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Adenylyl Cyclase Superactivation Induced by Long-Term Treatment with Opioid Agonist Is Dependent on Receptor Localized within Lipid Rafts and Is Independent of Receptor Internalization

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ABSTRACT

Long-term opioid agonist treatment results in adenylyl cyclase superactivation. A recent "RAVE" theory implicates a direct correlation between the ability of agonist to induce receptor internalization and the magnitude of adenylyl cyclase superactivation. We decided to test such a theory by examining the adenylyl cyclase superactivation after long-term activation of μ-opioid receptor (MOR) in an EcR293 cell model. We examined the magnitudes of adenylyl cyclase superactivation in the presence of naloxone after long-term treatment with morphine, etorphine, and methadone, three agonists reported to have differential activities in promoting MOR internalization. It can be shown that the magnitudes of adenylyl cyclase superactivation after treating with these three agonists, although different, were dependent on MOR density. Blunting MOR internalization with the dominant-negative mutant of dynamin, K44E, did not alter the magnitude of either morphine- or etorphine-induced adenylyl cyclase superactivation. In the presence of diprenorphine, the magnitude of adenylyl cyclase superactivation after etorphine treatment was identical to that observed with morphine. It could be demonstrated further that adenylyl cyclase superactivation is dependent on the cell surface-located MOR. Sucrose gradient fractionation demonstrated the colocalization of MOR and adenylyl cyclase V/VI with caveolin-1, a marker for lipid rafts. After long-term agonist treatment, the majority of MOR remained at the lipid rafts. Methyl-β-cyclodextrin (MβCD) completely blunted the adenylyl cyclase superactivation and agonist-induced receptor internalization. These MβCD actions were reversed by incubating the cells with cholesterol. Thus, the adenylyl cyclase superactivation is not dependent on agonist-induced receptor internalization. Rather, the location of MOR at lipid rafts is an absolute requirement for the observed adenylyl cyclase superactivation.

Among the several second messenger systems that are regulated by opioid agonist, the short-term inhibition of adenylyl cyclase activity and subsequent reduction in the intracellular cAMP level have been studied extensively (Sharma et al., 1975, 1977). When the receptors are undergo long-term activation, there is a gradual loss in agonist inhibition of adenylyl cyclase activity (receptor desensitization) and an eventual increase in adenylyl cyclase activity observable only after agonist washout or the addition of an antagonist such as naloxone (Sharma et al., 1977; Law et al., 1982; Taylor and Fleming, 2001). This phenomenon, referred to as adenylyl cyclase superactivation (also termed adenylyl cyclase overshoot, superactivity, or sensitization), is thought to represent a possible biochemical substratum for the development of opiate tolerance and dependence, commonly observed on prolonged exposure to opiate drugs (Varga et al., 2003a). Moreover, it has been suggested that such regulation of adenylyl cyclase could be a general means of cellular adaptation to the activation of opioid receptors (Avidor-Reiss et al., 1996; Chakrabarti et al., 1998b).

Although the exact mechanism for adenylyl cyclase superactivation after long-term agonist treatment has yet to be elucidated, altered protein phosphorylation, particularly the phosphorylation of adenylyl cyclase isoforms, has long been considered to underlie, at least in part, many of the physiological sequelae of opioid receptor persistent activation

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ABBREVIATIONS: MOR, μ-opioid receptor; PKC, protein kinase C; CHO, Chinese hamster ovary; DOR, δ-opioid receptor; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; MEM, minimum Eagle's medium; DMEM, Dulbecco's modified Eagle's medium; PA, ponasterone A; TCA, trichloroacetic acid; FACS, fluorescence-activated cell sorting; MβCD, methyl-β-cyclodextrin; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance.

(Chakrabarti et al., 1998a,b). In COS-7 cells cotransfected with MOR and various adenylyl cyclase isoforms, the adenylyl cyclase superactivation was adenylyl cyclase V-dependent (Avidor-Reiss et al., 1996, 1997). Furthermore, tyrosine kinase, PKC, and raf-1 were indicated to participate in the phosphorylation of adenylyl cyclase VI, leading to an increase of cAMP accumulation on CHO cells stably expressing the human δ-opioid receptor (DOR) (Varga et al., 2003a). The direct phosphorylation of the adenylyl cyclase in ileum myenteric plexus during long-term morphine treatment has been reported also (Chakrabarti et al., 1998b). The phosphorylation of adenylyl cyclase and other signaling molecules could be the key for observed adenylyl cyclase superactivation.

In addition to protein phosphorylation, Whistler and von Zastrow have proposed the "RAVE" theory to be the basis for adenylyl cyclase superactivation (Whistler et al., 1999). According to this theory, opioid agonist, such as morphine, that does not induce rapid receptor endocytosis, is more addictive and produces a greater magnitude of adenylyl cyclase superactivation than an agonist such as etorphine that induces rapid receptor endocytosis. Their hypothesis was supported by the alteration in adenylyl cyclase superactivation magnitudes induced by morphine and etorphine in cells that expressed MOR/DOR receptor chimera and the ability to alter morphine in vivo tolerance in the presence of a low concentration of [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (Whistler et al., 1999; Finn and Whistler, 2001; He et al., 2002).

However, the RAVE theory could not explain two issues that were reported with long-term opioid agonist treatment. Although clinical studies have indicated that methadone is as addictive as morphine (Kreek, 2000), the RAVE theory predicts that methadone has a lower magnitude of adenylyl cyclase superactivation and is less addictive than morphine because of its ability to induce opioid receptor endocytosis (Whistler et al., 1999; Finn and Whistler, 2001). Another issue is that previous studies with DOR have indicated that receptor internalization required the dissociation of the receptor oligomers into monomers and that opioid receptors did not "drag" each other into the cell during long-term agonist treatment (Cvejic and Devi, 1997; Law et al., 2005). With DOR, the magnitude of adenylyl cyclase superactivation has been reported to be receptor concentration-dependent (Law et al., 1994). Thus, the observed differences in adenylyl cyclase superactivation magnitudes with different opioid receptor mutants or chimeras used to develop the RAVE theory could be a reflection of differences in receptor densities.

Membrane microdomains known as lipid rafts/caveolae have been found to be enriched with a variety of signaling proteins (Lisanti et al., 1994). It is noteworthy that many components of the cAMP-mediated signal transduction cascade have been shown biochemically and morphologically to concentrate within lipid rafts/caveolae. G protein-coupled receptors (GPCRs) (Dupree et al., 1993; Ostrom et al., 2001), G protein α and $\beta\gamma$ subunits, adenylyl cyclase, nitric-oxide synthase, and several protein kinases, such as protein kinase A, PKCα, Src tyrosine kinase, and phosphoinositide-3 kinase, are a few examples of signaling molecules that are observed in such microdomains (Sargiacomo et al., 1993; Smart et al., 1994, 1995; Feron et al., 1996, 1997; Li et al., 1996a; Mineo et al., 1998; Razani and Lisanti, 2001; Harder and Engelhardt, 2004). An important facet is that caveolins, the structural protein components of caveolae, in serving as protein scaffold, could restrict dynamic interactions of membrane proteins, thus regulating the phosphorylation and dephosphorylation equilibrium (Harder and Engelhardt, 2004).

Therefore, the current study was undertaken to examine the role of MOR density and agonist-induced MOR internalization on the agonist-dependent adenylyl cyclase superactivation. We could demonstrate that MOR-mediated adenylyl cyclase superactivation was not agonist-dependent but cell surface receptor concentration-dependent. In particular, adenylyl cyclase superactivation requires MOR to be localized within the lipid rafts/caveolin microdomains of the cell surface membranes. Hence, our studies do not support the proposed RAVE hypothesis as the mechanism for adenylyl cyclase superactivation.

Materials and Methods

Plasmids and Cells. The rat MOR in pCDNA3 was tagged with human hemagglutinin (HA) epitope (YPYDVPDYA) and was transfected into human embryonic kidney (HEK) 293 cells. Stable clones were selected with Geneticin (G418) at 1 mg/ml. One of the HEK293 clones stably expressing MOR determined to have the B_{max} and K_d values for [^{3}H]diprenorphine of 6.9 pmol/mg of protein and 0.9 ± 0.1 nM, respectively, was used in the current study (El Kouhen et al., 1999). These HEK293 cells were cultured in MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml G418 under humidified atmosphere at 5% CO_2.

For cells in which MOR level could be induced, HA-MOR was subcloned into the HIND3/XbaI sites of the ecdysone-inducible expression vector pINDsp1 (Invitrogen, Carlsbad, CA). The resulting constructs were transfected into EcR293 cells derived from HEK293 cells stably expressing the heterotrimeric ecdysone receptor (VgEcR) and the retinoid X receptor and obtained from the manufacturer (Invitrogen). The cells were selected with G418 (1 mg/ml), and the cells stably expressing the inducible HA-MOR were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml G418, and 100 μg/ml Zeocin under humidified atmosphere at 10% CO_2. Studies were performed 48 h after the addition of various concentrations of the inducing agent ponasterone A (PA).

Radioligand Binding Assays. Opioid receptor binding assays were carried out as described by Law et al. (1983). Protein concentrations were determined by the method of Lowry et al. (1951). Receptor density (B_{max}) and K_d values for [^{3}H]diprenorphine binding were calculated using the Prism program (GraphPad Software Inc., San Diego, CA).

Intracellular cAMP Level. Approximately 4×10^4 cells/well were seeded in 96-well plates 24 h before the assay. After the cells were treated with 1 μM morphine, etorphine, or methadone for the indicated time intervals, the medium was removed and replaced with 100 μl of reaction buffer [0.5 mM 3-isobutyl-1-methylxanthine and 10 μM forskolin in Krebs-Ringer-HEPES buffer (110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.4) with or without agonist or serial dilution of antagonist. After sealing the plates with HotSeal (Diversified Biotech, Boston, MA), the plates were incubated at 37°C for 15 min. Afterward, the plates were placed in a water bath at 85 to 90°C for 5 min so as to lyse the cells and to release the intracellular cAMP. After centrifuging the plates at 500*g* for 2 min, the amount of cAMP in 4 μl of the supernatant was determined with the AlphaScreen cAMP detection kit (BioSignal, Montreal, QC, Canada) according to the manufacturer's instructions and as described previously (Claude-Geppert et al., 2005). Luminescence was measured with the α-Fusion (PerkinElmer Life and Analytical Sciences, Boston, MA)

plate reader. Data are means ± S.E.M. obtained from five to 10 independent experiments, with each drug concentration carried out in quintuplets.

Detergent-Free Preparation of Lipid Rafts/Caveolae and Immunoblotting. The isolation of lipid rafts/caveolae in the current studies was adapted from Li et al. (1996b). At confluence, HEK293 cells stably expressing HA-MOR were exposed to saline or to 1 μM agonist for 4 h. Two milliliters of 500 mM sodium carbonate, pH 11, at 4°C was added, and the cells were detached with a cell scraper. The cells were homogenized with three 10-s bursts of a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY) at the maximum setting, followed by one 30-s burst at setting 4 and one 30-s burst at setting 8 of a sonicator equipped with a microprobe (Heat Systems-Ultrasonics, Inc., Plainview, NY). The homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in modified Barth's solution at pH 6.8 and placed at the bottom of an ultracentrifuge tube. The lysate was then overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose, both prepared in modified Barth's solution containing 250 mM sodium carbonate at pH 11. The discontinuous gradient was centrifuged at 39,000 rpm for 16 to 20 h in a SW41 rotor. One-milliliter fractions were collected, and the total proteins in each fraction were precipitated with the addition of 5% trichloroacetic acid (TCA). The resulting pellets were washed with acetone to remove excess TCA and were resuspended in Laemmli buffer. All the proteins from each fraction were separated on a 10% SDS-polyacrylamide gel. Afterward, the separated proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and the membrane was blocked in a blocking solution of 10% dry milk and 1% Tween 20 in Tris-buffered saline. Western analyses were carried with mouse anti-HA (1:2000), mouse monoclonal anti-caveolin-1 (1:5000), and rabbit anti-adenylyl cyclase V/VI (1:500), respectively. Primary antibody was probed with alkaline phosphatase-conjugated secondary antibodies (1:5000). Proteins bands were detected by the addition of the ECF substrate and fluorescence of the bands determined with Storm 860 (GE Healthcare). Band intensities were quantified and analyzed using ImageQuant (GE Healthcare). To normalize the Western analyses from separate runs, the relative percentage of the proteins in each fraction was obtained by dividing the total pixels from individual gradient fractions by the sum of the pixels from all the fractions.

Fluorescence Flow Cytometry. The MOR located on the plasma membrane was quantified by fluorescence-activated cell sorting (FACS) analysis. In brief, EcR293 cells were treated with 2 μM PA for 48 h. Then, cells were treated with 1 μM morphine, methadone, or etorphine for 4 h so as to induce receptor internalization. Before the addition of antibodies, EcR293 cells were rinsed twice with serum-free DMEM. Then, the cells were incubated at 4°C for 60 min in serum-free DMEM with anti-HA (1:500 dilution) antibody. Afterward, the cells were washed twice with serum-free DMEM and incubated with Alexa488-labeled goat anti-mouse IgG secondary antibody (1:400 dilution) at 4°C for an additional 1 h. The cells were then washed and fixed with 3.7% formaldehyde before quantifying the receptor immunofluorescence with FACS (FACScan; BD Biosciences, Palo Alto, CA). Fluorescence intensity of 10,000 cells was collected for each sample. CellQuest software (BD Biosciences) was used to calculate the mean fluorescence intensity of the cell population. All FACS analyses were conducted three times with triplicate in each experiment.

Materials. Cell culture reagents, including DMEM, MEM, fetal bovine serum, and G418, were purchased from Invitrogen. The ecdysone-inducible mammalian expression system, the EcR293 cells, the synthetic insect hormone PA, and the antibiotic Zeocin were purchased from Invitrogen. Morphine, etorphine, and methadone were supplied by the National Institute on Drug Abuse (Bethesda, MD). The following manufacturers supplied the various antibodies: mouse monoclonal anti-hemagglutinin protein (HA1.1) was from Covance (Richmond, CA); rabbit anti-caveolin-1 was from BD Biosciencies PharMingen (San Diego, CA), and rabbit anti-adenylyl cyclase V/VI was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [^{3}H]Diprenorphine (39 Ci/mmol) was supplied by GE Healthcare. Other chemicals and buffers were purchased from Sigma-Aldrich (St. Louis, MO).

Results

Time-Dependent Receptor Desensitization and Adenylyl Cyclase Superactivation in EcR293 Cells Expressing MOR. In a previous study, we successfully used the ecdysone-inducible mammalian expression system to control DOR expression in EcR293 cells (Law et al., 2000). We could demonstrate that the rate of DOR desensitization was dependent on the receptor level. To examine similar questions on agonist-induced MOR desensitization, we established EcR293 cells in which the MOR expression is regulated by the synthetic insect hormone PA. When the EcR293 cells were treated with various concentrations of PA for 48 h, the amount of MOR expressed on the cell surface was shown to be PA concentration-dependent. In the absence of PA, this EcR293 clone expressed 0.027 ± 0.003 pmol/mg of protein of MOR. Maximal MOR expression of 1.59 ± 0.0.71 pmol/mg of protein was observed with 5 μM PA for 48 h (Fig. 1A). Reducing the PA concentration to 2, 0.5, and 0.2 μM resulted in a MOR expression level of 1.41 ± 0.10, 0.68 ± 0.04, and 0.32 ± 0.013 pmol/mg of protein, respectively. Increasing the PA treatment time to 72 h did not alter the level of MOR being expressed. Thus, for subsequent experiments, EcR293 cells were cultured with PA for 48 h before the initiation of opioid agonist treatment.

When EcR293 cells were cultured with 2 μM PA for 48 h and then exposed to 1 μM morphine for up to 5 h, receptor desensitization and adenylyl cyclase superactivation could be observed (Fig. 1B). After 5 h of morphine treatment, 1 μM morphine maximally inhibited adenylyl cyclase activity by 12 ± 5.3%, which was 19% of the short-term inhibition level (Fig. 1B). In parallel, the adenylyl cyclase superactivation, as measured in the presence of 10 μM naloxone after morphine treatment, reached the maximum of 320 ± 65% above the control level after 4 h of treatment. Therefore, for subsequent adenylyl cyclase superactivation studies, EcR293 cells after culturing in the presence of 2 μM PA, were treated with agonists for 4 h to induce both receptor desensitization and adenylyl cyclase superactivation.

Adenylate Cyclase Superactivation Was Dependent on Receptor Density on the Cell Surface and Not on the Agonist Ability to Induce Receptor Internalization. Previous reports suggest that the ability of agonist to induce MOR endocytosis predicts the maximal level of adenylyl cyclase superactivation. An agonist such as morphine that does not induce rapid receptor internalization exhibits higher level of adenylyl cyclase superactivation than an agonist such as etorphine that induces receptor endocytosis (Finn and Whistler, 2001). However, our previous studies with CHO cells expressing different levels of DOR, and with NG108-15 and N18TG2 cells expressing endogenous DOR, indicated that DOR-mediated adenylyl cyclase superactivation was dependent on receptor densities and independent of agonist (Law et al., 1982, 1994). Whether MOR-mediated adenylyl cyclase superactivation has properties similar to that of DOR is unknown. Hence, in the present experiments,

EcR293 cells were treated with PA concentrations of 0.2, 0.5, and 2 μM for 48 h to induce different MOR level. Afterward, the short- and long-term effects of morphine, etorphine, or methadone were examined. As summarized in Table 1, morphine-, etorphine-, and methadone-mediated inhibitions of forskolin-stimulated intracellular cAMP production in EcR293 cells were dependent on receptor density. With the increase of MOR level from 0.32 to 0.68 pmol/mg of protein after 0.2 and 0.5 μM PA treatment, respectively, both the potencies and maximal inhibition level of these three agonists were observed to increase with an increase in receptor numbers. A further increase in the receptor level after 2 μM PA treatment to 1.4 pmol/mg of protein continued to increase the potencies of the three agonists tested. However, only the maximal inhibition levels of morphine and methadone were increased (Table 1). At 2 μM PA, the maximal inhibition levels observed with these three agonists were not significantly different from each other. Hence, at least with the inhibition of adenylyl cyclase activities, morphine, etorphine, and methadone can be considered to be agonists with equal efficacies at the highest receptor density tested.

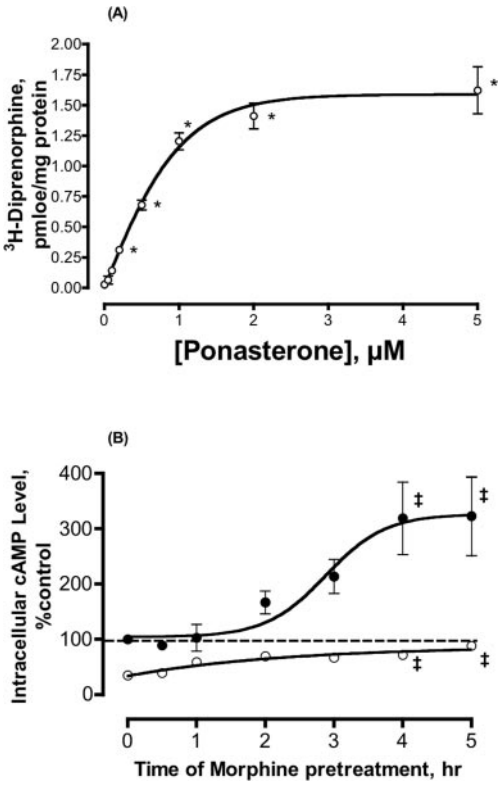

Fig. 1. Ponasterone A concentration-dependent increase in HA-MOR level and subsequent long-term morphine effects in EcR293 cells. In A, EcR293 cells were treated with various concentrations of PA for 48 h. Afterward, whole cell binding assays with 2 nM [^{3}H]diprenorphine were carried out to determine the level of MOR expression. The values represent the averages of triplicate binding assays from three separate 25-mm plates. In B, EcR-293 cells were treated with 2.0 μM PA for 48 h. Cells were pretreated then with vehicle or 1.0 μM morphine for the indicated time. The ability of 1.0 μM morphine to inhibit the 10 μM forskolin-stimulated intracellular cAMP production (○) or the amount of intracellular cAMP produced in the presence of 10 μM forskolin and 10 μM naloxone (●) was determined as described under *Materials and Methods*. The values represent means ± S.E.M. from three separate experiments. *, $p \leq 0.001$ compared with PA = 0 μM control; ‡, $p \leq 0.004$ compared with without morphine treatment.

When the EcR293 cells were cultured with different concentrations of PA and subsequently treated with 1 μM morphine, etorphine, or methadone for 4 h, the adenylyl cyclase superactivation observed in the presence of 10 μM naloxone seemed to be different among the three agonists. As summarized in Fig. 2A, at 2 μM PA, the magnitude of adenylyl cyclase superactivation observed after etorphine treatment was significantly lower than that observed after morphine or methadone pretreatment. Although there was MOR density dependence in the magnitude of superactivation, the differences in the adenylyl cyclase superactivation magnitudes between etorphine and morphine or methadone were observed at all three receptor levels (Table 2). The difference in the adenylyl cyclase superactivation magnitude between etorphine and morphine seemed to correlate with the abilities of these agonists to induce MOR internalization. As shown in Fig. 2B, 4 h of etorphine exposure resulted in a 51 ± 2.8% decrease of MOR cell surface level, whereas 4 h of morphine treatment only elicited a 7 ± 1.4% reduction in the receptor level. Although methadone caused a 26 ± 3.9% decrease in the cell surface receptor, the magnitude of adenylyl cyclase superactivation after long-term methadone exposure was similar to that observed with morphine (Fig. 2; Table 1). These observations contradict the RAVE hypothesis, which predicts a lower magnitude of adenylyl cyclase superactivation for methadone as was reported by Whistler and coworkers (Whistler et al., 1999; Finn and Whistler, 2001). A noticeable difference between methadone and morphine is the naloxone concentration needed to induce 50% (EC_{50}) maximal adenylyl cyclase superactivation. At 2 μM PA, the naloxone EC_{50} value to induce morphine-mediated adenylyl cyclase superactivation was determined to be 49 ± 1.5 nM, whereas the naloxone EC_{50} value to induce methadone-mediated adenylyl cyclase superactivation was 311 ±

TABLE 1

Effect of opioid receptor density on the inhibition of forskolin-stimulated intracellular cAMP production

EcR293 cells stably expressing HA-MOR were cultured in the presence of 0.2, 0.5, and 2.0 μM PA for 48 h in 96-well plates. Then, the abilities of various concentrations of morphine, etorphine, and methadone to inhibit 10 μM forskolin-stimulated intracellular cAMP production were determined as described under *Materials and Methods*. The concentrations of agonists to elicit half-maximal inhibition were determined using the GraphPad Prism 4 curve-fitting program. The values represent means ± S.E.M. of three independent concentration-dependent curves carried out in triplicates. The values in parentheses are the calculated maximal inhibition level (as percentages) achieved by these agonists in various receptor levels induced by different PA concentrations.

[Ponasterone]	IC_{50}		
	Morphine	Etorphine	Methadone
		nM	
0.2 μM	42 ± 3.9$^{\#}$ (39 ± 3.6)	5.9 ± 1.2* (52 ± 3.6)	44 ± 4.5 (56 ± 3.1)
0.5 μM	28 ± 5.5$^{\#}$ (49 ± 2.2)	5.2 ± 1.5‡ (60 ± 2.5)	24 ± 2.9 (55 ± 1.6)
2.0 μM	19 ± 2.7 (70 ± 7.9)	1.0 ± 0* (64 ± 5.4)	17 ± 2.0 (67 ± 0.5)

* $p \leq 0.001$ and $^{‡}p \leq 0.01$ when IC_{50} values of etorphine were compared with those of morphine or methadone using ANOVA.
$^{\#}$ $p \leq 0.03$ when the morphine maximal inhibition level was compared with that of etorphine or methadone using ANOVA.

30 nM. At the lower concentrations of naloxone, there were dramatic differences in the magnitudes of adenylyl cyclase superactivation in EcR293 cells treated with morphine or with methadone (Fig. 2A).

Because methadone could induce MOR internalization and morphine could not, the similarity between the magnitude of adenylyl cyclase superactivation after long-term morphine and methadone treatment suggested that ability of the ligand to induce MOR endocytosis could not be the basis for superactivation. The dissociation between receptor internalization and magnitude of adenylyl cyclase superactivation could be demonstrated further by the use of the dominant-negative Dynamin mutant (DynK44E). DynK44E has been used to block the clathrin-coated pit-mediated internalization of GPCR (Zhang et al., 1996). A similar mutant has been used to impede opioid agonist-induced endocytosis of MOR, DOR, and κ-opioid receptor (Chu et al., 1997; Li et al., 1999; Whistler and von Zastrow, 1999). When DynK44E was transfected to HEK293 cells expressing HA-MOR, after 4 h of 1 μM etorphine pretreatment, 92 ± 6.6% of MOR remained at the cell surface as determined by FACS analysis (Fig. 3A). In contrast, in cells transfected with wild-type Dynamin, the same etorphine treatment internalized 40 ± 2.8% of the receptor. As expected, morphine did not induce MOR internalization in cells transfected with either Dynamin or DynK44E.

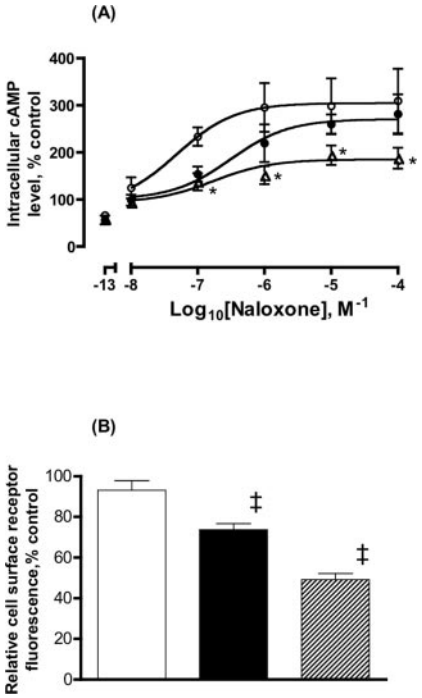

Fig. 2. Agonist-dependent (A) adenylyl cyclase superactivation (A) and receptor internalization (B) in EcR293 cells. In A, the cells were treated either with vehicle or with 1 μM morphine (○), 1 μM methadone (●), and 1 μM etorphine (△) for 4 h. Afterward, the medium was aspirated, and the cells were washed three times before determining the amount of intracellular cAMP produced in the presence of 10 μM forskolin and various concentrations of naloxone as described under *Materials and Methods*. The values represent means ± S.E.M. from three independent experiments performed in triplicate. In B, the percentage of receptor remained on the cell surface after 4 h of 1 μM morphine (□), 1 μM methadone (■), or 1 μM etorphine (▨) treatment was determined by FACS analysis as described under *Materials and Methods*. The values represent means ± S.E.M. from three separate determinations. *, $p \leq 0.03$ when morphine-treated cells were compared with those treated with etorphine using one-way analysis of variance (ANOVA). ‡, $p \leq 0.02$ when etorphine- or methadone-treated cells were compared with the morphine-treated cells using unpaired *t* test.

When the magnitude of adenylyl cyclase superactivation was measured in cells transfected with wild-type Dynamin, to our surprise, there was no difference in the magnitudes observed in cells treated with etorphine or with morphine for 4 h (Fig. 3B). In the presence of 10 μM naloxone, the adenylyl cyclase activities were determined to be 183 ± 8.9 and 176 ± 7.6% above control level in cells treated with morphine and etorphine, respectively. It is noteworthy that blockade of receptor internalization with DynK44E did not alter the magnitude of adenylyl cyclase superactivation in cells treated with morphine or etorphine. The magnitudes remained 192 ± 4.0 and 184 ± 4.8% above the control level in cells treated with morphine and etorphine, respectively (Fig. 3B). Because the RAVE theory predicts that the absence of receptor internalization should result in a higher magnitude of superactivation, the absence of adenylyl cyclase superactivation increase after blockade of MOR endocytosis by DynK44E clearly could not explain the difference between etorphine and morphine by the RAVE theory.

One possible explanation in the dramatic difference between the adenylyl cyclase superactivation magnitudes in cells treated with morphine and etorphine could be the difference in accessibility of the agonist-receptor complexes to the antagonist naloxone. There is no doubt the MOR population responsible for adenylyl cyclase superactivation is lo-

TABLE 2

Effect of opioid receptor density on the adenylyl cyclase superactivation level after chronic agonist treatment

EcR293 cells stably expressing HA-MOR were cultured in the presence of 0.2, 0.5, and 2.0 μM PA for 48 h in 96-well plates. Then, the cells were treated with either 1 μM morphine, etorphine, or methadone for 4 h. Then, the abilities of various concentrations of naloxone to induce adenylyl cyclase superactivation were determined as described under *Materials and Methods*. The concentrations of antagonists to elicit half-maximal superactivation and the maximal superactivation level were determined using the GraphPad Prism 4 curve-fitting program. The values represent means ± S.E.M. of three independent concentration-dependent curves carried out in triplicates. The values in parentheses are the calculated EC_{50} values of naloxone to elicit half-maximal superactivation in various receptor levels induced by different concentrations of PA.

[Ponasterone]	Maximal Superactivation Level		
	Morphine	Etorphine	Methadone
		%	
0.2 μM	149 ± 3.8 (41 ± 14)	109 ± 3.0* (930 ± 240)#	151 ± 4.5 (51 ± 6.9)
0.5 μM	262 ± 7.3 (3900 ± 690)	161 ± 7.5‡ (7400 ± 1900)	253 ± 4.7 (5030 ± 453)
2.0 μM	305 ± 2.3 (49 ± 1.5)†	185 ± 6.7* (175 ± 35)	271 ± 5.3 (311 ± 30)

* $p \leq 0.001$ and ‡ $p \leq 0.01$ when the maximal adenylyl cyclase superactivation levels in cells treated with etorphine were compared with those in cells treated with morphine and methadone using ANOVA.

$p \leq 0.006$ when the EC_{50} value of naloxone to elicit adenylyl cyclase superactivation in etorphine-treated cells was compared with that of morphine and methadone.

† $p \leq 0.001$ when the EC_{50} value of naloxone to elicit adenylyl cyclase superactivation in cells treated with morphine was compared with that in cells treated with etorphine or methadone using ANOVA.

cated at the cell surface. The use of a cell-impermeable antagonist, naloxone methiodide, to induce morphine-mediated or etorphine-mediated adenylyl cyclase superactivation resulted in similar magnitudes when naloxone was used to induce the superactivation (Fig. 4). It is noteworthy that when the corresponding antagonist for etorphine, diprenorphine, was used to induce adenylyl cyclase superactivation, instead of a 188 ± 22% increase in adenylyl cyclase activity in the presence of naloxone after long-term etorphine treatment, a 350 ± 17% increase in adenylyl cyclase activity was observed in the presence of diprenorphine. The magnitude of adenylyl cyclase superactivation in response to etorphine pretreatment was identical to that when the EcR293 cells were pretreated with morphine or methadone (Fig. 4). Thus, the magnitude of adenylyl cyclase superactivation depends on the ability of opioid antagonist to completely displace the agonist from the receptor and is dependent on the MOR level and not on the ability of the agonist to promote receptor internalization.

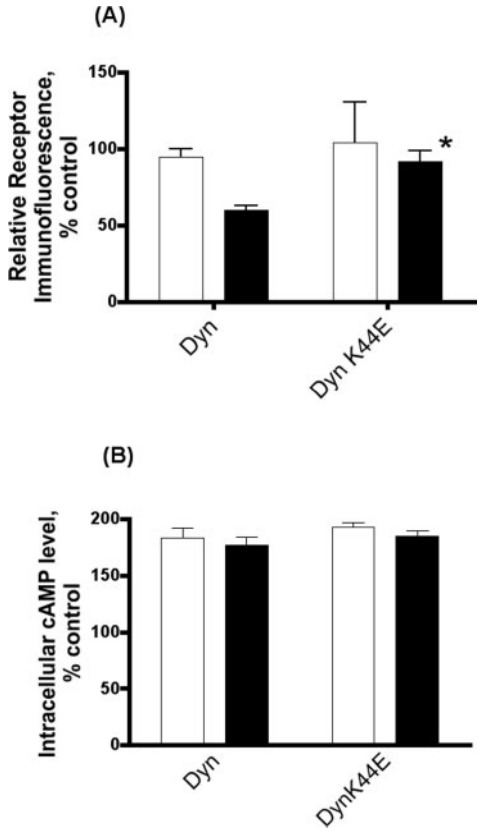

Fig. 3. Effect of dominant-negative Dynamin mutant on receptor internalization (A) and adenylyl cyclase superactivation (B). HEK293 cells cotransfected with HA-MOR and Dynamin or dominant-negative Dynamin mutant (DynK44E). In A, the percentage of receptor remained on the cell surface after 4 h of 1 μM morphine (□) and 1 μM etorphine (■) treatment was determined by FACS analysis as described under *Materials and Methods*. The values represent means ± S.E.M. from three separate determinations. In B, the cells were treated either with vehicle, with 1 μM morphine (□), or with 1 μM etorphine (■) for 4 h. Afterward, the medium was aspirated, and the cells were washed three times before determining the amount of intracellular cAMP produced in the presence of 10 μM forskolin and 10 μM naloxone as described under *Materials and Methods*. The values represent means ± S.E.M. from three independent experiments performed in triplicate. *, $p \leq 0.01$ when the etorphine-treated cells transfected with wild-type dynamin were compared with the cells transfected with dynamin K44E using unpaired *t* test.

Requirement of MOR to Localize in Lipid Rafts/Caveolae Microdomains for the Adenylyl Cyclase Superactivation. The ability of diprenorphine and not naloxone to elicit similar magnitude of adenylyl cyclase superactivation after long-term etorphine and morphine treatment suggests that MOR is sequestered in plasma membrane microdomains that could distinguish the differences in physical properties of these two opioid antagonists. Furthermore, such domains must contain signaling molecules that could transduce the receptor signals in the superactivation of adenylyl cyclase after long-term agonist treatment. Compartmentation of signaling molecules by scaffolding proteins, which place multiple related signaling molecules to specific intracellular sites such as the lipid rafts/caveolae, has been demonstrated within the cAMP-signaling pathway (Schwencke et al., 1999). Colocalization of GPCRs such as β_2-adrenergic receptor within the lipid rafts/caveolae has been reported previously (Ostrom et al., 2001). Thus, a possible explanation for the observed MOR-mediated adenylyl cyclase superactivation is the colocalization of MOR with signaling molecules within the lipid rafts/caveolae domains of the plasma membrane. Hence, caveolin-enriched membrane fractions were obtained from HEK293 cells stably expressing HA-MOR using sucrose density gradient centrifugation. The proteins from the 1-ml fractions of these gradients were separated with SDS-PAGE and the presence of HA-MOR, caveolin-1 as well as adenylyl cyclase V/VI isoenzyme, the enzyme demonstrated to be involved in adenylyl cyclase superactivation (Avidor-Reiss et al., 1997). When the band intensities were quantified and analyzed using ImageQuant software, HA-MOR-enriched membrane could be observed in multiple fractions (Fig. 5A). The averages from multiple Western blots indicated that fractions 4 and 5 contained the highest level of HA-MOR immunoreactivities. These fractions were also those that contained the highest level of caveolin-1 and adenylyl cyclase V/VI immunoreactivity (Fig. 5, D and G). Because caveolin has been used as the marker for lipid rafts/caveolae domains (Lisanti et al., 1994), these data suggest that fractions 4 and 5 must reflect the lipid rafts/caveolae domains of HEK293 cells, and the majority of the HA-MOR and adenylyl cyclase V/VI resided within this domain.

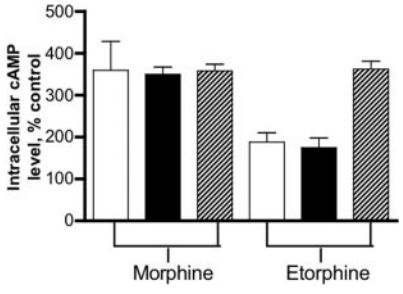

Fig. 4. Antagonist-dependent adenylyl cyclase superactivation after long-term agonist treatment. HEK293 cells stably expressing HA-MOR were pretreated with 1 μM morphine or 1 μM etorphine for 4 h, the medium was aspirated, and the cells were washed three times before determining the amount of intracellular cAMP produced in the presence of 10 μM forskolin and 1 μM naloxone (□), 1 μM naloxone methiodide (■), or 1 μM diprenorphine (▨) as described under *Materials and Methods*. The values represent means ± S.E.M. from three independent experiments performed in triplicate. *, $p \leq 0.002$ compared with diprenorphine-induced AC superactivation in cells treated with etorphine and that induced by naloxone or naloxone methiodide.

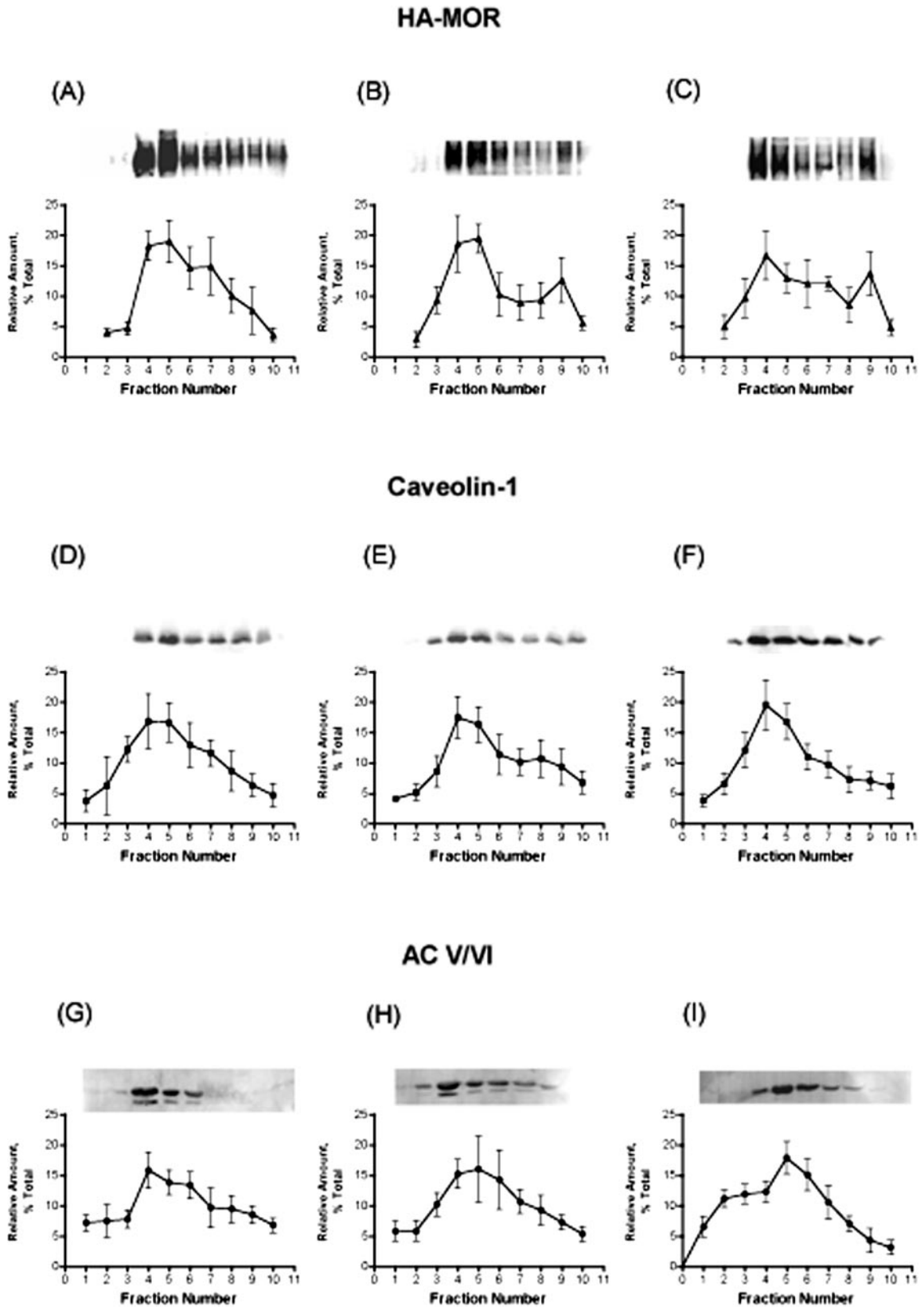

Fig. 5. Colocalization of MOR, caveolin-1, and adenylyl cyclase typeV/VI in sucrose gradients. HEK293 cells stably expressing MOR were pretreated with vehicle (A, D, and G), 1 μM morphine (B, E, and H), or 1 μM etorphine (C, F, and I) for 4 h. Caveolin-enriched membrane fractions were purified on the basis of their selective resistance to sodium carbonate buffer and their buoyancy density in sucrose gradients as described under *Materials and Methods*. Total proteins from the first 10 fractions were precipitated with TCA, and pellets were resuspended in sample treatment buffer after acetone extraction and subjected to SDS-PAGE analysis. The presence of HA-MOR, caveolin-1, and adenylyl cyclase V/VI enzyme was determined by Western analyses using respective antibodies. Top, representative immunoblots of these proteins. Bottom, relative intensity of individual bands quantitated with the ImageQuant software from three to four sucrose gradients.

When the HEK293 cells were exposed to 1 μM morphine or etorphine for 4 h and the localization of HA-MOR was examined, the opioid receptor was determined to localize predominantly at the lipid rafts/caveolae fractions (i.e., fraction 4 and 5) (Fig. 5, B and C), similar to cells not treated with agonist. These were the same fractions that caveolin-1 and adenylyl cyclase V/VI immunoreactivities were the highest. In contrast to the control cells, after morphine or etorphine treatment, a second HA-MOR peak was observed in fraction 9, where the immunoreactivities of caveolin-1 or adenylyl cyclase V/VI did not exhibit a peak (Fig. 5, E, F, H, and I). This second peak could not represent membrane fractions from intracellular compartment, because morphine could not induce MOR endocytosis, whereas etorphine could. The gradient profiles from both agonist treatments exhibited a HA-MOR peak at the same fraction. Because the adenylyl cyclase V/VI immunoreactivity peaked at the lipid rafts/caveolae fractions, the receptors that localized in these microdomains were probably responsible for the adenylyl cyclase superactivation.

To demonstrate the role of lipid rafts/caveolae in the agonist-induced adenylyl cyclase superactivation, HEK293 cells stably expressing HA-MOR were treated with the raft-disrupting agent methyl-β-cyclodextrin (MβCD). By extracting cholesterol within the lipid rafts/caveolae domains and subsequently restoring the cholesterol content within the same domains, the roles of signaling molecules within such microdomains in the GPCR signaling have been demonstrated (Triantafilou et al., 2002; Marwali et al., 2003). When HEK293 cells were treated with 100 μM MβCD for 1 h before the fractionation on sucrose gradient, instead of localizing at fractions 4 and 5 in the gradient, the HA-MOR immunoreactivities peaked at fractions 7 and 8 (Fig. 6A). Maximal caveolin-1 level was observed in similar fractions. Incubation of the cells with cholesterol for 1 h after MβCD treatment relocalized the HA-MOR and caveolin-1 immunoreactivities within fractions 4 and 5 (Fig. 6A). It is clear that these data indicated that HA-MOR localization within the lipid rafts/caveolae microdomains of HEK293 cells could be disrupted with MβCD and restored with cholesterol.

When the ability of agonist to elicit long-term effects after MβCD treatment was examined, we could demonstrate that the localization of HA-MOR within the lipid rafts/caveolae microdomains was an absolute requirement. As shown in Fig. 6B, the disruption of lipid rafts/caveolae domains with MβCD treatment completely blocked the ability of etorphine to induce receptor internalization. Regardless of whether the HEK293 cells were treated with MβCD or not, morphine could not induce MOR internalization. It is noteworthy that when the HEK293 cells were treated with 1 μM morphine for 4 h first to elicit maximal adenylyl cyclase superactivation and then were treated with MβCD for an additional hour in the presence of morphine before the determination of adenylyl cyclase superactivation, adenylyl cyclase superactivation was not observed regardless of the naloxone concentration used (Fig. 6C). This absence of adenylyl cyclase superactivation after MβCD treatment paralleled the decrease of HA-MOR and caveolin-1 content within fractions 4 and 5 of the sucrose gradient. Similar results were observed with MβCD disruption of lipid rafts/caveolae domains in cells subjected to long-term with etorphine. Incubating the cells with cholesterol after MβCD treatment for 1 h resulted in the restoration of the agonist-induced adenylyl cyclase superactivation, correlating with the relocalization of HA-MOR and caveolae-1 to fractions 4 and 5 of the gradient (Fig. 6C). Thus, these data suggested that the adenylyl cyclase superactivation observed after long-term agonist treatment required the localization of MOR within the lipid rafts/caveolae domains and was not dependent on the ability of agonist to induce receptor internalization.

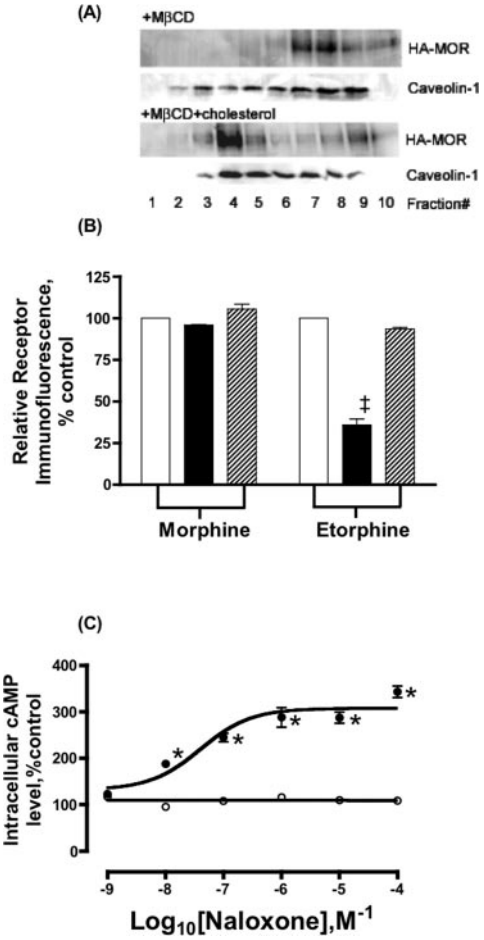

Fig. 6. Effect of MβCD on MOR distribution in lipid rafts/caveolin (A), receptor internalization (B), and adenylyl cyclase superactivation after long-term agonist treatment (C). In A, HEK293 cells stably expressing HA-MOR were treated with 100 μM MβCD for 1 h or treated with 1 mg/ml water-soluble cholesterol for 2 h after MβCD. Then, the lipid rafts/caveolin-enriched membrane fractions were purified on sucrose density gradients as described under *Materials and Methods*. Aliquots from the first 10 fractions were subjected to SDS-PAGE, and the presence of MOR and caveolin-1 were determined by Western analyses using respective antibodies. In B, HEK293 cells stably expressing HA-MOR were pretreated with vehicle (□), 1 μM morphine, or 1 μM etorphine for 4 h, followed by treatment with vehicle (■) or with 100 μM MβCD (▨) for 1 h. Then, the percentage of receptor remaining on the cell surface was determined by FACS analysis as described under *Materials and Methods*. The values represent means ± S.E.M. from three separate determinations. In C, HEK293 cells were treated with 1 μM morphine for 4 h. Then, the cells were treated with 100 μM MβCD treatment for 1 h in the presence of morphine. Afterward, the HEK293 cells were incubated with either 1 mg/ml water-soluble cholesterol (●) or with vehicle (○) for 2 h. The amount of intracellular cAMP produced in the presence of 10 μM forskolin and various concentrations of naloxone was measured in these cells as described under *Materials and Methods*. The values represent means ± S.E.M. from three independent experiments performed in triplicate. ‡, $p \leq 0.001$ when receptor levels after etorphine treatment in control cells were compared with that in MβCD-treated cells. *, $p \leq 0.002$ when the AC superactivation levels in MβCD-treated cells were compared with that in MβCD/cholesterol-treated cells.

Discussion

Many hypotheses regarding the mechanism of adenylyl cyclase superactivation after long-term opioid agonist treatment have been proposed. Different laboratories have suggested the coupling of opioid receptor to G_s proteins (Crain and Shen, 1996; Ammer and Schultz, 1997, 1998; Tso and Wong, 2001), the involvement of G$\beta\gamma$ subunits in activating the adenylyl cyclase (Avidor-Reiss et al., 1996), increase in the receptor constitutive activities (Chavkin et al., 2001; Liu et al., 2001), or the phosphorylation of the adenylyl cyclase (Chakrabarti et al., 1998b, 2001) as the mechanism for the observed adenylyl cyclase superactivation. One hypothesis among the many that have gained recent attention is the RAVE theory proposed by Whistler and von Zastrow (Whistler et al., 1999; Alvarez et al., 2001). In their theory, the degree of opioid tolerance and dependence is proportional to the ability of the agonist to internalize the receptor. Agonists that could induce rapid receptor internalization will have lower magnitude of receptor desensitization and adenylyl cyclase superactivation. The RAVE theory is based mainly on the different magnitudes of adenylyl cyclase superactivation between etorphine or methadone and morphine and on the ability of etorphine or methadone to induce MOR internalization, whereas morphine cannot (Whistler and von Zastrow, 1999). Such observed differences and the proposed RAVE created a "methadone paradox" (Kieffer and Evans, 2002). Although methadone could promote receptor internalization and morphine could not (Fig. 2B), clinically methadone and morphine are equally addictive (Kreek, 2000). Furthermore, morphine could induce MOR to internalize in cultured striatal neurons and in the nucleus accumbens dendrites but not the cell bodies of nucleus accumbens neurons (Haberstock-Debic et al., 2003, 2005). This raises the question of whether agonist-induced receptor internalization or the RAVE theory has any role in the drug addiction processes.

In our current studies, there is no difference in the adenylyl cyclase superactivation magnitude in cells treated with methadone or with morphine (Fig. 2; Table 2). The apparent difference between our studies and those reported could stem from the amount of residual agonist remaining at the receptor during the adenylyl cyclase assays. In our studies, a higher concentration of naloxone was needed to elicit similar adenylyl cyclase superactivation responses in cells treated with methadone compared with those treated with morphine. Thus, in situations when adenylyl cyclase activity was measured after repeated washings to remove bound agonist as in the reported study (Whistler et al., 1999), the adenylyl cyclase superactivation magnitude could be masked by residual agonist present.

The basis for the proposed RAVE theory is the differential ability of various agonists to induce receptor internalization. There are unequivocal results suggesting that etorphine, and not morphine, induced rapid MOR internalization (Keith et al., 1996, 1998; Whistler et al., 1999). Because the magnitude is dependent on receptor density (Table 2), it is not surprising that the magnitude of adenylyl cyclase superactivation after etorphine pretreatment was ~50% of that observed after morphine pretreatment, considering that etorphine could induce receptor internalization but morphine could not. However, concluding from our current data, the correlation between receptor internalization and adenylyl cyclase superactivation magnitude does not exist. Our conclusion is based on two observations: 1) Although methadone could induce receptor internalization but morphine could not, the adenylyl cyclase superactivation magnitudes induced by these two agonists were similar in all three different MOR levels (Fig. 2; Table 2). 2) The adenylyl cyclase superactivation magnitude after morphine or etorphine pretreatment was not altered when the agonist-induced receptor endocytosis was blunted with the dominant-negative Dynamin mutant K44E (Fig. 3). If the receptor endocytosis could affect the magnitude because of resensitizing the receptor, then by preventing the etorphine-induced receptor endocytosis with DynK44E, adenylyl cyclase superactivation magnitude should be increased, which was not observed. It is noteworthy that in the cells overexpressing either the wild-type or the dominant-negative mutant of Dynamin, adenylyl cyclase superactivation magnitude after long-term morphine treatment was similar to those observed after etorphine treatment. Although the dynamin role in adenylyl cyclase superactivation has yet to be determined, it is clear that under such conditions, the putative RAVE index among opioid agonists disappears. As discussed below, the lipid rafts/caveolae-located MOR is critical for the observed adenylyl cyclase superactivation. Because DynK44E has been known to block the lipid rafts/caveolae located proteins' endocytosis (Nabi and Le, 2003), it is tempting to postulate that the observed effects of Dynamin and Dyn K44E on adenylyl cyclase superactivation are caused by the trafficking of the proteins within the lipid rafts/caveolae domain.

The absence of any RAVE index among opioid agonists could be demonstrated further by our studies with different opioid antagonists. The magnitudes of adenylyl cyclase superactivation after etorphine and morphine pretreatment were similar if a hydrophobic high-affinity antagonist, diprenorphine, was used (Fig. 4). Unlike the studies in which Dynamin was overexpressed, etorphine remained active in promoting MOR internalization, whereas morphine could not. The similarity between the adenylyl cyclase superactivation magnitudes suggests that MOR must be more accessible to diprenorphine than to naloxone after etorphine pretreatment. The inability to wash away etorphine completely or to compete for the etorphine completely with naloxone could account for the observed difference in adenylyl cyclase superactivation magnitudes observed. By fractionating the HEK293 cells in a discontinuous sucrose gradient, we did not observe the difference in cellular location of MOR before and after agonist treatment, whether the agonist was etorphine or morphine. It is noteworthy that most if not all of the MOR immunoreactivity was observed in fractions that were enriched in caveolin-1, the major protein component of lipid rafts/caveolae in HEK293 cells (Sotgia et al., 2002). Although morphine and etorphine exhibited differential action in promoting MOR endocytosis, a second peak at fraction 9 was observed in cells treated by these two agonists. The location of MOR in this peak, therefore, could not reflect the internalized receptor. Because fractionations were carried out with cells treated with agonist for >4 h, the translocation of the internalized receptor to lysosome for degradation would eliminate the appearance of secondary receptor representing the internalized receptor. As demonstrated with the MβCD studies, MOR localized at fractions 4 and 5 of the sucrose

gradient was responsible for the observed adenylyl cyclase superactivation. Hence, the difference in observed adenylyl cyclase superactivation magnitudes after etorphine and morphine pretreatment in the presence of naloxone might reflect the varied distribution of the agonist-receptor complexes within the microdomains lipid rafts/caveolae.

Lipid rafts/caveolae were first defined in the 1950s as cholesterol-rich Triton X-100-insoluble domains within the plasma membrane. It has been identified as a key microdomain that concentrates signaling components, particularly the cAMP signaling pathway (Schwencke et al., 1999). For example, adenylyl cyclase V was identified to colocalize with β-adrenergic receptor in caveolin-enriched membrane fractions in cardiomyocytes (Ostrom et al., 2001). The activation of ErK1/2 by β_2-adrenergic receptor required the interaction of Src kinase, which is also enriched in lipid rafts/caveolae domains, facilitated by the G$\beta\gamma$ subunits with the β-arrestin molecule (Luttrell et al., 1996, 1997, 1999). The phosphorylation of dynamin by Src regulated the agonist-induced receptor internalization (Ahn et al., 2002). DynK44E has been known to block endocytosis of proteins within lipid rafts/caveolae (Nabi and Le, 2003). Thus, the recruitment and the modification of the signaling molecules within the lipid rafts/caveolae domains could modify the eventual signaling of the GPCRs. In our current study, adenylyl cyclase V/VI and MOR are shown to colocalize in the caveolin-1 enriched fractions. Adenylyl cyclase V is one of the adenylyl cyclase isoforms that has been demonstrated to be involved in opioid agonist-induced superactivation (Avidor-Reiss et al., 1996, 1997). In both the guinea pig ileum myenteric plexus and CHO cells, long-term agonist treatment resulted in the phosphorylation of adenylyl cyclase molecules (Chakrabarti et al., 1998b). In CHO cells stably expressing the human DOR, tyrosine kinase, PKC, and raf-1 were reported to be involved in phosphorylation of adenylyl cyclase V/VI and resulted in the increase of cAMP accumulation (Varga et al., 1999, 2003a,b). These data and others suggest that the colocalization of MOR and other signaling molecules such as protein kinases could be responsible for the eventual adenylyl cyclase superactivation. Our current studies indicate that MOR is localized within the lipid rafts/caveolae microdomains and the disruption of the microdomains could completely blunt the adenylyl cyclase superactivation response. The disruption of lipid rafts/caveolae microdomains has affected the signaling of many GPCRs (Xue et al., 2004; Bari et al., 2005; Jiao et al., 2005; Monastyrskaya et al., 2005; Nguyen et al., 2005; Quinton et al., 2005). Thus, the blunting of adenylyl cyclase superaction after long-term agonist treatment could be caused by the attenuation of initial MOR activation. However, this could not be the case because in our current treatment paradigm, MβCD did not disrupt lipid rafts/caveolae microdomains until after long-term agonist treatment. Besides, the MβCD effect could be reversed by cholesterol, restoring the maximal adenylyl cyclase superactivation level. These studies indicate that localization of MOR within the lipid rafts/caveolae is the key for adenylyl cyclase superactivation.

If lipid rafts/caveolae location is the key, then a putative model for adenylyl cyclase superactivation can then be proposed to resolve the dichotomy of adenylyl cyclase superactivation. The mechanism for opioid receptor desensitization is analogous to that of other GPCRs within the rhodopsin subfamily. Involvement of agonist-induced receptor phosphorylation and the recruitment of β-arrestin molecules in MOR desensitization have been well documented (Whistler and von Zastrow, 1998; Bohn et al., 2000). Although morphine could not induce MOR internalization, using the fibroblasts from the β-arrestin knockout mice, Bohn et al. (2004) could demonstrate the association of the receptor with the β-arrestin molecules. Thus, the association of β-arrestin with the opioid receptor during long-term agonist treatment would terminate the receptor signaling. At the same time, β-arrestin could function as a scaffolding molecule. Recruitment and activation of protein kinases such as Src kinase by β_2-adrenergic receptor is dependent on β-arrestin (Luttrell et al., 1999). The activated Src kinase could directly phosphorylate the adenylyl cyclase molecule or could recruit in lipid rafts/caveolae a series of newly tyrosine-phosphorylated substrates (Lee et al., 2002), resulting in adenylyl cyclase superactivation. The recruited molecules and the dynamic interactions of these proteins regulated by lipid rafts/caveolae domains could result in the alteration in the equilibria of protein phosphorylation and dephosphorylation. Such equilibrium dynamics could manifest in the putative increase in the "constitutive" opioid receptor activities reported after long-term agonist treatment. In our current model, the recruitment of β-arrestin molecule by agonist-activated receptor will blunt the receptor signal, leading to receptor desensitization. β-Arrestin leads to the association of Src kinase with the agonist-receptor complex that results in the eventual increase in the adenylyl cyclase activation or a "switch" in opioid receptor signaling when the original signal has been turned off. The details of such a model require future investigation in the roles of β-arrestin and Src kinase in MOR activation that lead to adenylyl cyclase superactivation. If the roles of β-arrestin and Src kinase could be established, by altering the Src kinase content and/or activities, the significance of the in vitro adenylyl cyclase superactivation on the in vivo responses to long-term MOR activation could then be examined.

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