Sub-Cellular localization of mu-opioid receptor \(G_s\) signaling

Sumita Chakrabarti, Andrew Chang and Alan R Gintzler

Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, New York 11203 (SC, AC, ARG)
a) Running title: MOR G_α_ signaling and membrane microdomains

b) Corresponding Author: Dr. Alan Gintzler

Box 8, Department of Biochemistry
SUNY Downstate Medical Center
450 Clarkson Ave.
Brooklyn, NY 11203
Tel: 718 270 2129
Fax: 718 270 3316
E-mail: alan.gintzler@downstate.edu

c) text pages: 28
tables: none
figures: 6
references: 30
abstract: 226 words
introduction: 442 words
discussion: 1523 words

d) Abbreviations: MOR, µ-opioid receptor; MOR-CHO, Chinese hamster ovary cells stably transfected with µ-opioid receptors; AC, adenylyl cyclase; CTX, Cholera toxin; IP, immunoprecipitate;
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 \(^{35}\text{S}\)GTP\(_\gamma\)S binding to G\(_{\text{S}}\) that was abolished by blocking MOR with naloxone. This unequivocally demonstrates the long debated functionality of the previously described association of MOR with G\(_{\text{S}}\). Several complimentary observations indicate the relevance of caveolae to MOR-coupled G\(_{\text{S}}\) signaling. (1) In MOR-CHO membranes, sufentanil stimulated the translocation of G\(_{\text{S}}\) into Triton insoluble membrane compartments. (2) Sufentanil enhanced the co-immunoprecipitation of G\(_{\text{S}}\) and adenylyl cyclase (AC) with caveolin-1 (a marker for caveolae) from the Triton insoluble membrane fraction of spinal cord as well as MOR-CHO. (3) MOR blockade (via naloxone) or G\(_{\text{S}}\) inactivation (via Cholera toxin) abolished both the increased trafficking of G\(_{\text{S}}\) into the Triton insoluble membrane fraction of MOR-CHO and the augmented co-immunoprecipitation from spinal cord membranes of G\(_{\text{S}}\) and AC with caveolin-1. This indicates that these events occurred subsequent to activation of MOR and G\(_{\text{S}}\). Strikingly, lesser-phosphorylated G\(_{\text{S}}\), which preferentially couple to MOR (Chakrabarti and Gintzler, 2007; Chakrabarti et al., 2005; Shy et al., 2008)), are concentrated in caveolae underscoring their relevance to MOR G\(_{\text{S}}\) signaling. MOR-stimulated trafficking of G\(_{\text{S}}\) and AC into caveolae as well as the likelihood of increased MOR G\(_{\text{S}}\) coupling within caveolae could suggest that they contain the downstream effectors for MOR G\(_{\text{S}}\) AC signaling.
Introduction

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

Interaction of MOR with $G_{sa}$ is a prerequisite for its transduction of MOR-stimulated signaling. Nevertheless, demonstration of their association does not unequivocally indicate that MOR functionally couples to $G_{sa}$. Validation of functional inferences drawn from the co-immunoprecipitation (co-IP) of MOR and $G_{sa}$ requires quantification of a parameter that is a direct indicator of $G_{sa}$ activation by MOR, e.g., stimulation of [$^{35}$S]GTPγS 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 $G_{sa}$. Diminished $G_{sa}$ 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 increased association of MOR with $G_{sa}$ (Chakrabarti and Gintzler, 2007). Phosphorylation state is inversely related to hydrophobicity; decreasing phosphorylation augments lipid solubility. Thus,
the inverse relationship between $G_{\alpha \text{S}}$ phosphorylation and MOR association could suggest that MOR $G_{\alpha \text{S}}$ signaling occurs predominantly in lipid-rich membrane micro domains.

Caveolae are one such subcellular compartment that has received considerable attention regarding their ability to serve as organizing foci for cellular signal transduction. Caveolae are a subset of lipid rafts, re-named 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 proteins (>90% of the cellular content of caveolin is present in caveolae (Li et al., 1995). These bind signaling molecules such as $G$ protein-coupled receptors, 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 $G_{\alpha \text{S}}$ activation by MOR and to define the membrane micro-domains in which they occur. Results not only definitively demonstrate dose-dependent stimulation of $[^{35}\text{S}]\text{GTP}_\gamma \text{S}$ binding to $G_{\alpha \text{S}}$ by sufentanil, a MOR-selective agonist, but provide cross-validating data that underscore the relevance of caveolae to MOR $G_\alpha$ signaling.
Materials and Methods

Cell Culture and Membrane preparation. Chinese Hamster Ovary (CHO) cells stably transfected with MOR (MOR-CHO) (Chakrabarti et al., 2005) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing high glucose and L-glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 units/ml penicillin/streptomycin and 100 µg/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 Benzamidine, 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, Nα-p-tosyl-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride (PMSF), and complete cocktail inhibitor tablet/50 ml. Cells were homogenized in the same buffer and centrifuged at 1,000 x g, 4°C for 10 min. Supernatants obtained from the low speed spin were centrifuged at 105,000 g for 1h at 4°C. Membrane fractions obtained were re-suspended 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 % Na-deoxycholate and 0.2% Na-dodecyl sulfate, in the same HEPES buffer containing protease inhibitors, 10% glycerol and 150 mM NaCl. After centrifugation (16,000 x g for 15 min
at 4°C), clear supernatants were used for Protein Assay (Bradford), Western analyses and immunoprecipitation (IP). For caveolin IP, purified mouse monoclonal anti-caveolin-1 antibody (BD Biosciences; San Jose, CA; 1µl /100 µg protein) was used together with pre-washed Protein G-agarose (50 µl; 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 µl of sample buffer (15 min at 85°C). Samples separated on 4-12% gradient Bis-Tris gels (Invitrogen) were electro-transferred onto nitrocellulose membranes and used for either autoradiography (to assess \(^{32}\)P incorporation) or Western analyses.

**Assessment of relative phosphorylation state of \(G_{\text{as}}\) populations.** We determined the magnitude of \(^{32}\)P incorporation into \(G_{\text{as}}\) immunoprecipitated with anti-caveolin and \(G_{\text{as}}\) antibodies, i.e., back phosphorylation. This was used to reflect the relative degree of phosphorylation of each population of \(G_{\text{as}}\). \(G_{\text{as}}\) was immunoprecipitated from solubilized MOR-CHO membranes as described previously (Chakrabarti et al, 2005). Following IP, the protein A agarose beads containing the antigen-antibody complexes were washed and re-suspended in a kinase buffer containing 20 mM HEPES, pH 7.6, 10 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM EGTA, 0.25% bovine serum albumin (BSA), 1 mM DTT, 10% glycerol, complete protease inhibitor cocktail, 1 tablet/50 mL, 100 µM 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\(_{\text{cat}}\) (20 mU/reaction; Calbiochem, San Diego, CA) and \([\gamma^{32}\text{P}]-\text{ATP} \) (2.5 µCi/reaction; PerkinElmer) and incubated for 2h 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 electro-transferred onto a nitrocellulose membrane. Phosphorylated \(G_{\text{as}}\)
subunits were visualized by autoradiography, quantitated using PhosphorImager analysis software (ImageQuant TL) and identified as Gsα proteins using anti-Gsα antibodies on a Western blot.

**Western analysis.** Gsα and Caveolin proteins were visualized using 1:5,000 dilution of a polyclonal rabbit anti-Gsα antibody raised against the C-terminus of Gsα (generously provided by Dr. J. Hildebrandt, Medical University of South Carolina) and a polyclonal rabbit anti-caveolin antibody raised against human caveolin (1-97 aa), respectively. AC was visualized using a 1:1,000 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, Du¨sseldorf, Germany; (Mollner and Pfeuffer, 1988). The secondary antibody utilized was either a peroxidase-labeled donkey anti-rabbit or anti-mouse antibody (Amersham/GE Healthcare, Piscataway, NJ). Antibody-substrate complex was visualized using a Supersignal West Dura Chemiluminescence detection kit (Pierce, Rockford, IL). Specificity of Gsα and AC Western signals had been previously demonstrated (Chakrabarti 2005b; Chakrabarti, 1998b) in MOR-CHO membranes and was therefore not repeated in the current study. The use of both monoclonal and polyclonal anti-caveolin antibodies differing in their epitope specificities (aa 1-21, which is unique to caveolin-1, vs. 1-97 aa, respectively) validated the identity of the caveolin Western blot signal. The product of the IP, obtained using the monoclonal antibody, was analyzed by Western blotting with the polyclonal antibody. Sample pairs, obtained from opioid naïve 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 using Syngene software in GeneGnome.

**Detection of MOR agonist-stimulated [35S]GTPγS binding via antibody capture and**
scintillation proximity assay (SPA). $[^{35}S]GTP\gamma S$ binding was detected and quantified using antibody capture in combination with a scintillation proximity assay (SPA). Unlike conventional $[^{35}S]GTP\gamma S$ binding assays which cannot distinguish among the various $G\alpha$ subunits that bind $[^{35}S]GTP\gamma S$, the antibody capture strategy enables the detection of $[^{35}S]GTP\gamma S$ that is bound to specific $G\alpha$ protein subunits, e.g., $G_{s\alpha}$. In the current iteration of this approach, MOR-CHO membranes were permeabilized with 0.5% 3-[[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (Sigma, St. Louis, MO) for 30 min on ice. Agonist (sufentanil, 1-1000 nM) induced dose-dependent increase in $[^{35}S]GTP\gamma S$ binding to $G_{s\alpha}$ was determined using membranes (300 µg) incubated in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 5 mM MgCl$_2$, 0.1 µM GDP, and 0.1 µCi (500 pM) $[^{35}S]GTP\gamma S$ in the presence and absence of opioid agonist sufentanil (30°C for 30 min). Membranes were pre-incubated with agonists and antagonists for 15 min at 30°C followed by the addition of $[^{35}S]GTP\gamma S$ (DeLapp et al., 1999). Reactions were terminated on ice, centrifuged (15 min at 16,000 rpm at 4°C) and supernatants discarded. Membranes were solubilized by using 1% n-Dodecyl β-D-glucopyranoside, 0.5% Na-deoxycholate and 0.2% Na-dodecyl sulfate in 50mM Tris buffer (pH 7.4), agitated for 1h at room temperature and incubated with anti-$G_{s\alpha}$ antibodies for an additional 2h at room temperature. The reaction mixture was added to 96 well plates, the bottom of which was impregnated with scintillant (‘flash plates’; PerkinElmer) and coated with anti-rabbit IgG antibodies. Nonspecific binding was detected in the presence of 10 µM GTP$\gamma$S. The (rabbit) anti-$G_{s\alpha}$ antibodies, included in the reaction mixture bound to $G_{s\alpha}[^{35}S]GTP\gamma S$, thereby promoting its proximity to the scintillant impregnated secondary antibody-coated well surface. This allowed the short range electrons emitted from the $[^{35}S]GTP\gamma S$ to excite the impregnated fluorophor and emit light, which was quantified using a Microbeta Jet counter (PerkinElmer). Importantly, since the anti-$G_{s\alpha}$ antibodies did not recognize other $G$ protein subunits, that are present, only $[^{35}S]GTP\gamma S$ bound $G_{s\alpha}$ is brought into proximity with the scintillant impregnated well bottom.
Thus, quantification of basal and agonist-stimulated \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding to G_{\text{S} \alpha} subunits would not be confounded by \[^{35}\text{S}]\text{GTP} \gamma \text{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_{\text{S} \alpha} antibodies or using non-immune 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 \(^{35}\text{S}\text{GTP} \gamma \text{S} binding to G_{\text{S} \alpha} \). A non-linear regression analysis (Graph pad Prism Software) was used to generate the corresponding ED\(_{50}\) values for Sufentanil.
Results

Effect of sufentanil on [\textsuperscript{35}S]GTP\gamma S binding to G\textsubscript{sa}. The MOR-selective agonist sufentanil produced a concentration-dependent (1-1000 nM) stimulation (~40-100%) of [\textsuperscript{35}S]GTP\gamma S binding to G\textsubscript{sa} (Spearman correlation coefficient of 0.65, p=0.002; Fig. 1). In membranes obtained from MOR-CHO, maximal stimulation was 100.5±12.8% (n=3-6; p<0.05) with an ED\textsubscript{50} of 31.4 nM (Fig.1). To validate the identity of the receptor mediating the stimulation of [\textsuperscript{35}S]GTP\gamma S binding, the assay was conducted in the presence of the MOR antagonist naloxone (10 µM). The presence of this antagonist abolished sufentanil (1 µM) stimulation of [\textsuperscript{35}S]GTP\gamma S binding. In order to eliminate the possibility that results were confounded by the recognition of G\textsubscript{i}/G\textsubscript{o} proteins by the anti-G\textsubscript{sa} antibodies, IP obtained using the anti-G\textsubscript{sa} antibodies were subjected to Western analysis using anti-G\textsubscript{i}/G\textsubscript{o} antibodies. No G\textsubscript{i}/G\textsubscript{o} were detected indicating that [\textsuperscript{35}S]GTP\gamma S binding to G\textsubscript{ia}/G\textsubscript{oa}, the predominant G proteins that couple to MOR, were not a likely confound. Importantly, sufentanil (1 µM) also stimulated [\textsuperscript{35}S]GTP\gamma S binding to G\textsubscript{sa} in membranes obtained from spinal cord (111.8±26%, n=3; p<0.05). Collectively, these results indicate the functionality of the previously demonstrated association of MOR with G\textsubscript{sa} (Chakrabarti and Gintzler, 2007; Chakrabarti et al., 2005).

Decreased phosphorylated G\textsubscript{sa} preferentially partitions with caveolin. We previously reported that de-phosphorylation of G\textsubscript{sa} 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\textsubscript{sa} signaling, we investigated if decreased phosphorylated G\textsubscript{sa} preferentially partitioned in this membrane micro-domain. Since caveolin is a marker for lipid membrane rafts/caveolae, we compared the phosphorylation state of the G\textsubscript{sa} that co-immunoprecipitated with caveolin vs. the phosphorylation state of G\textsubscript{sa} that was
immunoprecipitated using anti-\(\text{G}_{\text{sa}}\) antibodies (which should reflect the composite phosphorylation state of \(\text{G}_{\text{sa}}\) among the total population). Phosphorylation state was assessed by quantifying the magnitude of back phosphorylation, in which the level of \(^{32}\text{P}\) incorporation is inversely proportional to the pre-existing degree of phosphorylation. Fig. 2 illustrates that the magnitude of \(^{32}\text{P}\) incorporation into the \(\approx 48\) kDa molecular mass \(\text{G}_{\text{sa}}\) that co-immunoprecipitated with caveolin was 145±24% (p<0.05) greater than that incorporated into \(\text{G}_{\text{sa}}\) immunoprecipitated with anti-\(\text{G}_{\text{sa}}\) antibodies. Note that this is an underestimate of the actual increment in back phosphorylation since the \(\text{G}_{\text{sa}}\) protein content of the caveolin-1 IP was significantly less (37%; p<0.05, n=3) than that present in \(\text{G}_{\text{sa}}\) IP. Interestingly, back phosphorylation of the \(\approx 52\) kDa molecular mass \(\text{G}_{\text{sa}}\) that co-immunoprecipitated with anti-caveolin-1 antibodies was also substantially greater than that of the \(\text{G}_{\text{sa}}\) that co-immunoprecipitated with anti-\(\text{G}_{\text{sa}}\) antibodies (note the markedly greater protein content of the \(\approx 52\) kDa molecular mass species in \(\text{G}_{\text{sa}}\) IP vs. caveolin-1 IP). However, since levels of the protein corresponding to the \(\approx 52\) kDa molecular mass autoradiographic signal were barely detectable in caveolin-1 IP, differences in \(^{32}\text{P}\) incorporation were not quantified. Importantly, the anti-\(\text{G}_{\text{sa}}\) antibody employed in these studies does not distinguish among differentially phosphorylated forms of \(\text{G}_{\text{sa}}\) (Chakrabarti and Gintzler, 2007).

**Acute sufentanil stimulates the translocation of \(\text{G}_{\text{sa}}\) into triton-insoluble membrane compartments of MOR-CHO.** Quantification of changes in the content of \(\text{G}_{\text{sa}}\) 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 micro-domains in nonionic detergents. Acute activation of MOR in MOR-CHO membranes by sufentanil (1 \(\mu\text{M}, 10\) min at 30°C) produced a significant increase (\(\approx 53±8.8\%\); p<0.02) in the content of \(\text{G}_{\text{sa}}\) in the Triton insoluble fraction of MOR-CHO membranes (Fig. 3; lane 2 vs. lane 1). The sufentanil-induced
increase in $G_{\text{sst}}$ content was reduced by ~80% following pretreatment with naloxone (10 μM), indicating that MOR activation was a prerequisite for sufentanil-induced translocation of $G_{\text{sst}}$ (Fig. 3, lane 3 vs. 2, p<0.05). Interestingly, a 24 h pretreatment with Cholera toxin (1 μg/ml), abolished sufentanil stimulation of $G_{\text{sst}}$ translocation to the Triton insoluble membrane fraction (Fig. 3, lane 5 vs. lane 4). This indicates that it occurred subsequent to its activation.

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

**Acute sufentanil increases the co-IP of $G_{\text{sa}}$ and AC with caveolin-1 from spinal cord membranes.** In order to determine if the stimulation of $G_{\text{sst}}$ translocation by MOR observed in MOR-CHO generalized to complex integrated neuronal tissue, we investigated the effect of MOR activation on the association of $G_{\text{sa}}$ and caveolin-1 in rat spinal cord. Following acute sufentanil (1 μM), the content of $G_{\text{sa}}$ (48 kDa) in caveolin-1 IP obtained from Triton insoluble and soluble fractions, increased by 106.6 + 31.1% and 43.3 + 21.9 %, respectively (Fig. 5, left panel, lane 2 vs. lane 1 and lane 5 vs. lane 4, respectively; n=5; p<0.05 for both). The ≈45 kDa $G_{\text{sa}}$ 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 µM naloxone indicating its mediation by MOR (Fig. 5, left panel, lane 3 vs. lane 2 and lane 6 vs. lane 5, respectively). 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.

Adenylyl cyclase (AC), which is located outside of and within caveolae, is a primary target for $G_{s\alpha}$. In order to explore if sufentanil-stimulated translocated $G_{s\alpha}$ interacts with AC prior to 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 anti-caveolin-1 monoclonal antibody. Concomitant with $G_{s\alpha}$, acute sufentanil produced an increase in the co-immunoprecipitation of AC with caveolin-1 from the triton insoluble membrane fraction of rat spinal cord (69.6±19.66%; Fig. 5, right panel, lane 2 vs. lane 1; n=5; p<0.02), which was abolished by naloxone pre-treatment (Fig. 5, right panel, lane 3 vs. 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) $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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 (Allen et al., 2005; Huang et al., 1999). 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 principle of the three isoforms of caveolin, 21-24-kDa integral membrane proteins. Caveolins are the principal structural component 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 caveoli (Li et al., 1995); essentially 100% of the caveolin-1 present in the Triton insoluble fraction is associated 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 immunoprecipitate 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 $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ to heterotrimeric guanine nucleotide binding proteins (G proteins) by an agonist selective for a particular G protein-coupled receptor (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 $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ 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 $G_{si}$ (Chakrabarti et al., 2005) as well as from 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 (Gintzler and Xu, 1991; Shen and Crain, 1990; Szucs et al., 2004; Wang and Gintzler, 1997; Xu et al., 1989).

MOR-stimulated $^{35}\text{S}GTP^\gamma\text{S}$ binding to $G_{si}$ was assessed using a MOR overexpression system in which the density of MOR is $\approx 3.63$ pmol/mg protein (Szucs et al., 2004) vs. $\approx 500$ fmol/mg protein in the superficial lamina of the spinal cord (Stevens and Seybold, 1995). It is unlikely, however, that the observed MOR $G_{si}$ coupling results from the $\approx 6$-fold increment in MOR density since it would be expected to reduce the normally occurring 100:1 ratio of $G_{si}$ 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 $G_{si}$ membrane translocation into caveolae. This indicates its mediation by MOR. MOR-coupled $G_{si}$ translocation is also abolished by CTX. Long term treatment (e.g., 24h) with CTX is well known to cause down
regulation of Gs\(\alpha\) (Wedegaertner et al., 1996). This effect of CTX does not confound data interpretation since the ability of CTX to abolish the sufentanil-stimulated increment in the co-IP of Gs\(\alpha\) with caveolin was assessed relative to the co-IP of Gs\(\alpha\) that was obtained from CTX-treated cells in the absence of sufentanil. Importantly, the effects of CTX are generally ascribed to ADP-ribosylation of Gs\(\alpha\); CTX does not ADP-ribosylate Gi or Go, in the absence of agonist as in the current experiments).

In addition to inhibiting the intrinsic GTPase activity of Gs\(\alpha\), (Cassell and Selinger, 1977; Gill and Meren, 1978), CTX-catalyzed ADP ribosylation of Gs\(\alpha\) also uncouples Gs\(\alpha\)-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 Gs\(\alpha\) translocation into the triton-insoluble membrane fraction not only provides independent validation of functional MOR Gs coupling but also that MOR-stimulated translocation of Gs\(\alpha\) occurs subsequent to its activation. A remaining conundrum is whether or not MOR-stimulated Gs\(\alpha\) translocation into caveolae reflects the termination of its signaling (Li et al., 1995) or if caveolae are the membrane microdomain in which MOR Gs\(\alpha\) signaling occurs. The sufentanil-stimulated concomitant increase in Triton insoluble caveolin-associated AC as well as Gs\(\alpha\) (see below) would suggest the latter.

The current demonstration of MOR activation-dependent translocation of Gs\(\alpha\) to lipid membrane rafts/caveolae in MOR-CHO and spinal cord is consistent with reported interactions between Gs\(\alpha\) and caveolin in the absence of G\(\beta\gamma\) subunits (Li et al., 1995) and with earlier findings that (1) Gs\(\alpha\) (as well as Gi\(\alpha\) and Go\(\alpha\)) can directly bind to caveolin (Li et al., 1995). Activation-dependent trafficking of Gs\(\alpha\) to caveolae is not unique to this G protein subunit; selective activation of Gq\(11\) and Gi\(3\) also increases the content of their respective G\(\alpha\) subunits in caveolin IP (Murthy and Makhlouf, 2000).
The source of the translocated \( G_{\alpha i} \) following acute sufentanil remains unresolved. In the present experiments, we were not able to observe reciprocal changes in the \( G_{\alpha i} \) content of the Triton soluble and insoluble membrane fractions following sufentanil. This could result from the differential size of these two pools of \( G_{\alpha i} \). Since the pool of \( G_{\alpha i} \) in the Triton soluble membrane fraction is \( \approx 7X \) larger than that present in the Triton insoluble fraction, a decrease in the \( G_{\alpha i} \) 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 \( G_{\alpha i} \) 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 \( G_{\alpha i} \) that co-immunoprecipitated from Triton insoluble vs. soluble fractions could indicate a decrement in the \( G_{\alpha i} \) content of the Triton soluble pool following sufentanil since the amount of \( G_{\alpha i} \) that is bound to caveolin (the content of which should remain constant) is heavily influenced by the mass action effects of the size of the \( G_{\alpha i} \) pool.

Interestingly, the \( G_{\alpha i} \) that co-IPs with caveolin can be back phosphorylated more than a fold greater than that of the general population. Notably, the phosphorylation sites on \( G_{\alpha i} \) are not recognized by the anti-\( G_{\alpha i} \) antibody (Chakrabarti and Gintzler, 2007). It is, thus, highly unlikely that its presence during the back phosphorylation would interfere with \(^{32}\)P incorporation and thereby confound the inference that lesser phosphorylated \( G_{\alpha i} \) preferentially localizes to lipid rafts/caveolae. This is of particular relevance to MOR \( G_{\alpha i} \) signaling since MOR is not only present in caveolae (Zhao et al., 2006) but has increased association with de-phosphorylated \( G_{\alpha i} \) (Chakrabarti and Gintzler, 2007). The ability of MOR to augment the trafficking of AC
together with $G_{\alpha}$ to caveoli, which concentrate $G_{\alpha}$ that preferentially associate with MOR, underscore that caveoli are a micro-domain within which MOR-coupled $G_{\alpha}$ AC signaling occurs.

This is underscored by the association of two pools of $G_{\alpha}$ with caveolae, the lesser phosphorylated $G_{\alpha}$ that is concentrated in caveolae, which would be expected to interact with MOR present within that membrane compartment, and the $G_{\alpha}$ that is translocated into caveolae together with AC subsequent to MOR activation. These data emphasize the centrality to MOR $G_{\alpha}$ 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 $G_{\alpha}$ subunits and their association with caveolin remain controversial. One school of thought purports that $G_{\alpha}$ subunits remain bound to the membrane when they are activated; activation of $G_{\alpha}$ 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 as well as $G_{\alpha}$ into caveolae suggests that they contain the down stream targets for MOR-coupled $G_{\alpha}$-AC signaling. This supposition is undergirded by the association of both the protein kinase A catalytic subunit and its regulatory subunit $R_{I\alpha}$ with lipid rafts (Vang et al., 2001) as is A-kinase-anchoring proteins (Tasken and Aandahl, 2004), which target protein kinase A to specific subcellular compartments. Thus, MOR-coupled translocation of $G_{\alpha}$ and AC into caveolae could enable spatial and temporal constraints on MOR $G_{\alpha}$-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 G\textsubscript{\alpha} proteins in an inactive GDP-bound state (Couet et al., 1997; Li et al., 1996; Li et al., 1995; Murthy and Makhlouf, 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 influenced by the particular GPCR and G protein to which it is coupled and the associated signaling pathway(s).

In summary, we demonstrate the relevance of caveolae, a subpopulation of membrane lipid rafts, to MOR-coupled G\textsubscript{s} signaling (see figure 6). Since increased MOR G\textsubscript{\alpha\textsubscript{s}} signaling is associated with the formation of opioid tolerance (Chakrabarti and Gintzler, 2007; Chakrabarti et al., 2005; Shy et al., 2008) and caveolae are generally considered to be important sub-cellular domains specifying the formation of signaling complexes, understanding the caveolae-associated substrates for MOR G\textsubscript{\alpha\textsubscript{s}} AC signaling could provide unexpected insights into critical sub-cellular determinants of tolerance and the related phenomenon of addiction.
References


Footnotes

This work was supported by the National Institutes of Health [Grant R01DA012251-08 to ARG].
Legends for figures:

**Figure 1.** Sufentanil stimulates $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding to $G_{\text{sa}}$ in MOR-CHO. Sufentanil stimulation of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding to $G_{\text{sa}}$ was quantified using a scintillation proximity assay in which MOR-CHO membranes (300 µg) were incubated in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 5 mM MgCl$_2$, 0.1 µM GDP, and 0.1 µCi (500 pM) $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ in the presence and absence of Sufentanil (0.001-1 µM; 30°C for 30 min) as described in Materials and Methods ($n=3$-6). $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding in the presence of 10 µM unlabeled GTP$^\gamma$S was defined as nonspecific binding. Sufentanil stimulation of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding to $G_{\text{sa}}$ was completely abolished by the presence of the MOR antagonist, naloxone (10µM), which unambiguously reveals functional MOR $G_\text{s}$ coupling. ($n=3$; *=p<0.05)

**Figure 2.** Assessment of $G_{\text{sa}}$ phosphorylation states via back phosphorylation. Autoradiography of $^{32}\text{P}$ incorporated into $G_{\text{sa}}$ immunoprecipitated with either anti-caveolin (lane 1) or anti-$G_{\text{sa}}$ (lane 2) antibodies was assessed. IP(s) were incubated (2h at 30°C) with PKC$_{\text{cat}}$ and $[^{32}\text{P}]\text{ATP}$ as described in Methods. Samples were resolved by gel electrophoresis (4–12% Bis-Tris gel), electro-transferred onto a nitrocellulose membrane, which was exposed to Phosphorimager for autoradiography. The same nitrocellulose membranes were immunoblotted with anti-$G_{\text{sa}}$ antibodies to quantify $G_{\text{sa}}$ immunoprecipitated with anti-caveolin antibodies (lane 3) or anti-$G_{\text{sa}}$ antibodies (lane 4). $n=3$; p<0.05. $G_{\text{sa}}$ immunoprecipitated with anti-caveolin antibodies contained less $G_{\text{sa}}$ protein than that obtained with anti-$G_{\text{sa}}$ antibodies but incorporated ~2-fold more $^{32}\text{P}$. This indicates that caveolin preferentially interacts with $G_{\text{sa}}$ of lower phosphorylation states.
**Figure 3.** Sufentanil induces translocation of $G_{s\alpha}$ into triton-insoluble membrane compartments.

High speed centrifugation was used to obtain the Triton insoluble membrane fraction (30 min, 105,000g at 4°C), which was washed in 20mM HEPES buffer (pH 7.4) and solubilized with a mixture of detergents (1% n-Dodecyl β-D-glucopyranoside, 0.5% Na-deoxycholate and 0.2% Na-dodecyl sulfate and 10% glycerol) containing a cocktail of protease inhibitors. Western analyses employing 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 µM, 10 min at 30°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 vs. lane 1). Sufentanil-induced translocation of $G_{s\alpha}$ was reduced by ~80% following pretreatment with naloxone (10 µM; #=p<0.05; lane 3 vs. 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 following a 24 h pretreatment with 1 µg/ml CTX (compare lane 5, CTX + sufentanil, vs. 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.

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

**Figure 5.** MOR activation increased the co-immunoprecipitation of $G_{s\alpha}$ and AC with caveolin-1 from the Triton insoluble membrane fraction of spinal cord. MOR was activated, triton insoluble and soluble membrane fractions were obtained and subjected to caveolin-1 immunoprecipitation as described above. Parallel Western analyses were used to quantify $G_{s\alpha}$ and AC in separate aliquots of the caveolin-1 IP obtained from control and sufentanil-stimulated samples. $G_{s\alpha}$ and AC values were normalized using the content of caveolin-1 in each IP. Although sufentanil increased the co-immunoprecipitation 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 co-immunoprecipitated from the Triton insoluble fraction was ≈2.5-fold greater than that obtained from the triton soluble fraction. Sufentanil also stimulated the co-immunoprecipitation of AC (~70%, n=5; p<0.02) with caveolin-1 but only from the Triton insoluble membrane fraction. Tri-IS (Triton insoluble); Tri-SOL (Triton soluble).

**Figure 6.** Schematic representation of the relevance of caveolae to MOR signaling via $G_{s\alpha}$. MOR is known to be present in caveolae, which also concentrate lesser-phosphorylated $G_{s\alpha}$. Since diminished phosphorylation of $G_{s\alpha}$ promotes its association with MOR, we envision caveolae to be a membrane micro-domain in which there is enhanced MOR $G_{s\alpha}$ signaling. Additionally, in parallel, MOR-coupled activation of $G_{s\alpha}$ results in it’s 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 micro-domains.
Figure 1

![Graph showing % Change Gsα [35]GTPγS Binding versus Sufentanil [nM]. The graph includes data points for different concentrations of Sufentanil, with error bars indicating variability. There is a notable increase in % Change at higher concentrations, marked with an asterisk (*) indicating statistical significance compared to Suf + Nx.]
Figure 2

[Image of autoradiogram and Western blot with labeled bands for Cav-IP and Gαs-IP]
Figure 3

![Graph showing % Increase in Triton Insoluble fraction for Suf, Suf + Nx, Suf + CTX.]

- **Suf**
- **Suf + Nx**
- **Suf + CTX**

**Legend:**
- *: Significant difference from control
- #: Significant difference from Suf + Nx

**Image:**
- Tri-LS
- kDa 51
- Gα
Sufentanil induced increase in Gsα in Caveolin IP (%control)

Figure 4
Sufentanil induced increase in Gsα in Caveolin-1 IP (%control)

Sufentanil induced increase in AC in Caveolin-1 IP (%control)

Figure 5