Heterodimerization has been shown to modulate the ligand binding, signaling, and trafficking properties of G protein-coupled receptors. However, to what extent heterodimerization may alter agonist-induced phosphorylation and desensitization of these receptors has not been documented. We have recently shown that heterodimerization of sst2A and sst3 somatostatin receptors results in inactivation of sst2 receptor function (Pfeiffer, M., Koch, T., Schröder, H., Klutzny, M., Kirsch, S., Kreienkamp, H. J., Höllt, V., and Schulz, S. (2001) J. Biol. Chem. 276, 14027–14036). Here we examine dimerization of the sst2A somatostatin receptor and the μ-opioid receptor, members of closely related G protein-coupled receptor families. In coimmunoprecipitation studies using differentially epitope-tagged receptors, we provide direct evidence for heterodimerization of sst2A and MOR1 in human embryonic kidney 293 cells. Unlike heteromeric assembly of sst2A and sst3, sst2A-MOR1 heterodimerization did not substantially alter the ligand binding or coupling properties of these receptors. However, exposure of the sst2A-MOR1 heterodimer to the sst2A-selective ligand L-779,976 induced phosphorylation, internalization, and desensitization of sst2A as well as MOR1. Similarly, exposure of the sst2A-MOR1 heterodimer to the μ-selective ligand [d-Ala²,Me-Phe⁴,Gly⁵-ol]enkephalin induced phosphorylation and desensitization of both MOR1 and sst2A but not internalization of sst2A. Cross-phosphorylation and cross-desensitization of the sst2A-MOR1 heterodimer were selective; they were neither observed with the sst2A-sst3 heterodimer nor with the endogenously expressed lysophosphatidic acid receptor. Heterodimerization may thus represent a novel regulatory mechanism that could either restrict or enhance phosphorylation and desensitization of G protein-coupled receptors.

Recent biochemical, biophysical, and functional studies suggest that G protein-coupled receptors (GPCRs)³ can assemble as homo- or heterodimeric complexes (1, 2). Heterodimerization has been shown to alter both ligand binding affinity and signaling efficacy of GPCRs (1, 2). δ- and κ-opioid receptors form stable heterodimers with ligand binding and signaling properties resembling that of the κ2 receptor (3). Formation of heterodimers between the sst1 and sst2 somatostatin receptors has been found to modulate the pharmacology and signaling of both receptors (4). The γ-aminobutyric acid receptor B is unique in that heterodimerization of the nonfunctional γ-aminobutyric acid receptors B1 and B2 is required for native affinity for ligands and complete functional activity (5–9). Heteromeric assembly of fully functional AT1 angiotensin II and B2 bradykinin receptors results in increased efficacy of angiotensin II and decreased efficacy of bradykinin (10).

Heterodimerization has also been shown to alter endocytotic trafficking of GPCRs (3, 4, 10, 11). The κ-δ heterodimer exhibited a decrease in agonist-mediated receptor endocytosis (3). Oligomerization of δ- and κ-opioid receptors with the distantly related β,β-adrenergic receptor results in increased and decreased receptor endocytosis, respectively (11). AT1-B2 heterodimerization induced a switch to a clathrin- and dynamin-dependent endocytotic pathway for both receptors (10). Signaling of GPCRs is often terminated by phosphorylation of intracellular serine and threonine residues. After phosphorylation of the receptor, arrestins are frequently recruited to the plasma membrane, at which they facilitate endocytosis by serving as scaffolding proteins that bind to clathrin. Although changes in trafficking have been clearly documented, agonist-induced phosphorylation and desensitization of these GPCR heterodimers has not been examined.

We have recently shown that the sst2A and sst3 somatostatin receptors exist as constitutive homodimers when expressed alone and as constitutive heterodimers when coexpressed in human embryonic kidney (HEK) 293 cells (12). Whereas the sst2A-sst3 heterodimer behaved like the sst2A homodimer, it did not reproduce the pharmacological characteristics of the sst3 homodimer, suggesting that physical interaction of sst3 with sst2A induced functional inactivation of the sst3 subtype (12). Here we report that the sst2A receptor also forms stable heterodimers with the μ-opioid receptor (MOR1), a member of a closely related GPCR family. Unlike that observed for the sst2A-sst3 heterodimer, sst2A-MOR1 heterodimerization did not significantly affect the ligand binding or coupling properties but promoted cross-modulation of phosphorylation, internalization, and desensitization of these receptors.

EXPERIMENTAL PROCEDURES

Materials—The sst₂-selective ligand L-779,976 and the sst₃-selective ligand L-796,778 were kindly provided by Dr. Susan Rohrer (13) (Merck). The radioligand [¹²⁵I-Tyr¹⁴]SS-14 (74 TBq/mmol) was from Amersham Biosciences, and [⁹⁹mTc]DIDAMO was from PerkinElmer Life Sciences. Mouse monoclonal anti-T7 tag antibody was obtained from Novagen (Madison, WI), rat monoclonal anti-HA tag antibody was from
Cross-desensitization of sst<sub>2A</sub>-MOR1 Heterodimers

Table I: Ligand binding properties of sst<sub>2A</sub>, MOR1, and sst<sub>2A</sub>-MOR1 receptors

| Ligand | K<sub>D</sub> (nM) | B<sub>max</sub> (fmol/mg protein) |
|--------|-----------------|-------------------------------|
| T7sst2A-HAMOR1 | 0.48 ± 0.08 | 2458 ± 418 |
| T7sst2A | 0.17 ± 0.02 | 820 ± 19 |
| T7sst2A-HAMOR1 | 1.20 ± 0.07 | 2986 ± 135 |
| HAMOR1 | 1.22 ± 0.14 | 574 ± 20 |

Table II: Ligand binding and signaling properties of sst<sub>2A</sub>-MOR1 heterodimers

| Ligand | K<sub>D</sub> (nM) | B<sub>max</sub> (fmol/mg protein) |
|--------|-----------------|-------------------------------|
| T7sst2A | 0.02 ± 0.002 | 0.04 ± 0.006 |
| T7sst2A-HAMOR1 | >1,000 | >1,000 |
| HAMOR1 | >1,000 | 6.51 ± 0.06 |
| DAMGO | 1,000 | 8.65 ± 1.12 |

Roche Molecular Biochemicals, and polyclonal rabbit anti-T7 and anti-HA antibodies were from Gramsch Laboratories (Schwabhausen, Germany). In addition, rabbit anti-sst<sub>2A</sub> antibody (6291), guinea-pig anti-sst<sub>2A</sub> antibody (GP3), rabbit anti-MOR1 antibody (9998), and rabbit anti-sst<sub>3</sub> antibody (7986) were used and have been characterized extensively (12, 14–16). All polyclonal rabbit antibodies 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—The wild-type rat sst<sub>2A</sub> receptor was tagged at its amino terminus with the T7 epitope tag sequence MASMTQLAEPDYA using polymerase chain reaction and subcloned into a pcDNA3.1 expression vector (Invitrogen) containing a neomycin resistance cassette. The wild-type rat µ-opioid receptor MOR1 was tagged at its amino terminus with the HA epitope tag sequence YPYDVPDYA using polymerase chain reaction and subcloned into a pcDNA3.1 expression vector (Invitrogen) containing a neomycin resistance cassette as described previously (17). HEK 293 cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 10% CO2. The cells were first transfected with plasmids containing the neomycin resistance using the calcium phosphate precipitation method. Stable transfectants were selected in the presence of 500 µg/ml G418 (Invitrogen). To generate lines coexpressing two differentially epitope-tagged receptors, the cells were subjected to a second round of transfection using FuGENE 6 (Roche Diagnostics) and selected in the presence of 500 µg/ml G418 and 1 µg/ml puromycin (Sigma). Three clones expressing T7sst<sub>2A</sub> alone, six clones expressing HAMOR1 alone, and four clones coexpressing T7sst<sub>2A</sub> 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 ~1:1 ratio of sst<sub>2A</sub> and MOR1 were selected. Finally, double immunofluorescent staining was performed to validate that sst<sub>2A</sub> and MOR1 were coexpressed within the same cells. The B<sub>max</sub> and K<sub>D</sub> values of the cells that were used throughout this study are given in Table I.

Immunoprecipitation and Western Blot Analysis—Stably transfected HEK 293 cells were plated onto poly-L-lysine-coated 150-mm dishes and

![Figure 1: Characterization of sst<sub>2A</sub>-MOR1 heterodimers by coimmunoprecipitation](image)
were placed in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, chemiluminescence detection system. When indicated, the membranes were lysed in radioimmune precipitation 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 phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 10 μg/ml bacitracin) for 1 h on ice. Alternatively, the cells were lysed in radiolimmune precipitation 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) as described below. The receptor proteins were then immunoprecipitated with 100 μl of protein A-agarose beads preloaded with 10 μg of anti-HA, anti-T7, or anti-sst2A (6291) antibodies. Immunocomplexes were eluted from the beads using SDS sample buffer for 20 min at 60 °C. The amount of receptor in each sample was calculated as the function of receptor expression times the total protein content of the solubilized fraction of each sample subjected to immunoprecipitation. The receptor content of each sample was normalized to the sample with the least receptor content by dilution with sample buffer. The samples were then subjected to 8% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The extent of phosphorylation of receptor monomers was quantitated using a Fuji PhosphorImaging system and BAS 1000 software.

Immunocytochemistry—The cells were grown on poly-L-lysine-treated coverslips overnight and then exposed to agonists. The cells were fixed and permeabilized as described (12). For single immunofluorescence, the cells were then incubated with either mouse monoclonal anti-T7, rat monoclonal anti-HA, affinity-purified rabbit anti-sst2A (6291), or anti-MOR1 (9998) antibodies at a concentration of 1 μg/ml for 12 h at 4 °C, followed by detection using an enhanced chemiluminescence detection system. When indicated, the membranes were placed in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 55 °C and subsequently reprobed.

Whole Cell Phosphorylation Assays—The cells expressing T7sst2A, T7sst3, or HAMOR1 alone as well as cells coexpressing T7sst2A and Mycst3 or T7sst2A and HAMOR1 were plated onto 100-mm dishes and grown to 80% confluence. The cells were exposed to the cross-linking cocktail (6291) antibodies. Immunocomplexes were eluted from the beads using SDS sample buffer for 20 min at 60 °C and resolved by SDS-PAGE. After electrophoretog, membranes were incubated with either mouse monoclonal anti-T7, rat monoclonal anti-HA, affinity-purified rabbit anti-sst2A (6291), or anti-MOR1 (9998) antibodies at a concentration of 1 μg/ml for 12 h at 4 °C, followed by detection using an enhanced chemiluminescence detection system. When indicated, the membranes were placed in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 55 °C and subsequently reprobed.

The receptor proteins of each sample were then immunoprecipitated with 100 μl of protein A-agarose beads preloaded with 10 μg of anti-HA, anti-T7, or anti-sst2A (6291) antibodies. Immunocomplexes were eluted from the beads using SDS sample buffer for 20 min at 60 °C. The amount of receptor in each sample was calculated as the function of receptor expression times the total protein content of the solubilized fraction of each sample subjected to immunoprecipitation. The receptor content of each sample was normalized to the sample with the least receptor content by dilution with sample buffer. The samples were then subjected to 8% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The extent of phosphorylation of receptor monomers was quantitated using a Fuji PhosphorImaging system and BAS 1000 software.

Immunocytochemistry—The cells were grown on poly-L-lysine-treated coverslips overnight and then exposed to agonists. The cells were fixed and permeabilized as described (12). For single immunofluorescence, the cells were then incubated with either mouse monoclonal anti-T7, rat monoclonal anti-HA, affinity-purified rabbit anti-HA, affinity-purified rabbit anti-sst2A (6291), or affinity-purified rabbit anti-MOR1 (9998) antibody at a concentration of 1 μg/ml in Tris/phosphate-buffered saline and 1% normal goat serum overnight. Bound primary antibody was detected with biotinylated secondary antibodies (1:100; Vector, Burlingame, CA) followed by cyanine 3.18-conjugated streptavidin (Amersham Biosciences). For double immunofluorescence, the cells were incubated either with a mixture of rat monoclonal anti-HA and affinity-purified rabbit anti-sst2A (6291) or affinity-purified rabbit anti-HA and guinea pig anti-sst2A (GPS) antibodies. Bound primary antibodies were detected with biotinylated anti-rabbit antibodies, followed by a mixture of cyanine 2.18-conjugated streptavidin and cyanine 5.18-conjugated anti-rat or anti-guinea pig antibodies (1:200, Jackson ImmunoResearch, West Grove, PA). The cells were then dehydrated, cleared in xylol, and permanently mounted in DPX (Fluka, Neu-Ulm, Germany).

Male Wistar rats (n = 3, 200–250 g; Tierzucht, Schönwalde, Germany) were deeply anesthetized with chloral hydrate and transcardially perfused with Tyrode’s solution followed by Zamboni’s fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The brains were rapidly dissected and post-fixed in the same fixative for 2 h at room temperature. For all animal procedures ethical approval was sought prior to the experiments according to the requirements of the German National Act on the Use of
Experimental Animals. The tissue was cryoprotected by immersion in 30% sucrose before sectioning using a freezing microtome. Free-floating sections (30–40 μm) were incubated with a mixture of guinea pig anti-sst2A (GP3) and rabbit anti-MOR1 antibody (9998). The subcellular distribution of receptor proteins was examined by confocal microscopy. Top panels, sst2A is shown in red. Middle panels, MOR1 is shown in green. Bottom panels, overlay of sst2A (red) and MOR1 (green).

Note that in untreated cells both sst2A and MOR1 were almost exclusively confined to the plasma membrane revealing extensive colocalization. Exposure to the sst2A-selective agonist L-779,976 induced robust cointernalization of sst2A and MOR1. Similarly, treatment with the PKC activator PMA also promoted endocytosis of both sst2A and MOR1. In contrast, the MOR1-selective agonist DAMGO promoted selective endocytosis of MOR1, whereas sst2A remained at the plasma membrane. Shown are representative results from one of three independent experiments performed in duplicate. Scale bar, 20 μm.

Cross-desensitization of sst2A-MOR1 Heterodimers

Measurements of cAMP Accumulation—Transfected cells were seeded at a density of 1.5 × 10^5/well onto poly-l-lysine-treated 24-mm 12-well dishes. On the next day, the cells were incubated in the presence or absence of 100 nM L-779,976 or 100 nM DAMGO in OPTIMEM 1. The medium was then removed and replaced with 0.5 ml of serum-free RPMI medium containing 25 μM forskolin or 25 μM forskolin plus either L-779,976 or DAMGO in concentrations ranging from 10^{-12} to 10^{-6} M.

Fig. 3. Comparison of agonist-induced endocytosis of sst2A-MOR1 heterodimers by confocal microscopy. HEK 293 cells coexpressing T7sst2A and HAMOR1 were either left untreated (Control) or exposed to 100 nM L-779,976 (sst2-selective agonist), 1000 nM DAMGO (MOR1-selective agonist), or 100 nM PMA (PKC activator) for 30 min. The cells were subsequently fixed and fluorescently labeled with a mixture of guinea pig anti-sst2A antibody (GP3) and rabbit anti-MOR1 antibody (9998). The subcellular distribution of receptor proteins was examined by confocal microscopy. Top panels, sst2A is shown in red. Middle panels, MOR1 is shown in green. Bottom panels, overlay of sst2A (red) and MOR1 (green).
Phospho-ERK1/2 levels were normalized to total ERK1/2 per lane and expressed as the fold ERK1/2 phosphorylation over the basal value of untreated cells.

Data Analysis—Data from ligand binding, cAMP, and ERK assays were analyzed by nonlinear regression curve fitting using GraphPad Prism 5.0 software. Statistical analysis was carried out using the two-tailed paired t test or two-way analysis of variance followed by the Bonferroni test. p values < 0.05 were considered to be statistically significant.

RESULTS

Heterodimerization of sst2A and MOR1—To directly examine the association between the sst2A and the MOR1 receptor, we stably coexpressed T7-tagged sst2A receptors and HA-tagged MOR1 receptors in HEK 293 cells. Saturation binding experiments revealed that these cells coexpressed ~1:1 ratio of somatostatin binding sites (B_{max} 2,458 ± 418 fmol/mg membrane protein) and DAMGO binding sites (B_{max} 2,986 ± 135 fmol/mg membrane protein) (Table I). When membrane extracts from these cells were prepared with detergent buffer and immunoprecipitated using the rat anti-HA antibody, the carboxyl-terminal anti-sst2A antibody (6291) detected a single band migrating at 160 kDa, suggesting that this band represents a T7sst2A-

HAMOR1 heterodimer (Fig. 1A). Immunoprecipitation of sst2A-MOR1 heterodimers was facilitated when cells were preincubated with the cross-linking agent bis(sulfosuccinimidyl) suberate (Fig. 1A). In contrast, no bands were detectable in immunoprecipitates prepared under identical conditions from cells expressing only T7sst2A or HAMOR1 or from a mixture of T7sst2A- and HAMOR1-expressing cells. These data strongly suggest that sst2A-MOR1 heterodimers pre-existed in cells prior to cell lysis and were not artificially formed during sample preparation. When the blot shown in Fig. 1A was stripped and reprobed with rabbit anti-HA antibody, MOR1 monomers and dimers were revealed in immunoprecipitates from HAMOR1 cells, from T7sst2A-HAMOR1 cells, and from a mixture of T7sst2A- and HAMOR1 cells (Fig. 1A’). To test the stability of sst2A-MOR1 heterodimers under conditions used for whole cells phosphorylation assays, the cells were lysed in radioimmune precipitation buffer, immunoprecipitated using anti-HA antibody, and detected with anti-sst2A antibody. As shown in Fig. 1B, sst2A-MOR1 heterodimers were not detectable under these conditions. When the blot shown in Fig. 1B was stripped and reprobed with rabbit anti-HA antibody, it was apparent that cell lysis in SDS-containing radioimmune precipitation buffer in the absence of cross-linking agents leads to nearly complete dissociation of sst2A-MOR1 heterodimers (Fig. 1B’).

Ligand Binding and Signaling Properties of the sst2A-MOR1 Heterodimer—We compared the ligand binding properties of sst2A-MOR1 heterodimers with those of sst2A and MOR1 homodimers by examining the ability of the selective agonists to compete with [125I-Tyr11]SS-14 or [3H]DAMGO binding in membranes prepared from cells expressing either T7sst2A or HAMOR1 or coexpressing both T7sst2A and HAMOR1 (Fig. 2). The results in Table I show that T7sst2A-HAMOR1 cells had a 2-fold lower affinity for the sst2-selective agonist L-779,976 than T7sst2A cells. In contrast, HAMOR1 and T7sst2A-

HAMOR1 cells had similar high affinities for the µ-selective agonist DAMGO. Moreover, L-779,976 did not compete with [3H]DAMGO binding, and DAMGO did not compete with [125I-Tyr11]SS-14 binding in membranes prepared from T7sst2A-HAMOR1 cells. Ligand binding assays also revealed that T7sst2A cells had no affinity for DAMGO and that HAMOR1 cells had no affinity for L-779,976. The activation of somatostatin and opioid receptors by agonists results in decreased levels of intracellular cAMP as well as in a rapid and transient stimulation of ERK1/2 phosphorylation. As shown in Table II, the sst2-selective agonist L-779,976 produced 2–4-fold more
robust responses in cells coexpressing T7sst2A and HAMOR1 compared with cells expressing T7sst2A alone. Conversely, the MOR1-selective agonist DAMGO produced slightly more robust responses in cells coexpressing T7sst2A and HAMOR1 as compared with cells expressing HAMOR1 alone. In contrast, L-779,976 neither inhibited forskolin-stimulated cAMP accumulation nor activated ERK1/2 in HAMOR1 cells. Similarly, DAMGO showed no significant functional responses in T7sst2A cells. Although small changes in L-779,976 binding and signaling were detected, these findings suggest that two separate binding pockets were formed by the sst2A-MOR1 heterodimer and that the ligand binding and coupling properties of the sst2A and MOR1 receptors were not substantially altered after heterodimerization.

Endocytotic Trafficking of the sst2A-MOR1 Heterodimer—We next examined the effect of sst2A-HAMOR1 heterodimerization on receptor endocytosis using HEK 293 cells expressing either T7sst2A or HAMOR1 or coexpressing both T7sst2A and HAMOR1. The cells were exposed to either 100 nM L-779,976, 1000 nM DAMGO, or 100 nM PMA for 30, 60, 120, or 180 min at 37 °C. The cells were subsequently fixed, permeabilized, and fluorescently labeled with T7sst2A- and/or HAMOR1-specific antibodies. The subcellular distribution of receptor proteins was then analyzed by confocal microscopy. As depicted in Fig. 2, both sst2A and MOR1 receptors were predominantly confined to the plasma membrane in the absence of agonist. Treatment with L-779,976 but not with DAMGO induced an accumulation of sst2A receptors in vesicle-like structures within the cytoplasm in cells expressing T7sst2A alone. Exposure to DAMGO but not to L-779,976 promoted internalization of MOR1 receptors in cells expressing HAMOR1 alone. Activation of protein kinase C (PKC) by phorbol esters is known to stimulate heterologous phosphorylation of both the sst2A and the MOR1 receptor (18, 19). However, PMA induced only the internalization of sst2A but not of MOR1 in cells expressing these receptors alone (Fig. 2). In coexpressing cells both sst2A and MOR1 were seen at the cell surface, revealing extensive colocalization (Fig. 3, Control). Interestingly, after 30 min of L-779,976 exposure, both the sst2A and the MOR1 receptor underwent robust internalization (Fig. 3, L-779,976). Similarly, after treatment with PMA, sst2A and MOR1 were also cointernalized (Fig. 3, PMA).

FIG. 5. Agonist-induced cross-desensitization of coupling to adenylate cyclase of sst2A-MOR1 heterodimers. A and B, HEK 293 cells coexpressing T7sst2A and HAMOR1 were incubated in the presence or absence of 100 nM L-779,976 or 100 nM DAMGO for 6 h. The cells were washed, and inhibition of forskolin-stimulated cAMP accumulation by various concentrations of L-779,976 or DAMGO was determined. C and D, HEK 293 cells coexpressing T7sst2A and HAMOR1 were incubated in the presence or absence of 100 nM L-779,976 or 100 nM DAMGO for 0, 0.5, 1, 2, 4, or 6 h. The cells were washed, and inhibition of forskolin-stimulated cAMP accumulation by 10 nM L-779,976 or 10 nM DAMGO was determined. Statistical analysis of the time-course of L-779,976-mediated desensitization (C) revealed that L-779,976 significantly (p < 0.05) attenuated both L-779,976- and DAMGO-dependent responses after 1, 2, 4, and 6 h of preincubation (two-way analysis of variance followed by the Bonferroni test). Statistical analysis of the time course of DAMGO-mediated desensitization (D) revealed that DAMGO significantly (p < 0.05) attenuated DAMGO-dependent responses after 0.5, 1, 2, 4, and 6 h of preincubation as well as L-779,976-dependent responses after 2, 4, and 6 h of preincubation (two-way analysis of variance followed by the Bonferroni test). The values represent the means ± S.E. from four separate measurements performed in triplicate. Where error bars are not apparent the S.E. values were smaller than symbol size.
Desensitization of the sst<sub>2A</sub>-MOR1 Heterodimer—We then examined agonist-induced desensitization of the sst<sub>2A</sub>-MOR1 heterodimer. Cells coexpressing T7sst<sub>2A</sub> and HAMOR1 were preincubated in the presence or absence of either 100 nM L-779,976 or 100 nM DAMGO for 0.5, 1, 2, 4, or 6 h. The medium was removed, and the ability of either L-779,976 or DAMGO to inhibit forskolin-stimulated cAMP accumulation was determined. Interestingly, the sst<sub>2A</sub>-dependent responses of the sst<sub>2A</sub>-MOR1 heterodimer underwent a rapid time-dependent loss of coupling to adenylate cyclase upon preincubation with either L-779,976 or DAMGO with a maximum desensitization at 6 h (Fig. 5). Conversely, the MOR1-dependent responses of the sst<sub>2A</sub>-MOR1 heterodimer underwent a rapid time-dependent loss of coupling to adenylate cyclase upon preincubation with either DAMGO or L-779,976 with a maximum desensitization at 6 h (Fig. 5). The L-779,976-induced desensitization of the sst<sub>2A</sub>-MOR1 heterodimer followed a similar time-course as that of the sst<sub>2A</sub> homodimer (12). In contrast, DAMGO did not bind, activate, or desensitize the sst<sub>2A</sub> homodimer (Table II). Similarly, the DAMGO-induced desensitization of the sst<sub>2A</sub>-MOR1 heterodimer followed a time course similar to that of the MOR1 homodimer (16). In contrast, L-779,976 did not bind, activate, or desensitize the MOR1 homodimer (Table II).

We also examined the desensitization of mitogenic signaling of the sst<sub>2A</sub>-MOR1 heterodimer. The cells coexpressing T7sst<sub>2A</sub> and HAMOR1 were preincubated in the presence or absence of either 100 nM L-779,976 or 100 nM DAMGO for 0.5, 1, 2, 4, or 6 h. The medium was removed, and the ability of either L-779,976, DAMGO or lysophosphatidic acid to stimulate ERK1/2 activity was determined. As depicted in Fig. 6, preincubation with either L-779,976 or DAMGO for 4 h significantly attenuated both sst<sub>2A</sub> and MOR1-dependent responses. In contrast, mitogenic signaling of the lysophosphatidic acid receptor, a third receptor that is endogenously expressed in this system, was unchanged under these conditions, suggesting that the sst<sub>2A</sub>-MOR1 heterodimer underwent homologous cross-desensitization under these conditions.

Phosphorylation of the sst<sub>2A</sub>-MOR1 Heterodimer—To delineate a mechanistic basis for the observed cross-desensitization of the sst<sub>2A</sub>-MOR1 heterodimer, we assessed whole cell receptor phosphorylation in response to both L-779,976 and DAMGO. As shown in Fig. 1, cell lysis in radioimmune precipitation buffer resulted in a nearly complete dissociation of receptor dimers, which enabled us to selectively analyze the phosphorylation level of the resulting receptor monomers. As depicted in Fig. 7 (A and B), L-779,976 induced a rapid and robust phosphorylation of the sst<sub>2A</sub> receptor monomer (~4.3-fold over basal). DAMGO produced a rapid and robust phosphorylation of the MOR1 receptor monomer (~5.9-fold over basal). Interestingly, L-779,976 also significantly increased phosphorylation of the MOR1 receptor monomer (~2.5-fold over basal). Conversely, DAMGO also significantly increased phosphorylation of the sst<sub>2A</sub> receptor monomer (~2.0-fold over basal), indicating that activation of the sst<sub>2A</sub> subunit of the sst<sub>2A</sub>-MOR1 heterodimer resulted in cross-phosphorylation of the MOR1 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 T7sst<sub>2A</sub> or HAMOR1 alone (Fig. 7, C and D). To elucidate the selectivity of the observed sst<sub>2A</sub>-MOR1 cross-phosphorylation, we examined agonist-induced phosphorylation of a sst<sub>2A</sub> heterodimer with different functional properties, namely the sst<sub>2A</sub>-sst<sub>3</sub> heterodimer. Like MOR1, the sst<sub>3</sub> receptor also forms stable heterodimers with the sst<sub>2A</sub> receptor. Unlike MOR1, the sst<sub>3</sub> receptor is functionally inactivated upon heterodimerization with sst<sub>2A</sub> (12). As shown in Fig. 8, whereas the sst<sub>3</sub>-selective agonist L-796,778 stimulated a robust phosphorylation of the sst<sub>3</sub> receptor monomer, it failed to increase phosphorylation of the sst<sub>3</sub> receptor monomer in cells coexpressing T7sst<sub>2A</sub> and Myc-sst<sub>3</sub>. Interestingly, the sst<sub>3</sub>-selective agonist L-796,778 promoted phosphorylation of the sst<sub>3</sub> receptor in cells expressing this receptor alone; however, it did not increase phosphorylation of the sst<sub>3</sub> or sst<sub>2A</sub> receptor monomers above basal levels in coexpressing cells. These findings suggest that the specific pattern of agonist-induced phosphorylation of heterodimeric receptors may largely depend on their functional properties. The loss of sst<sub>3</sub>-dependent binding and signaling of the sst<sub>2A</sub>-sst<sub>3</sub> heterodimer is associated with diminished L-796,778-induced phosphorylation of this receptor. In addition, sst<sub>2A</sub>-MOR1 cross-phosphorylation may provide a plausible explanation for homologous cross-desensitization of this heterodimer.

Colocalization of sst<sub>2A</sub> and MOR1 in Rat Brain—A major prerequisite for the physiological assembly of sst<sub>2A</sub>-MOR1 heterodimers is their coexpression in the same cells. We therefore examined the spatial relation between sst<sub>2A</sub> and MOR1 in the central nervous system, and serial rat brain sections were
processed for dual immunofluorescence and examined under a confocal microscope (Fig. 9). Both sst2A and MOR1 receptors were widely distributed throughout the central nervous system and mostly targeted to neuronal somata and dendrites. At low magnification it was apparent that immunoreactive sst2A and MOR1 receptors exhibited closely overlapping distributions in many brain stem regions including the locus coeruleus, spinal trigeminal nucleus, and superficial layers of the spinal cord dorsal horn (Fig. 9, C, I, and O). At high power magnification a high degree of colocalization of the two receptors was observed only in the locus coeruleus (Fig. 9F). In contrast, immunoreactive sst2A and MOR1 receptors were clearly confined to distinct neuronal somata and dendrites in the spinal trigeminal nucleus and the superficial layers of the spinal cord dorsal horn (Fig. 9, L and R).

DISCUSSION

The existence of homo- and heterodimers has been demonstrated for several GPCRs using coimmunoprecipitation, fluorescence and bioluminescence resonance energy transfer, and functional complementation techniques (3, 4, 10, 20–25). Heterodimers can be formed between members of both closely and distantly related GPCR families (5–11, 22, 26). We have previously shown that members of the somatostatin receptor family exist as constitutive homodimers when expressed alone and as constitutive heterodimers when coexpressed (12). In the present study, we explored the functional consequences of a physical interaction between the sst2A somatostatin receptor and the H9262- opioid receptor a member of a closely related GPCR family. The sst2A and the MOR1 receptor share 38% sequence homology. We find that the sst2A receptor forms heterodimers with the H9262-opioid receptor. The immunoprecipitation of HA-tagged MOR1 receptors results in coprecipitation of T7-tagged sst2A receptors only from coexpressing cells but not from a mixture of cells expressing these receptors separately, suggesting that sst2A-MOR1 heterodimers preexisted in these cells prior to cell lysis and were not artificially formed during sample preparation.

The physical interaction between sst2A and MOR1 has pro-
found consequences on the trafficking properties of these receptors. Whereas MOR1 was resistant to L-779,976- and PMA-induced endocytosis in cells expressing this receptor alone, it was readily internalized together with the sst2A receptor in response to both the sst2-selective agonist L-779,976 and the PKC activator PMA in coexpressing cells. This is in contrast to the trafficking properties of the sst2A-sst3 heterodimer (12). Whereas sst3 was readily internalized in the presence of L-796,778 in cells expressing this receptor alone, it was resistant to endocytosis mediated by the sst2-selective agonist L-779,976 and the PKC activator PMA in coexpressing cells. This is in contrast to the trafficking properties of the sst2A-sst3 heterodimer (12). Whereas sst3 was readily internalized in the presence of L-796,778 in cells expressing this receptor alone, it was resistant to endocytosis mediated by the sst2-selective agonist L-779,976, the sst3-selective agonist L-796,778, or the nonselective agonist SS-14 in cells coexpressing sst2A and sst3 (12). Thus it appears that heterodimerization differentially affects the properties of these closely related receptors, and this is unique for each heterodimeric complex.

Previous studies have reported similar effects of dimerization on the trafficking properties of GPCRs (3, 4, 10, 11). However, none of these studies has established a mechanistic basis for the observed differences. Phosphorylation of intracellular serine and threonine residues within the third intracellular loop and the carboxyl terminus is the initial step in the desensitization of opioid and somatostatin receptors (18, 19, 27–29). After phosphorylation, H9252-arrestins are rapidly recruited to the plasma membrane where they facilitate endocytosis via clathrin-coated pits and vesicles. The present study shows that activation of the sst2A subunit of the sst2A-MOR1 heterodimer resulted in cross-phosphorylation of the MOR1 subunit and vice versa. In contrast, the binding- and signaling-deficient sst3 subunit of the sst2A-sst3 heterodimer was resistant to phosphorylation induced by either sst2A- or sst3-selective agonists. The simplest explanation for our findings is that these heterodimers exist in a physically restrained conformation as proposed in the three-dimensional dimer model by Gouldson et al. (30). Both domain-swapped and contact dimer models support the involvement of transmembrane helices five and six as dimerization interface. Interestingly, the two models predict that the third intracellular loop originating from each monomer would be parallel within the dimer. Activation of one

![Image](http://www.jbc.org/)

**Fig. 8.** Agonist-induced phosphorylation of the sst2A-sst3 heterodimer. HEK 293 cells coexpressing T7sst2A and Mycsst3 or expressing either T7sst2A or T7sst3 alone were exposed to 100 nM SS-14, 100 nM L-779,976, or 1000 nM L-796,778 for 20 min, and whole cell receptor phosphorylation was determined as described under "Experimental Procedures." T7sst2A was immunoprecipitated (IP) with rabbit anti-T7 antibodies. Mycsst3 and T7sst3 were immunoprecipitated with rabbit anti-sst3 (7986) antibodies. A and C, autoradiographs from representative experiments are shown. B and D, 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 the lack of phosphorylation of the sst3 subunit of the sst2A-sst3 heterodimer in the presence of either the sst2-selective agonist L-779,976 or the sst3-selective agonist L-796,778. 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).
Given that both sst<sub>2A</sub> and MOR1 are phosphorylated upon activation of PKC by phorbol esters (18, 19), an alternative explanation exists in which the sst<sub>2A</sub>-MOR1 heterodimer may be subject to heterologous PKC-mediated phosphorylation. However, this hypothesis is unlikely, because sst<sub>3</sub> is also phosphorylated and internalized upon PMA-induced PKC activation in cells expressing sst<sub>3</sub> alone (not shown). The sst<sub>3</sub> subunit of the sst<sub>2A</sub>-sst<sub>3</sub> heterodimer would therefore be expected to undergo heterologous PKC-mediated phosphorylation independent of its ability to acquire an active binding and signaling conformation in coexpressing cells as well. Thus the lack of phosphorylation of the sst<sub>3</sub> subunit of the sst<sub>2A</sub>-sst<sub>3</sub> heterodimer in response to activation of the sst<sub>2A</sub> subunit argues against a heterologous phosphorylation of the sst<sub>2A</sub>-MOR1 heterodimer by PKC.

Although the sst<sub>2A</sub> subunit of the sst<sub>2A</sub>-MOR1 heterodimer underwent cross-phosphorylation and -desensitization in response to activation of the MOR1 subunit, it was not cointermediate with the MOR1 receptor. This suggests that the DAMGO-mediated cross-phosphorylation of the sst<sub>2A</sub> subunit may involve sites distinct from those involved in L-779,776- or PMA-induced phosphorylation. The specific pattern of DAMGO-induced phosphorylation leads to separation of the sst<sub>2A</sub>-MOR1 heterodimer at the plasma membrane and may thus facilitate desensitization of this receptor.

Cross-phosphorylation may provide a plausible explanation for homologous cross-desensitization of adenylyl cyclase and ERK1/2 signaling of the sst<sub>2A</sub>-MOR1 heterodimer. Conversely, lack of cross-phosphorylation could explain increased resistance to agonist-induced desensitization of the sst<sub>2A</sub>-sst<sub>3</sub> heterodimer (12). Interestingly, in a previous study cross-desensitization of somatostatin- and opioid-dependent signal transduction was noted upon expression of the µ-opioid but not the κ-opioid receptor in AtT-20 cells, which endogenously express the sst<sub>2A</sub> receptor (31). These findings underscore the importance of physical interactions in the differential modulation of a diverse array of GPCR functions.

sst<sub>2A</sub>-MOR1 heterodimerization could have functional relevance in vivo. sst<sub>2A</sub> and MOR1 receptors coexist and functionally interact in pain-processing pathways (14, 15). Studies have shown extensive cross-talk between opioid- and somatostatin-mediated analgesic responses (32, 33). A major prerequisite for the physiological assembly of heterodimeric GPCRs is their coexpression in the same cells. We observed a particularly high degree of colocalization between the sst<sub>2A</sub> and the µ-opioid receptor in the locus coeruleus a brain region known to be involved in the expression of the opioid withdrawal syndrome. It is possible that the attenuation of opioid withdrawal in humans by the sst<sub>2</sub>-preferring agonist octreotide may be due in part to the physical interactions of these two receptors in the locus coeruleus (34). However, further coimmunoprecipitation studies from rat brain tissue are necessary to elucidate whether physical interaction between sst<sub>2A</sub> and MOR1 receptors may also occur in vivo.

In conclusion, we provide biochemical and functional evidence for heterodimerization of the sst<sub>2A</sub> somatostatin and the µ-opioid receptor. We show that formation of heterodimers between somatostatin and opioid receptors selectively cross-modulates phosphorylation, internalization, and desensitization. Direct intramembrane protein-protein interactions may thus provide a novel regulatory mechanism that could either restrict or enhance the activation/deactivation cycle of G protein-coupled receptors.

Acknowledgments—We thank Dana Mayer, Evelyn Kahl, Diana Gericke, and Michaela Böck for excellent technical assistance.
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J. Biol. Chem. 2002, 277:19762-19772.
doi: 10.1074/jbc.M110373200 originally published online March 14, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110373200

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