Subcellular Localization of μ -Opioid Receptor G_s Signaling

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ABSTRACT

In membranes obtained from μ -opioid receptor (MOR) expressing Chinese hamster ovary (CHO) cells (MOR-CHO), the MOR-selective agonist sufentanil produced a concentration-dependent stimulation of guanosine 5'-O-(3-[35 S]thio)triphosphate binding to $G_s\alpha$ that was abolished by blocking MOR with naloxone. This unequivocally demonstrates the long-debated functionality of the previously described association of MOR with $G_s\alpha$. Several complementary observations indicate the relevance of caveolae to MOR-coupled $G_s\alpha$ signaling. 1) In MOR-CHO membranes, sufentanil stimulated the translocation of $G_s\alpha$ into Triton-insoluble membrane compartments. 2) Sufentanil enhanced the coimmunoprecipitation (co-IP) of $G_s\alpha$ and adenylyl cyclase (AC) with caveolin-1 (a marker for caveolae) from the Triton-insoluble membrane fraction of spinal cord

and MOR-CHO. 3) MOR blockade (via naloxone) or G_s inactivation (via cholera toxin) abolished both the increased trafficking of $G_s\alpha$ into the Triton-insoluble membrane fraction of MOR-CHO and the augmented co-IP from spinal cord membranes of $G_s\alpha$ and AC with caveolin-1. This indicates that these events occurred subsequent to activation of MOR and $G_s\alpha$. Strikingly, lesser-phosphorylated $G_s\alpha$, which preferentially couple to MOR (Mol Brain Res **135**:217–224, 2005; Mol Pharmacol **72**:753–760, 2007; Mol Pharmacol **73**:868–879, 2008), are concentrated in caveolae, underscoring their relevance to MOR $G_s\alpha$ signaling. MOR-stimulated trafficking of $G_s\alpha$ and AC into caveolae and the likelihood of increased MOR $G_s\alpha$ coupling within caveolae could suggest that they contain the downstream effectors for MOR $G_s\alpha$ AC signaling.

Pharmacological analyses (Xu et al., 1989; Shen and Crain, 1990; Gintzler and Xu, 1991; Cruciani et al., 1993; Wang and Gintzler, 1997; Szucs et al., 2004) and biochemical analyses (Chakrabarti et al., 2005; Chakrabarti and Gintzler, 2007; Shy et al., 2008) provide convergent evidence for μ -opioid receptor (MOR) signaling via G_s , which has been long debated among opioid researchers. Our recent demonstrations that MOR is present in $G_s\alpha$ immunoprecipitate (IP), obtained from a variety of MOR-expressing cell lines and spinal cord, and the content of MOR in $G_s\alpha$ IP increases after chronic morphine exposure underscores the putative relevance of MOR $G_s\alpha$ signaling to acute and chronic opioid responsiveness.

Interaction of MOR with $G_s\alpha$ is a prerequisite for its transduction of MOR-stimulated signaling. Nevertheless, demonstration of their association does not unequivocally indicate that MOR functionally couples to $G_s\alpha$. Validation of functional inferences drawn from the coimmunoprecipitation (co-IP) of MOR and $G_s\alpha$ requires quantification of a parameter

that is a direct indicator of $G_s\alpha$ activation by MOR, e.g., stimulation of [35 S]GTP γ S binding, and/or a direct consequence of it, e.g., increased association with adenylyl cyclase (AC), both of which have heretofore been lacking.

One striking characteristic of the association of MOR with G_s is its dependence on the phosphorylation state of $G_s\alpha$. Diminished $G_s\alpha$ phosphorylation, which results from either chronic morphine exposure (via increased protein phosphatase 2A activity) or in vitro pretreatment with protein phosphatase 2A (Chakrabarti and Gintzler, 2007), is causally associated with the increased association of MOR with $G_s\alpha$ (Chakrabarti and Gintzler, 2007). The phosphorylation state is inversely related to hydrophobicity, decreasing phosphorylation augments lipid solubility. Thus, the inverse relationship between $G_s\alpha$ phosphorylation and MOR association could suggest that MOR $G_s\alpha$ signaling occurs predominantly in lipid-rich membrane microdomains.

Caveolae are one such subcellular compartment that has received considerable attention because of their ability to serve as organizing foci for cellular signal transduction. Caveolae are a subset of lipid rafts, renamed membrane rafts, which are highly plastic, sterol-, sphingolipid-, and cholesterol-enriched membrane domains that compartmentalize cellular processes. As the name implies, caveolae are highly enriched with caveolin

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ABBREVIATIONS: MOR, μ -opioid receptor; CHO, Chinese hamster ovary; MOR-CHO, CHO stably transfected with MORs; AC, adenylyl cyclase; CTX, cholera toxin; IP, immunoprecipitate; co-IP, coimmunoprecipitation; GPCR, G protein-coupled receptor; DTT, dithiothreitol; Triton, Triton X-100; SPA, scintillation proximity assay; [35 S]GTP γ S, guanosine 5'-O-(3-[35 S]thio)triphosphate.

proteins (>90% of the cellular content of caveolin is present in caveolae; Li et al., 1995). They bind signaling molecules such as G-protein-coupled receptors (GPCRs), heterotrimeric G proteins, and G-protein-regulated effectors, thereby organizing signaling complexes and modulating interactions among them.

The current study was undertaken to investigate direct correlates of G_{α} activation by MOR and define the membrane microdomains in which they occur. Our results not only definitively demonstrate dose-dependent stimulation of [35 S]GTP γ S binding to G_{α} by sufentanil, a MOR-selective agonist, but provide cross-validating data that underscore the relevance of caveolae to MOR G_{α} signaling.

Materials and Methods

Cell Culture and Membrane Preparation. Chinese hamster ovary (CHO) cells stably transfected with MOR (MOR-CHO) were grown in Dulbecco's modified Eagle's medium containing high glucose and L-glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin/streptomycin, and 100 μ g/ml Geneticin (Mediatech) in a humidified atmosphere of 90% air and 10% CO₂ at 37°C. For membrane preparation, cells were washed thoroughly (twice, 15 ml each) with phosphate-buffered saline (pH 7.3) and harvested directly in 20 mM HEPES, pH 7.4, containing 10% sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT), protease inhibitors 1 mM benzamide, 0.2 mg/ml bacitracin, 2 mg/l aprotinin, 3.2 mg/l each of soybean trypsin inhibitor and leupeptin, 20 mg/l each of *N*-tosyl-L-phenylalanine chloromethyl ketone, Na-*p*-tosyl-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, and complete cocktail inhibitor tablet (50 ml). Cells were homogenized in the same buffer and centrifuged at 1000g at 4°C for 10 min. Supernatants obtained from the low-speed spin were centrifuged at 105,000g for 1 h at 4°C. Membrane fractions obtained were resuspended in the same HEPES buffer (pH 7.4) containing protease inhibitors without sucrose. Membranes were either stored at -80°C in aliquots or processed further. To stimulate MOR, sufentanil was incubated with the MOR-CHO membranes for 10 min at 30°C, after which it was incubated with 1% Triton X-100 (Triton; 30 min on ice). Sample preparations were centrifuged (105,000g for 30 min at 4°C) to separate the Triton-insoluble pellet from the Triton-soluble supernatant fraction. The pellet was washed again with the HEPES buffer, and the Triton-insoluble fraction was solubilized (by agitation, 60 min at 4°C) with a mixture of detergents, 1% *n*-dodecyl β -D-glucopyranoside, 0.5% sodium deoxycholate, and 0.2% sodium dodecyl sulfate, in the same HEPES buffer containing protease inhibitors, 10% glycerol, and 150 mM NaCl. After centrifugation (16,000g for 15 min at 4°C), clear supernatants were used for Bradford protein assay, Western analyses, and immunoprecipitation. For caveolin immunoprecipitation, purified mouse monoclonal anti-caveolin-1 antibody (BD Biosciences, San Jose, CA; 1 μ l/100 μ g protein) was used together with prewashed Protein G-agarose (50 μ l; Roche Molecular Biologicals, Indianapolis, IN) overnight at 4°C. The beads were washed in 20 mM HEPES buffer (pH 7.4) containing 1 mM each of DTT and EDTA, 150 mM NaCl, 0.05% *n*-dodecyl β -D-glucopyranoside, and the same protease inhibitors as mentioned above. IPs were eluted by heating samples in 30 μ l of sample buffer (15 min at 85°C). Samples separated on 4 to 12% gradient Bis-Tris gels (Invitrogen, Carlsbad, CA) were electrotransferred onto nitrocellulose membranes and used for either autoradiography (to assess 32 P incorporation) or Western analyses.

Assessment of Relative Phosphorylation State of G_{α} Populations. We determined the magnitude of 32 P incorporation into G_{α} immunoprecipitated with anticaveolin and G_{α} antibodies, i.e., back phosphorylation. This was used to reflect the relative degree of phosphorylation of each population of G_{α} . G_{α} were immunoprecipitated

from solubilized MOR-CHO membranes as described previously (Chakrabarti et al., 2005). After immunoprecipitation, the protein A agarose beads containing the antigen-antibody complexes were washed and resuspended in a kinase buffer containing 20 mM HEPES (pH 7.6), 10 mM MgCl₂, 1 mM CaCl₂, 1 mM EGTA, 0.25% bovine serum albumin, 1 mM DTT, 10% glycerol, 1 tablet/50 ml complete protease inhibitor cocktail, 100 μ M ATP, and the phosphatase inhibitors 0.1 mM sodium orthovanadate and 25 nM calyculin A to prevent endogenous phosphatases. Phosphorylation reactions were initiated by the addition of PKC_{catalytic} (20 mU/reaction; Calbiochem, San Diego, CA) and [γ - 32 P]ATP (2.5 μ Ci/reaction; PerkinElmer Life and Analytical Sciences, Waltham, MA) and incubated for 2 h at 30°C. The reactions were terminated by heating samples at 85°C for 15 min, resolved by gel electrophoresis (4–12% Bis-Tris gel), and electrotransferred onto a nitrocellulose membrane. Phosphorylated G_{α} subunits were visualized by autoradiography, quantitated by using PhosphorImager analysis software (ImageQuant TL; GE Healthcare, Piscataway, NJ), and identified as G_{α} proteins using anti- G_{α} antibodies on a Western blot.

Western Analysis. G_{α} and caveolin proteins were visualized by using 1:5000 dilution of a polyclonal rabbit anti- G_{α} antibody raised against the C terminus of G_{α} (generously provided by Dr. J. Hildebrandt, Medical University of South Carolina, Charleston, SC) and a polyclonal rabbit anticaveolin antibody raised against human caveolin (amino acids 1–97), respectively. AC was visualized by using a 1:1000 dilution of the monoclonal anti-AC antibody, BBC4 (generated against the carboxyl terminus common to most AC isoforms, generously provided by Dr. T. Pfeuffer, Heinrich Heine University, Dusseldorf, Germany; Mollner and Pfeuffer, 1988). The secondary antibody used was either a peroxidase-labeled donkey anti-rabbit or anti-mouse antibody (GE Healthcare). Antibody-substrate complex was visualized with a Supersignal West Dura Chemiluminescence detection kit (Pierce Chemical, Rockford, IL). Specificity of G_{α} and AC Western signals had been demonstrated previously (Chakrabarti et al., 1998, 2005) in MOR-CHO membranes and was therefore not repeated in the current study. The use of both monoclonal and polyclonal anticaveolin antibodies differing in their epitope specificities (amino acids 1–21, which is unique to caveolin-1, versus amino acids 1–97, respectively) validated the identity of the caveolin Western blot signal. The product of the IP, obtained with the monoclonal antibody, was analyzed by Western blotting with the polyclonal antibody. Sample pairs, obtained from opioid naive and acute sufentanil-treated MOR-CHO membranes, were processed, electrophoresed, and blotted in parallel. Control and experimental Western membranes were exposed concomitantly to GeneGnome (CCD camera; Syngene, Frederick, MD). Intensity of signal was quantified by using Syngene software in GeneGnome.

Detection of MOR Agonist-Stimulated [35 S]GTP γ S Binding via Antibody Capture and Scintillation Proximity Assay. [35 S]GTP γ S binding was detected and quantified by using antibody capture in combination with a scintillation proximity assay (SPA). Unlike conventional [35 S]GTP γ S binding assays that cannot distinguish among the various G_{α} subunits that bind [35 S]GTP γ S, the antibody capture strategy enables the detection of [35 S]GTP γ S that is bound to specific G_{α} protein subunits, e.g., G_{α} . In the current iteration of this approach, MOR-CHO membranes were permeabilized with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (Sigma-Aldrich, St. Louis, MO) for 30 min on ice. Agonist (sufentanil, 1–1000 nM)-induced dose-dependent increase in [35 S]GTP γ S binding to G_{α} was determined by using membranes (300 μ g) incubated in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 5 mM MgCl₂, 0.1 μ M GDP, and 0.1 μ Ci (500 pM) [35 S]GTP γ S in the presence and absence of opioid agonist sufentanil (30°C for 30 min). Membranes were preincubated with agonists and antagonists for 15 min at 30°C followed by the addition of [35 S]GTP γ S (DeLapp et al., 1999). Reactions were terminated on ice and centrifuged (15 min at 16,000 rpm at 4°C), and supernatants were discarded. Membranes were solubilized by using 1% *n*-dodecyl β -D-glucopyranoside,

0.5% sodium deoxycholate, and 0.2% sodium dodecyl sulfate in 50 mM Tris buffer (pH 7.4), agitated for 1 h at room temperature, and incubated with anti- $G_{s\alpha}$ antibodies for an additional 2 h at room temperature. The reaction mixture was added to 96-well plates, the bottom of which was impregnated with scintillant (flash plates; PerkinElmer Life and Analytical Sciences) and coated with anti-rabbit IgG antibodies. Nonspecific binding was detected in the presence of 10 μ M GTP γ S. The (rabbit) anti- $G_{s\alpha}$ antibodies included in the reaction mixture bound to $G_{s\alpha}$ [35 S]GTP γ S, thereby promoting its proximity to the scintillant-impregnated secondary antibody-coated well surface. This allowed the short-range electrons emitted from the [35 S]GTP γ S to excite the impregnated fluorophor and emit light, which was quantified by using a Microbeta Jet counter (PerkinElmer Life and Analytical Sciences). It is noteworthy that because the anti- $G_{s\alpha}$ antibodies did not recognize other G-protein subunits that are present only [35 S]GTP γ S-bound $G_{s\alpha}$ is brought into proximity with the scintillant-impregnated well bottom. Thus, quantification of basal and agonist-stimulated [35 S]GTP γ S binding to $G_{s\alpha}$ subunits would not be confounded by [35 S]GTP γ S binding to other G-protein subunits that may be present in even greater abundance (DeLapp et al., 1999). Specificity of this SPA method was determined by leaving out anti- $G_{s\alpha}$ antibodies or using nonimmune serum, which produced background-level counts.

Statistical Analysis. Significance of differences of mean values between two groups was determined by paired Student's *t* test. The Spearman correlation coefficient was used to assess dose relatedness of sufentanil stimulation of [35 S]GTP γ S binding to $G_{s\alpha}$. A nonlinear regression analysis (GraphPad Software Inc., San Diego, CA) was used to generate the corresponding ED₅₀ values for sufentanil.

Results

Effect of Sufentanil on [35 S]GTP γ S Binding to $G_{s\alpha}$.

The MOR-selective agonist sufentanil produced a concentration-dependent (1–1000 nM) stimulation (~40–100%) of [35 S]GTP γ S binding to $G_{s\alpha}$ (Spearman correlation coefficient of 0.65, $p = 0.002$; Fig. 1). In membranes obtained from MOR-CHO, maximal stimulation was $100.5 \pm 12.8\%$ ($n = 3$ –6; $p < 0.05$) with an ED₅₀ of 31.4 nM (Fig. 1). To validate the identity of the receptor mediating the stimulation of [35 S]GTP γ S binding, the assay was conducted in the presence of the MOR antagonist naloxone (10 μ M). The presence of this antagonist abol-

Fig. 1. Sufentanil stimulates [35 S]GTP γ S binding to $G_{s\alpha}$ in MOR-CHO. Sufentanil stimulation of [35 S]GTP γ S binding to $G_{s\alpha}$ was quantified by using a SPA in which MOR-CHO membranes (300 μ g) were incubated in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 5 mM MgCl₂, 0.1 μ M GDP, and 0.1 μ Ci (500 pM) [35 S]GTP γ S in the presence and absence of sufentanil (0.001–1 μ M; 30°C for 30 min) as described under *Materials and Methods* ($n = 3$ –6). [35 S]GTP γ S binding in the presence of 10 μ M unlabeled GTP γ S was defined as nonspecific binding. Sufentanil stimulation of [35 S]GTP γ S binding to $G_{s\alpha}$ was completely abolished by the presence of the MOR antagonist naloxone (10 μ M), which unambiguously reveals functional MOR G_s coupling. $n = 3$; * = $p < 0.05$.

ished sufentanil (1 μ M) stimulation of [35 S]GTP γ S binding. To eliminate the possibility that results were confounded by the recognition of $G_{i\alpha}$ / $G_{o\alpha}$ proteins by the anti- $G_{s\alpha}$ antibodies, IPs obtained using the anti- $G_{s\alpha}$ antibodies were subjected to Western analysis using anti- $G_{i\alpha}$ / $G_{o\alpha}$ antibodies. No $G_{i\alpha}$ / $G_{o\alpha}$ were detected, indicating that [35 S]GTP γ S binding to $G_{i\alpha}$ / $G_{o\alpha}$, the predominant G proteins that couple to MOR, were not a likely confound. Importantly, sufentanil (1 μ M) also stimulated [35 S]GTP γ S binding to $G_{s\alpha}$ in membranes obtained from spinal cord ($111.8 \pm 26\%$, $n = 3$; $p < 0.05$). Collectively, these results indicate the functionality of the previously demonstrated association of MOR with $G_{s\alpha}$ (Chakrabarti et al., 2005; Chakrabarti and Gintzler, 2007).

Decreased Phosphorylated $G_{s\alpha}$ Preferentially Partitions with Caveolin. We reported previously that dephosphorylation of $G_{s\alpha}$ increases its association with MOR (Chakrabarti and Gintzler, 2007). Therefore, as an initial indicator of the relevance of lipid membrane rafts/caveolae to MOR $G_{s\alpha}$ signaling, we investigated whether decreased phosphorylated $G_{s\alpha}$ preferentially partitioned in this membrane microdomain. Because caveolin is a marker for lipid membrane rafts/caveolae, we compared the phosphorylation state of the $G_{s\alpha}$ that coimmunoprecipitated with caveolin versus the phosphorylation state of $G_{s\alpha}$ that was immunoprecipitated using anti- $G_{s\alpha}$ antibodies (which should reflect the composite phosphorylation state of $G_{s\alpha}$ among the total population). Phosphorylation state was assessed by quantifying the magnitude of back phosphorylation, in which the level of 32 P incorporation is inversely proportional to the pre-existing degree of phosphorylation. Figure 2 illustrates that the magnitude of 32 P incorporation into the ~48-kDa $G_{s\alpha}$ that coimmunoprecipitated with caveolin was $145 \pm 24\%$ ($p < 0.05$) greater than that incorporated into $G_{s\alpha}$ immunoprecipitated with anti- $G_{s\alpha}$ antibodies. Note that this is an underestimate of the actual increment in back phosphorylation because the $G_{s\alpha}$ protein content of the caveolin-1 IP was significantly less (37%; $p < 0.05$, $n = 3$) than that present in $G_{s\alpha}$ IP. Interestingly, back phosphorylation of the ~52-kDa $G_{s\alpha}$ that coimmunoprecipitated with anticaveolin-1 antibod-

Fig. 2. Assessment of $G_{s\alpha}$ phosphorylation states via back phosphorylation. Autoradiography of 32 P incorporated into $G_{s\alpha}$ immunoprecipitated with either anticaveolin (lane 1) or anti- $G_{s\alpha}$ (lane 2) antibodies was assessed. IPs were incubated (2 h at 30°C) with PKC_{catalytic} and [γ - 32 P]ATP as described under *Materials and Methods*. Samples were resolved by gel electrophoresis (4–12% Bis-Tris gel) and electrotransferred onto a nitrocellulose membrane, which was exposed to a Phosphorimager for autoradiography. The same nitrocellulose membranes were immunoblotted with anti- $G_{s\alpha}$ antibodies to quantify $G_{s\alpha}$ immunoprecipitated with anticaveolin antibodies (lane 3) or anti- $G_{s\alpha}$ antibodies (lane 4). $n = 3$; $p < 0.05$. $G_{s\alpha}$ immunoprecipitated with anticaveolin antibodies contained less $G_{s\alpha}$ protein than that obtained with anti- $G_{s\alpha}$ antibodies but incorporated ~2-fold more 32 P. This indicates that caveolin preferentially interacts with $G_{s\alpha}$ of lower phosphorylation states.

ies was also substantially greater than that of the $G_{s\alpha}$ that coimmunoprecipitated with anti- $G_{s\alpha}$ antibodies (note the markedly greater protein content of the ≈ 52 -kDa species in $G_{s\alpha}$ IP versus caveolin-1 IP). However, because levels of the protein corresponding to the ≈ 52 -kDa autoradiographic signal were barely detectable in caveolin-1 IP, differences in ^{32}P incorporation were not quantified. Importantly, the anti- $G_{s\alpha}$ antibody used in these studies does not distinguish among differentially phosphorylated forms of $G_{s\alpha}$ (Chakrabarti and Gintzler, 2007).

Acute Sufentanil Stimulates the Translocation of $G_{s\alpha}$ into Triton-Insoluble Membrane Compartments of MOR-CHO. Quantification of changes in the content of $G_{s\alpha}$ in the Triton-insoluble membrane fraction was used as an initial indicator of its trafficking into lipid membrane rafts/caveolae based on the insolubility of these membrane microdomains in nonionic detergents. Acute activation of MOR in MOR-CHO membranes by sufentanil ($1 \mu M$, 10 min at $30^\circ C$) produced a significant increase ($\approx 53 \pm 8.8\%$; $p < 0.02$) in the content of $G_{s\alpha}$ in the Triton-insoluble fraction of MOR-CHO membranes (Fig. 3, lane 2 versus lane 1). The sufentanil-induced increase in $G_{s\alpha}$ content was reduced by $\sim 80\%$ after pretreatment with naloxone ($10 \mu M$), indicating that MOR activation was a prerequisite for sufentanil-induced

Fig. 3. Sufentanil induces translocation of $G_{s\alpha}$ into Triton-insoluble (Tri-IS) membrane compartments. High-speed centrifugation was used to obtain the Triton-insoluble membrane fraction (30 min, $105,000g$ at $4^\circ C$), which was washed in 20 mM HEPES buffer (pH 7.4) and solubilized with a mixture of detergents (1% *n*-Dodecyl β -D-glucopyranoside, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate, and 10% glycerol containing a cocktail of protease inhibitors). Western analyses using anti- $G_{s\alpha}$ antibodies were used to quantify the content of $G_{s\alpha}$. Acute activation of MOR in MOR-CHO membranes by sufentanil ($1 \mu M$, 10 min at $30^\circ C$) produced a significant increase ($\approx 53\%$; *, $p < 0.02$) in the content of $G_{s\alpha}$ in the Triton-insoluble fraction of MOR-CHO membranes (lane 2 versus lane 1). Sufentanil-induced translocation of $G_{s\alpha}$ was reduced by $\sim 80\%$ after pretreatment with naloxone ($10 \mu M$; #, $p < 0.05$; lane 3 versus 2), indicating that MOR activation was a prerequisite for sufentanil-induced translocation of $G_{s\alpha}$ into the Triton-insoluble membrane compartment. The $G_{s\alpha}$ translocation by sufentanil was eliminated after a 24-h pretreatment with $1 \mu g/ml$ CTX (compare lane 5, CTX + sufentanil, with lane 4, CTX alone). This indicates that sufentanil stimulation of $G_{s\alpha}$ translocation to the Triton-insoluble membrane compartment occurs subsequent to its activation. $n = 3-6$ for all determinations.

translocation of $G_{s\alpha}$ (Fig. 3, lane 3 versus lane 2, $p < 0.05$). Interestingly, a 24-h pretreatment with $1 \mu g/ml$ cholera toxin (CTX) abolished sufentanil stimulation of $G_{s\alpha}$ translocation to the Triton-insoluble membrane fraction (Fig. 3, lane 5 versus lane 4). This indicates that translocation of $G_{s\alpha}$ occurred subsequent to its activation.

Acute Sufentanil Increases the Co-IP of $G_{s\alpha}$ with Caveolin-1 from MOR-CHO. The Triton-insoluble membrane fraction contains lipid-rich membrane domains, in addition to caveolae. To validate that sufentanil was augmenting trafficking of $G_{s\alpha}$ into this membrane microdomain, we quantified the co-IP of $G_{s\alpha}$ with caveolin-1, the major structural protein of caveolae. Acute sufentanil significantly elevated the co-IP of both the 48- and 45-kDa forms of $G_{s\alpha}$ (106 ± 3.6 and $86.5 \pm 36\%$, respectively) with caveolin-1 when the IP was obtained from the Triton-insoluble membrane fraction (Fig. 4, lane 2 versus lane 1). In contrast, the content of $G_{s\alpha}$ in caveolin-1 IP obtained from the Triton-soluble membrane fraction was not affected by acute sufentanil (Fig. 4, lane 4 versus lane 3). This indicated that acute MOR activation stimulates the translocation of $G_{s\alpha}$ into the caveolae subtype of lipid raft.

Acute Sufentanil Increases the Co-IP of $G_{s\alpha}$ and AC with Caveolin-1 from Spinal Cord Membranes. To determine whether the stimulation of $G_{s\alpha}$ translocation by MOR observed in MOR-CHO generalized to complex integrated neuronal tissue, we investigated the effect of MOR

Fig. 4. MOR activation increased the co-IP of $G_{s\alpha}$ with caveolin-1 from the Triton-insoluble membrane fraction of MOR-CHO. MOR was stimulated by incubating MOR-CHO cell membranes with sufentanil ($1 \mu M$; $30^\circ C$ for 10 min). Triton-insoluble (Tri-IS) and Triton-soluble (Tri-SOL) fractions were separated by high-speed centrifugation as discussed for Fig. 3. Triton-insoluble fractions from unstimulated and sufentanil-activated MOR-CHO membranes were solubilized with a mixture of detergents as described and subjected to immunoprecipitation using an anti-caveolin-1 monoclonal antibody. Parallel Western analyses were used to quantify $G_{s\alpha}$ in the caveolin IPs obtained from control and sufentanil-stimulated samples. $G_{s\alpha}$ values were normalized by using the content of caveolin in each IP. Sufentanil increased $G_{s\alpha}$ co-IP in the Triton-insoluble fraction (lane 2 versus 1) but not in the Triton-soluble fraction (lane 4 versus 3). Quantification is shown for only the ≈ 48 -kDa $G_{s\alpha}$ because this molecular mass species is vastly more prominent than its smaller molecular mass variant.

activation on the association of $G_s\alpha$ and caveolin-1 in rat spinal cord. After acute sufentanil ($1\ \mu\text{M}$), the content of $G_s\alpha$ (48 kDa) in caveolin-1 IP obtained from Triton-insoluble and -soluble fractions increased by 106.6 ± 31.1 and $43.3 \pm 21.9\%$, respectively (Fig. 5, left, lane 2 versus lane 1 and lane 5 versus lane 4, respectively; $n = 5$; $p < 0.05$ for both). The ≈ 45 -kDa $G_s\alpha$ signal was not very prominent, which made quantification of its trafficking problematic. As was observed in membranes from MOR-CHO, sufentanil-stimulated translocation of $G_s\alpha$ was abolished by $1\ \mu\text{M}$ naloxone, indicating its mediation by MOR (Fig. 5, left, lane 3 versus lane 2 and lane 6 versus lane 5). Thus, MOR-coupled translocation of $G_s\alpha$ is a generalizable phenomenon and not idiosyncratic to a particular MOR-overexpressing cell line maintained in culture.

AC, which is located outside of and within caveolae, is a primary target for $G_s\alpha$. To explore whether sufentanil-stimulated translocated $G_s\alpha$ interacts with AC before its association with caveolae, we investigated whether or not MOR activation results in the parallel translocation of AC into caveolae. As was done for $G_s\alpha$, we quantified the content of AC in the IP obtained with anticaveolin-1 monoclonal antibody. Concomitant with $G_s\alpha$, acute sufentanil produced an increase in the co-IP of AC with caveolin-1 from the Triton-insoluble membrane fraction of rat spinal cord ($69.6 \pm 19.66\%$; Fig. 5, right, lane 2 versus lane 1; $n = 5$; $p < 0.02$), which was abolished by naloxone pretreatment (Fig. 5, right, lane 3 versus lane 2). Acute sufentanil did not significantly

alter the AC content of caveolin-1 IP obtained from the Triton-soluble membrane fraction.

Discussion

In this study, multiple parameters that reflect the activation of G_s were quantified to assess the functionality of the association of MOR with $G_s\alpha$, heretofore inferred but not directly established. These included stimulation by sufentanil of 1) [^{35}S]GTP γS binding to $G_s\alpha$, 2) the translocation of $G_s\alpha$ into a Triton-insoluble membrane compartment, and 3) the association of $G_s\alpha$ and AC with the caveolin-1 contained within the Triton-insoluble membrane fraction. $G_s\alpha$ and AC membrane translocation is considered to be reflective of their activation (Huang et al., 1999; Allen et al., 2005). Thus, these results not only validate the functionality of the previously established MOR $G_s\alpha$ association but also indicate that MOR G_s signaling spans multiple membrane microdomains and is intricately associated with caveolin (presumably caveolin-1).

Caveolin-1 is the principal of the three isoforms of caveolin, 21- to 24-kDa integral membrane proteins. Caveolins are the principal structural components of caveolae and also function as scaffolding proteins organizing a wide variety of signaling proteins (see Patel et al., 2008 for review). It is important to note that $>90\%$ of the caveolin-1 present in cells is localized to the caveolae (Li et al., 1995); essentially 100% of the caveolin-1 present in the Triton-insoluble fraction is associ-

Fig. 5. MOR activation increased the co-IP of $G_s\alpha$ and AC with caveolin-1 from the Triton-insoluble (Tri-IS) membrane fraction of spinal cord. MOR was activated, and Triton-insoluble and Triton-soluble (Tri-Sol) membrane fractions were obtained and subjected to caveolin-1 immunoprecipitation (Cav-ip) as described. Parallel Western analyses were used to quantify $G_s\alpha$ and AC in separate aliquots of the caveolin-1 IPs obtained from control and sufentanil-stimulated samples. $G_s\alpha$ and AC values were normalized by using the content of caveolin-1 in each IP. Although sufentanil increased the co-IP of the ≈ 48 -kDa $G_s\alpha$ with the caveolin-1 from Triton-insoluble and -soluble spinal membrane fractions, the increment in $G_s\alpha$ that coimmunoprecipitated from the Triton-insoluble fraction was ≈ 2.5 -fold greater than that obtained from the Triton-soluble fraction. Sufentanil also stimulated the co-IP of AC ($\sim 70\%$, $n = 5$; $p < 0.02$) with caveolin-1 but only from the Triton-insoluble membrane fraction.

ated with caveolae. Thus, caveolin serves as a marker protein for this membrane organelle. The current demonstration that acute MOR activation augments the content of both $G_{s\alpha}$ and AC in IP obtained from the Triton-insoluble membrane fraction using anti-caveolin-1 monoclonal antibody unambiguously indicates MOR-coupled translocation of $G_{s\alpha}$ and AC into membrane caveolae. Importantly, MOR-coupled translocation to caveolae of both $G_{s\alpha}$ and AC also occurred in spinal cord, eliminating any possibility that it is idiosyncratic to cells maintained in culture and/or overexpressing MOR.

Stimulation of the binding of [35 S]GTP γ S to heterotrimeric guanine nucleotide binding proteins (G proteins) by an agonist selective for a particular GPCR is a frequently used direct indicator of functional coupling of the cognate receptor to the G protein under study. G proteins cycle between GDP- and GTP-bound states, which is causally associated with G-protein quiescence and activation, respectively. The current demonstration of the ability of sufentanil to dose dependently stimulate [35 S]GTP γ S binding to $G_{s\alpha}$, which was abolished by naloxone, unambiguously and directly indicates that G_s is among the G proteins that functionally couple to MOR. This validates earlier indications of functional MOR G_s coupling, which was inferred from the co-IP of MOR with $G_{s\alpha}$ (Chakrabarti et al., 2005) and multiple reports of excitatory MOR-coupled effects that are resistant to pertussis toxin (and are thus not mediated via G_i/G_o) but sensitive to CTX (Xu et al., 1989; Shen and Crain, 1990; Gintzler and Xu, 1991; Wang and Gintzler, 1997; Szucs et al., 2004).

MOR-stimulated [35 S]GTP γ S binding to $G_{s\alpha}$ was assessed by using a MOR overexpression system in which the density of MOR is ≈ 3.63 pmol/mg protein (Szucs et al., 2004) versus ≈ 500 fmol/mg protein in the superficial lamina of the spinal cord (Stevens and Seybold, 1995). It is unlikely, however, that the observed MOR $G_{s\alpha}$ coupling results from the ≈ 6 -fold increment in MOR density because it would be expected to reduce the normally occurring 100:1 ratio of $G_{s\alpha}$ to GPCRs; lessening the excess of G_s relative to MOR would not be expected to favor MOR G_s interactions.

Prior treatment with naloxone abolishes sufentanil-stimulated $G_{s\alpha}$ membrane translocation into caveolae. This indicates its mediation by MOR. MOR-coupled $G_{s\alpha}$ translocation is also abolished by CTX. Long-term treatment (e.g., 24 h) with CTX is well known to cause down-regulation of $G_{s\alpha}$ (Wedegaertner et al., 1996). This effect of CTX does not confound data interpretation because the ability of CTX to abolish the sufentanil-stimulated increment in the co-IP of $G_{s\alpha}$ with caveolin was assessed relative to the co-IP of $G_{s\alpha}$ that was obtained from CTX-treated cells in the absence of sufentanil. Importantly, the effects of CTX are generally ascribed to ADP-ribosylation of G_s ; CTX does not ADP-ribo-

sylate G_i or G_o in the absence of agonist as in the current experiments.

In addition to inhibiting the intrinsic GTPase activity of $G_{s\alpha}$ (Cassell and Selinger, 1977; Gill and Meren, 1978), CTX-catalyzed ADP ribosylation of $G_{s\alpha}$ uncouples G_s -mediated signal transduction (Cassell and Selinger, 1977; Stadel and Lefkowitz, 1981; Wieland et al., 1994). Thus, the ability of CTX to abolish sufentanil stimulation of $G_{s\alpha}$ translocation into the Triton-insoluble membrane fraction not only provides independent validation of functional MOR G_s coupling but also indicates that MOR-stimulated translocation of $G_{s\alpha}$ occurs subsequent to its activation. A remaining conundrum is whether or not MOR-stimulated $G_{s\alpha}$ translocation into caveolae reflects the termination of its signaling (Li et al., 1995) or if caveolae are the membrane microdomain in which MOR $G_{s\alpha}$ signaling occurs. The sufentanil-stimulated concomitant increase in Triton-insoluble caveolin-associated AC and $G_{s\alpha}$ (see below) would suggest the latter.

The current demonstration of MOR activation-dependent translocation of $G_{s\alpha}$ to lipid membrane rafts/caveolae in MOR-CHO and spinal cord is consistent with reported interactions between $G_{s\alpha}$ and caveolin in the absence of $G\beta\gamma$ subunits (Li et al., 1995) and with earlier findings that $G_{s\alpha}$ (and $G_o\alpha$ and $G_i\alpha$) can directly bind to caveolin (Li et al., 1995). Activation-dependent trafficking of $G_{s\alpha}$ to caveolae is not unique to this G-protein subunit; selective activation of G_{q11} and G_{i3} also increases the content of their respective $G\alpha$ subunits in caveolin IP (Murthy and Makhlof, 2000).

The source of the translocated $G_{s\alpha}$ after acute sufentanil remains unresolved. In the present experiments, we were not able to observe reciprocal changes in the $G_{s\alpha}$ content of the Triton-soluble and -insoluble membrane fractions after sufentanil. This could result from the differential size of these two pools of $G_{s\alpha}$. Because the pool of $G_{s\alpha}$ in the Triton-soluble membrane fraction is approximately seven times larger than that present in the Triton-insoluble fraction, a decrease in the $G_{s\alpha}$ content of the Triton-soluble membrane fraction that corresponds in magnitude to the increase that occurs in the Triton-insoluble fraction would be difficult to detect by Western blotting. This notwithstanding, although sufentanil augmented ($\approx 90\%$) the co-IP of $G_{s\alpha}$ with caveolin from the Triton-insoluble membrane fraction of MOR-CHO, it failed to do so from the Triton-soluble membrane fraction. Analogous results were obtained from spinal cord. The differential sufentanil-induced increment in the $G_{s\alpha}$ that coimmunoprecipitated from Triton-insoluble versus -soluble fractions could indicate a decrement in the $G_{s\alpha}$ content of the Triton-soluble pool after sufentanil because the amount of $G_{s\alpha}$ that is bound to caveolin (the content of which should remain

Fig. 6. Schematic representation of the relevance of caveolae to MOR signaling via G_s . MOR is known to be present in caveolae, which also concentrate less-phosphorylated $G_{s\alpha}$. Because diminished phosphorylation of $G_{s\alpha}$ promotes its association with MOR, we envision caveolae to be a membrane microdomain in which there is enhanced MOR G_s signaling. In parallel, MOR-coupled activation of $G_{s\alpha}$ results in its trafficking into caveolae, which is accompanied by AC. These data strongly suggest the likelihood that downstream targets for MOR $G_{s\alpha}$ AC signaling are concentrated within discrete membrane microdomains.

constant) is heavily influenced by the mass action effects of the size of the G_sα pool.

Interestingly, the G_sα that coimmunoprecipitates with caveolin can be back-phosphorylated more than a fold greater than that of the general population. Notably, the phosphorylation sites on G_sα are not recognized by the anti-G_sα antibody (Chakrabarti and Gintzler, 2007). Thus, it is highly unlikely that its presence during the back phosphorylation would interfere with ³²P incorporation and thereby confound the inference that lesser phosphorylated G_sα preferentially localizes to lipid rafts/caveolae. This is of particular relevance to MOR G_sα signaling because MOR is not only present in caveolae (Zhao et al., 2006) but has increased association with dephosphorylated G_sα (Chakrabarti and Gintzler, 2007). The ability of MOR to augment the trafficking of AC together with G_sα to caveolae, which concentrate G_sα that preferentially associates with MOR, underscores that caveolae are a microdomain within which MOR-coupled G_sα AC signaling occurs.

This is underscored by the association of two pools of G_sα with caveolae, the lesser phosphorylated G_sα that is concentrated in caveolae, which would be expected to interact with MOR present within that membrane compartment, and the G_sα that is translocated into caveolae together with AC subsequent to MOR activation. These data emphasize the centrality to MOR G_sα signaling of lipid membrane rafts/caveolae, irrespective of whether their initial coupling occurred outside of or within them.

In general, the functional consequences of the localization in caveolae of Gα subunits and their association with caveolin remain controversial. One school of thought purports that Gα subunits remain bound to the membrane when they are activated; activation of Gα causes it to concentrate in subdomains of the plasma membrane but not to be released from the membrane (Huang et al., 1999). This formulation suggests that caveolae bring signaling molecules into proximity with downstream effectors and thereby facilitate signal transduction (Bhatnagar et al., 2004).

The current demonstration of MOR-coupled translocation of AC and G_sα into caveolae suggests that they contain the downstream targets for MOR-coupled G_s–AC signaling. This supposition is supported by the association of both the protein kinase A catalytic subunit and its regulatory subunit RIα with lipid rafts (Vang et al., 2001) as are A-kinase-anchoring proteins (Tasken and Aandahl, 2004), which target protein kinase A to specific subcellular compartments. Thus, MOR-coupled translocation of G_sα and AC into caveolae could enable spatial and temporal constraints on MOR G_sα-related signaling events that are of particular relevance to opioid antinociceptive and tolerance mechanisms. The ability of caveolae to spatially juxtapose signaling molecules and effectors could suggest substrates underlying acute and chronic opioid actions that have heretofore escaped attention.

The above formulation is in striking contrast to an opposing theory that purports that binding to caveolin maintains Gα proteins in an inactive GDP-bound state (Li et al., 1995, 1996; Couet et al., 1997; Murthy and Makhlof, 2000), thereby dampening GPCR signaling. Evidence of opposing effects of the association of signaling molecules with caveolin indicates that it is very problematic to generalize consequences of G-protein translocation to caveolae. The specific consequence of caveolin association on GPCR signaling is undoubtedly influ-

enced by the particular GPCR and G protein to which it is coupled and the associated signaling pathways.

In summary, we demonstrate the relevance of caveolae, a subpopulation of membrane lipid rafts, to MOR-coupled G_sα signaling (see Fig. 6). Because increased MOR G_sα signaling is associated with the formation of opioid tolerance (Chakrabarti et al., 2005; Chakrabarti and Gintzler, 2007; Shy et al., 2008) and caveolae are generally considered to be important subcellular domains specifying the formation of signaling complexes, understanding the caveolae-associated substrates for MOR G_sα AC signaling could provide unexpected insights into critical subcellular determinants of tolerance and the related phenomenon of addiction.

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