmunoprecipitation studies. The μ -opioid receptor was tagged with the HA epitope tag (HA-MOPr), and synaptophysin was tagged with the Myc epitope tag (Myc-Syp), both at the amino terminus. HEK293 cells with and without stable expression of HA-MOPr were transiently transfected with Myc-Syp expressing plasmid. Expression of HA-MOPr and/or Myc-Syp was examined by directly immunoblotting lysates from these cells with antibodies against HA and/or Myc epitope tag. A MOPr band migrating at approximately 75 kDa and a synaptophysin band migrating at approximately 38 kDa were detected (Fig. 1A, lanes 1 and 5, lysate). For coimmunoprecipitation, HA-MOPr receptors were precipitated from the lysates of HA-MOPr stably expressing cells and cells coexpressing HA-MOPr and Myc-Syp using anti-HA antibody. The resulting precipitates were immunoblotted with antibody directed against Myc epitope tag. As shown in Fig. 1A (lane 2, IP HA), Myc-tagged synaptophysin migrating at approximately 38 kDa was detected in immunoprecipitates from the cells coexpressing HA-MOPr and Myc-Syp, suggesting that MOPr is physically associated with synaptophysin in vivo. In contrast, no band was detectable in immunoprecipitates prepared under identical conditions from cells expressing only HA-MOPr or Myc-Syp or from a mixture of cells expressing HA-MOPr or Myc-Syp individually (Fig. 1A, lanes 3, 4, and 6, IP HA). This finding indicated that the MOPr-synaptophysin complex existed in cells before cell lysis and was not artificially formed during sample preparation.

Synaptophysin Binds Directly to the Third Intracellular Loop of MOPr. As suggested from the yeast two-hybrid screen, the third intracellular loop of the MOPr seems to be the important site for the interaction with synaptophysin. To confirm this suggestion, GST-affinity chromatography was carried out. The third intracellular loop of MOPr (3IL μ) was expressed as glutathione transferase fusion protein (GST-3IL μ). The purified GST and GST-3IL μ were then tested in GST pull-down experiments performed on purified

synaptophysin from transfected HEK293 cells expressing full-length synaptophysin. To ensure that approximately equal amounts of the eluted GST and GST fusion protein were loaded on the gel, nitrocellulose membranes were probed with anti-GST antibody (data not shown). As depicted in Fig. 1B, synaptophysin was precipitated by GST-3IL μ . Control experiments performed with GST alone yielded no precipitation. These results demonstrate that synaptophysin binds specifically to the third intracellular loop of the MOPr.

Analysis of the Interaction between the μ -Opioid Receptor and Synaptophysin by BRET Assay. Detergent solubilization of cells during coimmunoprecipitation studies could promote artificial aggregation of hydrophobic proteins such as transmembrane proteins. BRET is a nondestructive, living cell-based proximity assay, which overcomes the drawback of coimmunoprecipitation (Pfleger and Eidne, 2003). We

therefore additionally analyzed the interaction of MOPr with synaptophysin in living cells using BRET assay.

To assess the interaction of MOPr with synaptophysin and their interacting domain, fusion constructs linking Rluc to the receptor carboxyl terminus and linking GFP to the amino terminus of full-length synaptophysin (amino acids 1–307) or its truncation mutant (nSyp, amino acids 1–223; cSyp, amino acids 219–307 of synaptophysin) were cotransfected in HEK293 cells. The transfer of energy between the two partners was assessed after the addition of Deep Blue C. The BRET signal is determined by calculating the ratio of the light emitted by GFP fusion protein over the light emitted by Rluc or MOPr-Rluc. Expression level of GFP fusion proteins, MOPr-Rluc or Rluc alone was evaluated as described under *Materials and Methods*. There was no significant difference in the ratio of acceptor protein (GFP fusion protein) and donor protein (MOPr-Rluc or Rluc alone) (data not shown), indicating similar expression level for GFP-Syp, GFP-nSyp, and GFP-cSyp. As shown in Fig. 1C, a small BRET signal was obtained when GFP-Syp was coexpressed with Rluc alone (control). A significant BRET signal was detected in the combination of MOPr-Rluc with GFP-Syp or GFP-nSyp. And there was no significant BRET signal between MOPr-Rluc with GFP-cSyp. This indicates that the constitutive interaction between MOPr and synaptophysin also occurs in living mammalian cells.

Agonist Binding or Inhibition of Adenylate Cyclase by the μ -Opioid Receptor Is Not Affected by the Coexpression of Synaptophysin. To elucidate the potential function of the interaction between MOPr and synaptophysin, we stably coexpressed both amino-terminally HA-tagged MOPr and Myc-tagged synaptophysin in HEK293 cells. Coexpression of HA-MOPr and Myc-tagged synaptophysin was monitored by immunocytochemical analyses and Western blot, revealing that more than 90% of the cells coexpressed both proteins. Saturation binding experiments revealed no substantial differences between MOPr and MOPr-Syp expressing cells with respect to their affinities (K_D) to [3 H]DAMGO and their numbers of binding sites (B_{max}) (Table 1). For the functional property of μ -opioid receptors to inhibit adenylate cyclase, there was no significant difference on the maximal inhibition of forskolin-induced cAMP accumulation by the μ -agonist (Table 1). These indicate that synaptophysin has no substantial effect on the μ -opioid receptor for both agonist binding and functional inhibition of adenylate cyclase by the receptor.

Coexpression of Synaptophysin Augments μ -Opioid Receptor Trafficking. In HEK293 cells, μ -opioid receptor endocytosis was thought to be mediated via dynamin depen-

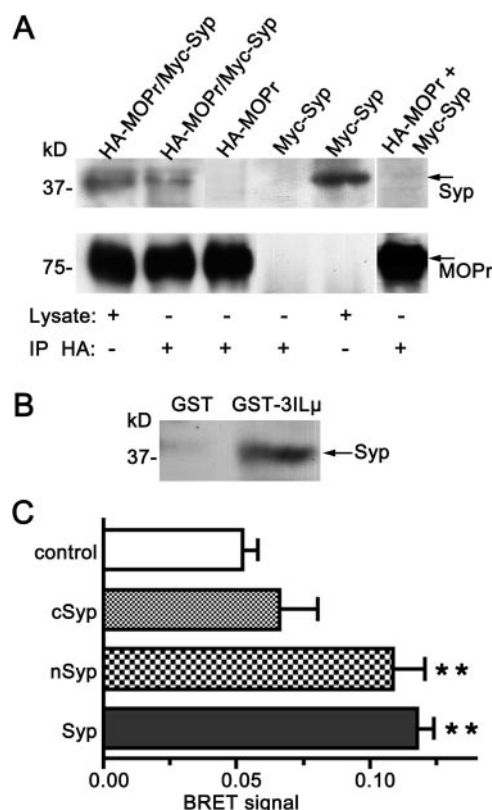


Fig. 1. Interaction of the μ -opioid receptor and synaptophysin. **A**, coimmunoprecipitation. Lysates from cells coexpressing HA-MOPr and Myc-Syp were extracted and either immunoblotted directly (lane 1, lysate) or immunoprecipitated using anti-HA antibody (lane 2, IP HA). In controls, lysates from cells expressing HA-MOPr (lane 3) or Myc-Syp (lanes 4 and 5) alone or their mixture (lane 6) were extracted and either immunoprecipitated using anti-HA antibody or immunoblotted directly. Arrows point to Syp and MOPr, respectively. **B**, in vitro binding between the third intracellular loop of MOPr (3IL μ) and synaptophysin. Purified Myc-tagged synaptophysin proteins were incubated with GST or GST-3IL μ beads in GST pull-down assay as described under *Materials and Methods*. After complete wash, precipitated protein was analyzed by Western blotting with anti-Myc antibody. **C**, BRET assay. Cells were transiently cotransfected with MOPr-Rluc in combination with GFP-Syp (full length), GFP-nSyp (amino acids 1–223 of Syp), or GFP-cSyp (amino acids 219–307, carboxyl cytoplasmic tail of Syp). A combination of Rluc and GFP-Syp was used as negative control (control). Cells were harvested 45 h after transfection. The energy transfer was initiated by addition of 5 μ M Deep Blue C, and BRET signal was assessed as described under *Materials and Methods*. Data are presented as mean \pm S.E.M. of three independent experiments performed in triplicate. **, $p < 0.01$ compared with negative control (ANOVA followed by Bonferroni's test).

TABLE 1

Binding characteristics of the μ -opioid receptor with and without coexpression of synaptophysin
 K_D and B_{max} for the binding of [3 H] DAMGO to μ -opioid receptors in stable cell line expressing HA epitope-tagged MOPr with or without coexpression of Myc epitope-tagged Syp in HEK293 cells were determined by Scatchard analyses. The effect of 5 μ M DAMGO on forskolin-stimulated cAMP accumulation was determined as described under *Materials and Methods*. Values shown are the mean \pm S.E.M. of three independent experiments performed in duplicate.

Stable Cell Lines	K_D	B_{max}	Maximal Inhibition of cAMP Accumulation
	nM	pmol/mg	%
MOPr	2.15 \pm 0.21	2.21 \pm 0.35	97.2 \pm 0.8
MOPr-Syp	2.08 \pm 0.35	1.95 \pm 0.21	96.0 \pm 2.1

dent clathrin-coated vesicle pathway. Overexpression of a dominant-negative mutant of dynamin I greatly inhibits μ -opioid receptor endocytosis (Zhang et al., 1998). Previous studies showed that synaptophysin interacts with dynamin I, which regulates the fission of endocytic vesicles (Daly et al., 2000; Daly and Ziff, 2002). To investigate whether synaptophysin influences μ -opioid receptor trafficking, Myc-tagged synaptophysin and HA-tagged MOPr were coexpressed in HEK293 cells, and their subcellular distribution was examined using confocal microscopy. In MOPr-transfected cells, the receptor was strictly localized in the cell membrane (Fig. 2A) (Finn and Whistler, 2001; Koch et al., 2003). However, as depicted in Fig. 2C, MOPr was both cytosolically and membranously localized and colocalized with synaptophysin in

cells coexpressing HA-MOPr and Myc-Syp. To test the possibility that there is an augmented constitutive trafficking of MOPr in cells with coexpression of synaptophysin, cells were exposed to 50 μ M monensin (a blocker of receptor recycling) for 1 h. As shown in Fig. 2D, MOPr was distributed mainly in cytosolic compartments in cells with high expression of synaptophysin, whereas MOPr was still mainly localized at the plasma membrane in adjacent cells with low expression of synaptophysin. In cells without overexpression of synaptophysin, monensin treatment induced the distribution of MOPr in the cytoplasm, but MOPr was still mainly localized at the plasma membrane (Fig. 2B). This observation indicates that the synaptophysin-mediated μ -opioid receptor accumulation in cytoplasm occurs in recycling vesicles.

The μ -opioid receptor and synaptophysin are generally transported to the processes in native neurons. In transfected cortical neurons coexpressing HA-MOPr and Myc-Syp, the μ -opioid receptor and synaptophysin were colocalized at the plasma membrane and in the cytoplasm in both of the cell body and processes (Fig. 2E). Consistent with this observation, electron microscopy in brain slice of nucleus accumbens revealed that μ -opioid receptors are distributed at the plasma membrane, in the cytoplasm of both dendrites and axons, and at the membrane of synaptic vesicles (Svingos et al., 1996).

Quantitative Analysis of μ -Opioid Receptor Trafficking. Confocal observation in Fig. 2D indicated that synaptophysin enhances the constitutive trafficking of μ -opioid receptors. To further confirm this finding, we quantified μ -opioid receptor trafficking by ELISA. As expected, there was an enhanced constitutive internalization of μ -opioid receptors in cells coexpressing synaptophysin ($11.15 \pm 1.99\%$ versus $28.00 \pm 2.36\%$ for MOPr and MOPr-Syp, respectively; Fig. 3A). Moreover, we found that synaptophysin also enhances the agonist-induced μ -opioid receptor internalization.

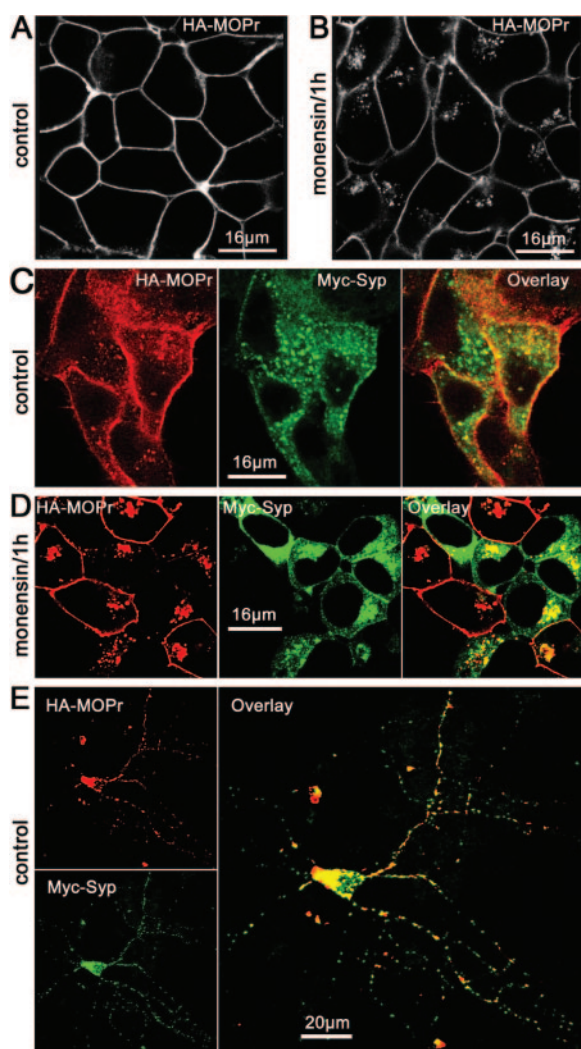


Fig. 2. A to D, role of synaptophysin in the subcellular distribution of μ -opioid receptors. HA-MOPr expressing cells with and without coexpression of Myc-Syp were either not treated (A and C) or treated with 50 μ M monensin for 1 h (B and D). Cells were subsequently fixed and subjected to immunofluorescent staining as described under *Materials and Methods*. Note that overexpression of synaptophysin augmented the constitutive trafficking of μ -opioid receptors. E, colocalization of MOPr and Syp in cotransfected primary cultured neurons. Newly prepared cortical cells (embryonic day 17) were grown on poly(D-lysine)-coated glass coverslips for 4 days, and then subjected to cotransfection with pCMV-HA-MOPr and pCMV-Myc-Syp plasmids. The receptor and synaptophysin were detected as described above. The overlay image was shown in large magnification (4-fold in comparison with single image). Shown are representative results from one of three independent experiments.

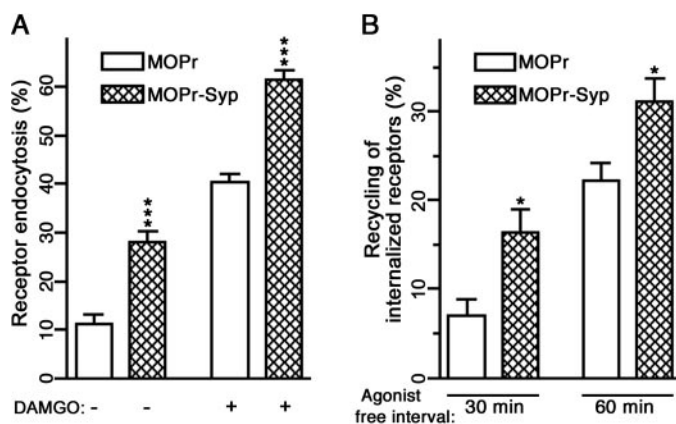


Fig. 3. Quantitative analysis of μ -opioid receptor trafficking. A, receptor endocytosis. HEK293 cells expressing HA-MOPr alone or coexpressing with Myc-Syp were surface-labeled with anti-HA antibody at 4°C for 1.5 h. Subsequently, cells were either not exposed or exposed to 1 μ M DAMGO at 37°C for 30 min, and then fixed. Receptor internalization, quantified as the percentage loss of cell surface receptors, was measured by ELISA as described under *Materials and Methods*. B, receptor recycling. After agonist-induced MOPr internalization to a stable level, the cells were exposed to 10 μ M naloxone for 30 and 60 min in agonist-free medium to allow receptor recycling. The recycling rate was estimated as a percentage of recovered surface receptors to internalized receptors as described under *Materials and Methods*. Data in A and B are presented as means \pm S.E.M. of three to five independent experiments performed in triplicate. *, $p < 0.05$; ***, $p < 0.001$ compared with MOPr alone in relative group (ANOVA followed by Bonferroni's test).

In the presence of 1 μ M DAMGO for 30 min, an augmented rate of μ -opioid receptor endocytosis by synaptophysin was detected ($40.43 \pm 1.62\%$ versus $61.48 \pm 1.95\%$ for MOPr and MOPr-Syp, respectively; Fig. 3A). As shown in Fig. 3B, we found that synaptophysin significantly increased the recycling of endocytosed receptors. These results provide evidences that synaptophysin enhances μ -opioid receptor trafficking in HEK293 cells.

Coexpression of Synaptophysin Truncation Mutants Blocks Agonist-Induced μ -Opioid Receptor Endocytosis. As shown above (Fig. 1C), the μ -opioid receptor interacted with the truncation mutant nSyp (amino acids 1–223 of synaptophysin), but not with cSyp (amino acids 219–307, carboxyl cytoplasmic tail of synaptophysin). A previous report showed that the carboxyl cytoplasmic tail of synaptophysin interacts with dynamin I and mediates the formation of a protein complex containing GTPase dynamin (Daly and Ziff, 2002). We assumed that the observed effect of synaptophysin on μ -opioid receptor endocytosis might be because of the synaptophysin-enhanced recruitment of dynamin to the plasma membrane during the budding of clathrin-coated vesicles. If this is true, agonist-induced μ -opioid receptor endocytosis should be decreased when the connection between receptor and dynamin via synaptophysin is disrupted. Indeed, after transient overexpression of both GFP-tagged truncation mutants (amino acids 1–223 and 219–307) of synaptophysin, the DAMGO-induced μ -opioid receptor endocytosis was almost completely blocked, whereas receptors in adjacent cells that did not express these mutants internalized efficiently in response to μ -agonist (Fig. 4, B and C). However, neither mutant causes a general impairment of dynamin-mediated endocytosis because the endocytosis of transferrin receptors (marked by Alexa Fluor 488-conjugated transferrin) was not affected by the expression of both mutants (Fig. 4, D and E). In addition, in cells transiently coexpressing GFP-tagged full-length synaptophysin, μ -opioid receptors were internalized efficiently under the same stimulation (Fig. 4A), suggesting GFP tag did not block agonist-induced receptor endocytosis. The negative effect of these mutants on μ -opioid receptor endocytosis was further confirmed by quantitative ELISA analysis in stable multiclonal lines with the coexpression of MOPr and synaptophysin truncation mutant (Fig. 4F). These findings indicate an essential role of synaptophysin in μ -opioid receptor internalization. In addition, the full-length of synaptophysin is required to enhance the internalization.

Synaptophysin Decreases Agonist-Induced Desensitization of the μ -Opioid Receptor. We have demonstrated that synaptophysin influences μ -opioid receptor internalization. Agonist-induced endocytosis of G protein-coupled receptors was reported to modulate receptor desensitization (Pak et al., 1996; Koch et al., 2004, 2005). To investigate the effect of synaptophysin on receptor desensitization, we exposed the cells expressing μ -opioid receptors with and without coexpression of synaptophysin to μ -agonist for various times and measured the receptor activity by determining the agonist-induced inhibition of forskolin-stimulated cAMP accumulation. As shown in Fig. 5A, prolonged exposure of μ -opioid receptors to the selective μ -agonist DAMGO led to a time-dependent decrease in the inhibition of cAMP accumulation. After 6 h of μ -agonist exposure, the μ -opioid receptor alone exhibited the desensitization with approximately 58% loss in

DAMGO-induced inhibition of cAMP accumulation, whereas overexpression of synaptophysin significantly decreased receptor desensitization, with only approximately 36% loss in the inhibition.

It is generally assumed that rapid receptor internalization contributes to rapid receptor desensitization. For investigat-

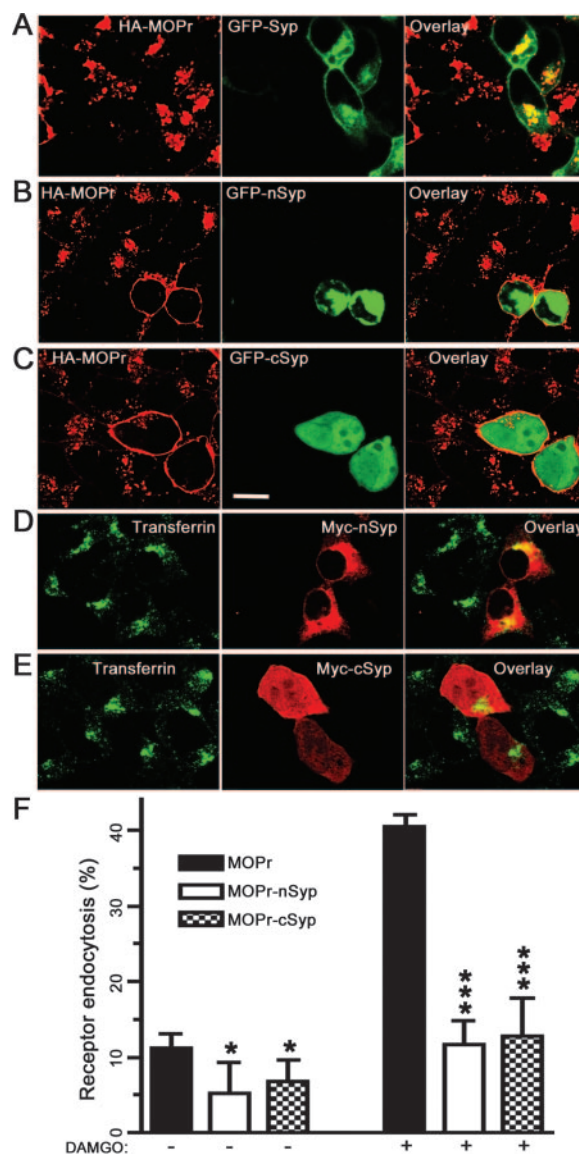


Fig. 4. Effect of synaptophysin truncation mutants on agonist-induced μ -opioid receptor internalization. A to E, cells with and without stable expression of HA-MOPr were transiently transfected with GFP-Syp, GFP-nSyp, GFP-cSyp, Myc-nSyp, or Myc-cSyp plasmid using calcium phosphate precipitation as described previously (Koch et al., 2003). Approximately 60 h after transfection, after the preincubation of 50 μ M monensin for 30 min, cells were exposed to 1 μ M DAMGO (A–C) or 5 μ g/ml Alexa Fluor 488-conjugated transferrin (Invitrogen) (D–E) in the presence of 50 μ M monensin for another 30 min at 37°C. Cells were subsequently fixed and subjected to immunofluorescent staining as described under *Materials and Methods*. The images were taken by confocal microscopy. Representative images from two independent experiments are shown. Scale bar, 8 μ m. F, in cells stably expressing HA-MOPr alone or coexpressing with GFP-nSyp or GFP-cSyp, receptor endocytosis was detected as described in Fig. 3. Data are presented as means \pm S.E.M. of three independent experiments performed in triplicate. *, $p < 0.05$; ***, $p < 0.001$ compared with MOPr alone, respectively (ANOVA followed by Bonferroni's test). Note that overexpression of synaptophysin truncation mutants significantly decreased μ -opioid receptor endocytosis.

ing whether the relatively slow desensitization of μ -opioid receptors with coexpression of synaptophysin is due to the augmented receptor recycling, we tested the effect of monensin, a blocker of receptor recycling, on receptor desensitization. As depicted in Fig. 5A, coapplication of monensin (mon) with DAMGO resulted in an enhanced μ -opioid receptor desensitization. The receptor was completely desensitized after 5-h DAMGO treatment in the presence of monensin. In the cells coexpressing MOPr and synaptophysin, a significantly faster desensitization was observed compared with cells expressing receptor alone in the presence of monensin (Fig. 5A). The finding indicated that the synaptophysin-mediated slow desensitization of μ -opioid receptors resulted from the enhanced-receptor recycling, which induces receptor reactivation.

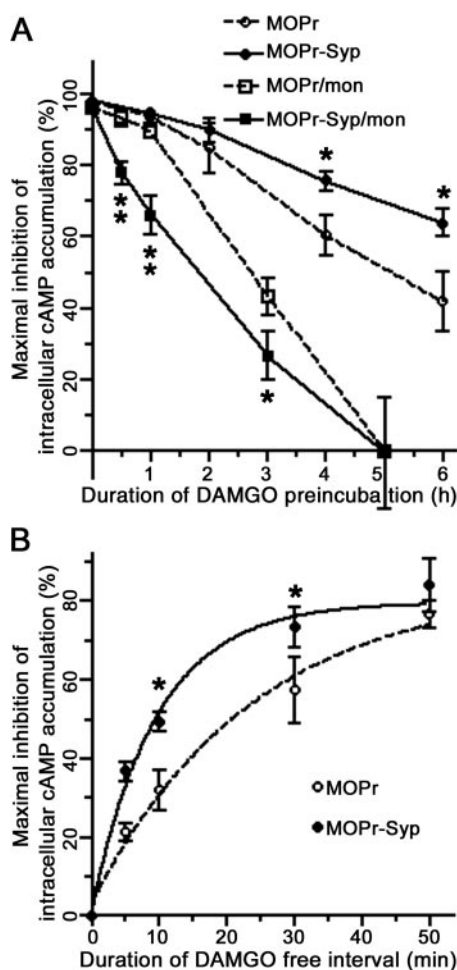


Fig. 5. Effect of synaptophysin on agonist-induced μ -opioid receptor desensitization and resensitization. **A**, DAMGO-induced receptor desensitization. Cells expressing HA-MOPr with and without coexpression of Myc-Syp were exposed to 5 μ M DAMGO in the presence or absence of 50 μ M monensin (mon) for the indicated time periods. After washing, cells were treated with forskolin or forskolin plus DAMGO for 15 min, and cAMP levels were determined as described under *Materials and Methods*. **B**, μ -opioid receptor resensitization. Cells were exposed to 5 μ M DAMGO plus 50 μ M monensin for 5 h followed by a drug-free interval of 0, 5, 10, 30, and 50 min. Then, the effect of DAMGO on forskolin-stimulated cAMP accumulation was measured as described above. DAMGO-induced inhibition of cAMP accumulation without a drug-free interval (0 min) was defined as 0%, whereas maximum inhibition of intracellular cAMP accumulation in cells that were not pretreated with DAMGO was defined as 100%. Values represent mean \pm S.E.M. of 3–4 independent measurements performed in duplicate. *, $p < 0.05$; **, $p < 0.01$ compared with MOPr alone (ANOVA followed by Bonferroni's test).

tion. Consistent with this opinion, overexpression of synaptophysin significantly increased μ -opioid receptor recycling (Fig. 3B) and reactivation (resensitization) (Fig. 5B).

Discussion

A large body of both in vitro and in vivo experimental data indicate that synaptophysin is involved in multiple, important aspects of *exo*-endocytosis of synaptic vesicles, including regulation of SNARE assembly into the fusion core complex, formation of the fusion pore initiating neurotransmitter release, activation of synaptic vesicle endocytosis and biogenesis. Synaptophysin has been found to interact in vitro with various nerve terminal proteins, which include dynamin I, adaptor protein 1 (AP-1), the v-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) vesicle-associated membrane protein 2/synaptobrevin II (VAMP2), the vesicular proton pump V-ATPase, and myosin V (for review, see Valtorta et al., 2004). Synaptophysin also binds to cholesterol; this interaction has been proposed to be essential for the biogenesis of synaptic-like microvesicles (Thiele et al., 2000). In the present study, synaptophysin was identified as a direct binding partner for the μ -opioid receptor, and influences receptor trafficking and signaling.

Synaptophysin was found to interact with full-length μ -opioid receptor but not with the receptor carboxyl cytoplasmic tail in yeast two-hybrid assay. This suggests that the μ -opioid receptor carboxyl tail is not the direct binding region for synaptophysin though this terminal tail has been shown to be an important domain for other μ -opioid receptor interacting protein (Koch et al., 2003; Onoprishvili et al., 2003). However, our experiments indicate that the third intracellular loop of the receptor interacts with synaptophysin because a μ -opioid receptor truncation mutant containing only this loop binds to synaptophysin in GST pull-down assay. An interaction between the μ -opioid receptor and synaptophysin was further confirmed by coimmunoprecipitation and BRET assay in HEK293 cells. Fluorescence studies using confocal microscopy provided evidences for a colocalization of the μ -opioid receptor and synaptophysin in both transfected HEK293 cells and primary cultured neurons. This is in line with the finding that a high degree of colocalization between endogenous μ -opioid receptor and synaptophysin was detected in mature synapses in both cultured cortical and hippocampal neurons (Liao et al., 2005). In addition, in the brain slice of the pallidum, the colocalization of the μ -opioid receptor and synaptophysin was also found (Olive et al., 1997). These findings provide a strong support for a direct interaction between the μ -opioid receptor and synaptophysin under physiological condition.

In transfected HEK293 cells coexpressing the μ -opioid receptor and synaptophysin, overexpression of synaptophysin has no effect on receptor properties such as agonist-binding and agonist-induced adenylate cyclase inhibition. However, the μ -opioid receptor shows colocalization with synaptophysin not only at the plasma membrane but also in the cytosolic compartments. This result is surprising because μ -opioid receptors are found mainly at cell surface in transfected HEK293 cells without overexpression of synaptophysin (Finn and Whistler, 2001; Koch et al., 2003). In transfected Chinese hamster ovary cells and in endocrine cell lines (PC-12 cells and RINm5F cells), synaptophysin is colocalized in the con-

stitutively recycling vesicles with transferrin receptors and follows the same trafficking pathway as transferrin receptors (Cameron et al., 1991). In addition, previous work (Keith et al., 1998) and our current observation (data not shown) show that μ -opioid and transferrin receptors share the same trafficking pathway. Based on these findings, it seems likely that the synaptophysin-enhanced μ -opioid receptor distribution in cytosolic compartments results from the synaptophysin-augmented constitutive receptor trafficking. This view is further supported by our observation that treatment with monensin (a blocker of receptor recycling) significantly increases the distribution of μ -opioid receptors in the cytoplasm. Furthermore, we additionally quantified the receptor trafficking and provided the direct evidence that overexpression of synaptophysin enhances the constitutive μ -opioid receptor trafficking. The synaptophysin-enhanced intracellular localization of μ -opioid receptors in HEK293 cells is in line with previous report that μ -opioid receptors are constitutively localized in both of the plasma membrane and the cytoplasm of native neurons (Svingos et al., 1996), which endogenously express synaptophysin.

Receptor endocytosis is thought to be initiated by the formation of receptor/ β -arrestin/AP-2/clathrin complexes. Subsequently, the rapid fission of budding vesicles depends essentially on dynamin, a GTPase that acts as a molecular scissor in sequestering the newly formed clathrin-coated vesicles away from the plasma membrane (Hinshaw, 2000; Slepnev et al., 2000; Claing et al., 2002). Synaptophysin has been shown to interact with dynamin I, and plays a role in the endocytosis of synaptic vesicles by recruiting dynamin to the vesicle membrane (Daly and Ziff, 2002). In squid giant terminal, disruption of the synaptophysin-dynamin interaction by microinjection of the carboxyl cytoplasmic tail of synaptophysin results in a block of vesicle endocytosis (Daly et al., 2000). In transfected 3T3 cells, deletion of this carboxyl tail has been found to impede internalization of the truncation mutant, although full-length synaptophysin is internalized efficiently (Linstedt and Kelly, 1991). These findings are consistent with a model in which the interaction between synaptophysin and dynamin facilitates the formation of endocytotic vesicles. Indeed, overexpression of synaptophysin significantly increases μ -opioid receptor internalization. It is thus reasonable to assume that synaptophysin augments μ -opioid receptor internalization by targeting dynamin to the plasma membrane during the formation of endocytotic vesicles. Consistent with this idea, overexpression of synaptophysin carboxyl tail, which is thought to disrupt the normal targeting of dynamin to the plasma membrane, is shown to almost completely block agonist-induced μ -opioid receptor internalization. Because the N-terminal fragment of synaptophysin (nSyp) also blocks MOPr internalization without affecting dynamin function, it is reasonable to suggest that binding of nSyp to MOPr prevents binding of endogenously expressed full-length synaptophysin to MOPr and subsequently impedes the receptor mediated targeting of dynamin to the plasma membrane. Transferrin receptor endocytosis was not affected by overexpression of either of the mutants, indicating that nSyp and cSyp do not lead to a general impairment of dynamin function.

Agonist-induced endocytosis of G protein-coupled receptors is a process that can contribute to functional desensitization of signal transduction by reducing the number of receptors

present at cell surface (Pak et al., 1996). However, in the present studies, the synaptophysin-augmented μ -opioid receptor endocytosis significantly decreases agonist-induced receptor desensitization. Although endocytosis enhances receptor desensitization, it also contributes to functional resensitization of signal transduction by promoting recycling of internalized receptors to cell surface (Ferguson et al., 1998; Lefkowitz et al., 1998; Law et al., 2000; Koch et al., 2004). After internalization, distinct G protein-coupled receptors are differentially sorted between the degradative and recycling pathways (Tsao and von Zastrow, 2000). Internalization of receptors is a rapid process and for the μ -opioid receptor, 30 min of DAMGO treatment resulted in a 40% loss of membrane receptors without markedly affecting functional activity at this time (Koch et al., 1998, 2005). The simplest explanation for this finding is that only 60% of intact MOPr are necessary for maximum inhibition of intracellular cAMP formation. The decreasing pool of receptors in the membrane after long-term agonist treatment may then be balanced by receptor recycling after internalization, counteracting fast receptor desensitization. For the μ -opioid receptor, it has been shown that agonist-induced receptor endocytosis is followed by a rapid receptor recycling and reactivation, thus reducing receptor desensitization (Finn and Whistler, 2001; He et al., 2002; Qiu et al., 2003; Koch et al., 2005). We therefore speculate that the observed slower rate of μ -opioid receptor desensitization after overexpression of synaptophysin is due predominantly to the synaptophysin-augmented receptor recycling and reactivation. Consistent with this speculation is that synaptophysin mediates faster DAMGO-induced receptor desensitization when the recycling pathway is blocked by monensin. This is also in line with the finding that the internalizing μ -agonist DAMGO leads to faster resensitization of μ -opioid receptors with the coexpression of synaptophysin.

After receptor internalization, the receptor might be recycled to cell surface, degraded in lysosomes, or transported for axonal targeting in neurons (Leterrier et al., 2006). Although it is clear that μ -opioid receptors are localized in both dendrites and axons, the mechanisms whereby this transportation occurs are still far from being understood. The AP-1 adaptor complex recruits clathrin to membranes in the *trans*-Golgi network to form transport vesicles (Seaman et al., 1996; Robinson and Bonifacino, 2001). Horikawa et al. (2002) reported that synaptophysin interacts with the AP-1 adaptor complex via γ -adaptin and provides adaptor sites for microtubule-based vesicle transport to axons and nerve terminals. Thus, synaptophysin may have multiple functional roles in μ -opioid receptor regulation. The transportation of newly synthesized μ -opioid receptors to neuronal processes might be involved in the complex formation of MOPr/Syp/AP-1. Regulation of specific synaptophysin function might be provided by protein kinases, because synaptophysin is known to be efficiently phosphorylated on both serine/threonine and in particular tyrosine residues (Horikawa et al., 2002; Evans and Cousin, 2005).

In conclusion, we have identified synaptophysin as a new μ -opioid receptor interacting protein, and demonstrated its role in receptor trafficking. Furthermore, the synaptophysin-augmented μ -opioid receptor trafficking attenuates agonist-induced receptor desensitization. It has been demonstrated in numerous studies that μ -opioid receptor desensitization

contributes to the development of opiate tolerance (Bohn et al., 2000; Finn and Whistler, 2001; He et al., 2002; Koch et al., 2004, 2005). We therefore suggest that the synaptophysin-augmented μ -opioid receptor trafficking decreases the development of opiate tolerance.

Acknowledgments

We thank Sandra Großbeilmann, Evelyn Kahl, Stefanie Holze, and Michaela Böx for excellent technical assistance.

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