Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Membrane Microviscosity Modulates μ-Opioid Receptor Conformational Transitions and Agonist Efficacy

*Paul J. Emmerson, *Mary J. Clark, *†Fedor Medzihradsky, and *Ann E. Remmers

*Departments of *Pharmacology and †Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.*

Abstract: The influence of membrane microviscosity on μ-opioid agonist and antagonist binding, as well as agonist efficacy, was examined in membranes prepared from SH-SY5Y cells and from a C6 glioma cell line stably expressing the rat μ-opioid receptor (C6μ). Addition of cholesteryl hemisuccinate (CHS) to cell membranes increased membrane microviscosity and reduced the inhibitory effect of sodium and guanine nucleotides on the affinity of the full agonists sufentanil and [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) for the μ-opioid receptor. Binding of the antagonists [^{3}H]naltrexone and [^{3}H]diprenorphine and the partial agonist nalbuphine was unaffected by CHS. The effect of CHS on agonist binding was reversed by subsequent addition of *cis*-vaccenic acid, suggesting that the effect of CHS is the result of increased membrane microviscosity and not a specific sterol–receptor interaction. CHS addition increased the potency of DAMGO to stimulate guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding by fourfold, whereas the potency of nalbuphine was unaffected. However, nalbuphine efficacy relative to that of the full agonist DAMGO was strongly increased in CHS-treated membranes compared with that in control membranes. Membrane rigidification also resulted in an increased efficacy for the partial agonists meperidine, profadol, and butorphanol relative to that of DAMGO as measured by agonist-stimulated GTPase activity in control and CHS-modified membranes. These findings support a regulatory role for membrane microviscosity in receptor-mediated G protein activation. **Key Words:** Opioid receptor—Opioid agonists—Efficacy—Membrane fluidity—Cholesterol.
J. Neurochem. **73,** 289–300 (1999).

Alterations in membrane lipid composition and biophysical properties may provide potential mechanisms by which a cell can regulate the function of integral membrane proteins. Protein function may be regulated by the direct binding of phospholipid, ionic headgroups, and hydrophobic acyl chains or through binding of cholesterol to the transmembrane domains (Fong and McNamee, 1987; Selinsky, 1992). Changes in lipid composition may also regulate protein function and protein–protein interactions through effects on membrane biophysical properties such as microviscosity, thus potentially affecting protein lateral diffusion or protein conformational changes (Stubbs and Smith, 1984; Yeagle, 1991; Selinsky, 1992). For example, the function of receptors coupled to adenylyl cyclase and possibly the enzyme itself are sensitive to alterations in membrane fluidity (Hanski et al., 1979; Depauw et al., 1990).

Determination of the lipid requirements for reconstitution of functional opioid (Hasegawa et al., 1987; Scheideler and Zukin, 1990), β-adrenergic (Ben-Arie et al., 1988), and muscarinic acetylcholine (Berstein et al., 1989) receptors illustrates the importance of specific interactions between these receptors and the lipid bilayer (Fong and McNamee, 1987). Also, the influence of membrane biophysical properties, e.g., lipid packing and mobility, on opioid receptor binding (Heron et al., 1981; Remmers et al., 1990; Lazar and Medzihradsky, 1992) and metarhodopsin conformational changes (Mitchell et al., 1992, 1996) illustrates the role of the membrane bilayer as a regulator of protein flexibility. Alterations in membrane microviscosity or composition have also been shown to modulate opioid receptor-mediated signal transduction (Abood et al., 1978; Law et al., 1983; Lazar and Medzihradsky, 1989).

Ligand binding affinity for the opioid receptor is an indicator of receptor conformation. The binding of opioid ligands is modulated by addition of sodium and guanine nucleotides (Blume, 1978; Ott and Costa, 1988). High-affinity binding of agonists to the receptor requires the association of receptor with G protein (Remmers and Medzihradsky, 1991*a*), and addition of sodium or gua-

Resubmitted manuscript received January 13, 1999; accepted February 11, 1999.

Address correspondence and reprint requests to Dr. A. E. Remmers at her present address: CNS Clinical Research, Parke-Davis, 2800 Plymouth Road, Ann Arbor, MI 48105, U.S.A.

The present address of Dr. P. J. Emmerson is Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637, U.S.A.

Abbreviations used: C6μ, C6 glioma cell line stably expressing the rat μ-opioid receptor; CHS, cholesteryl hemisuccinate; CTOP, D-Phe-Cys-Tyr-D-Tyr-Orn-Thr-Pen-Thr-NH_2; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; DPH, diphenylhexatriene; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; NEM, *N*-ethylmaleimide; PTX, pertussis toxin; TMA-DPH, trimethylammonium-diphenylhexatriene.

nine nucleotides dissociates this complex to convert the receptor to a conformation with low affinity for agonist (Blume, 1978; Childers and Snyder, 1980; Ott and Costa, 1988). In contrast, antagonist binding is increased by sodium and unchanged by further addition of guanine nucleotides, indicating an insensitivity to the presence of G protein (Simantov et al., 1976; Childers and Snyder, 1980; Nijssen and Childers, 1987). We have shown that rigidification of rat brain membranes reduced the effect of sodium on both opioid agonist and antagonist binding, suggesting that membrane biophysical properties can alter the sodium-induced conformational transitions of the μ-opioid receptor (Lazar and Medzihradsky, 1993). However, these experiments did not address receptor-mediated G protein activation in membranes with reduced fluidity.

In the current study, we have examined the effect of membrane rigidification on μ-opioid agonist binding and G protein activation in membranes from the SH-SY5Y neuroblastoma cell line and from a C6 glioma cell line stably expressing the rat μ-opioid receptor (C6μ) (Emmerson et al., 1996). Although the effects of temperature and GDP on agonist efficacy have been evaluated (Lorenzen et al., 1996; Breivogel et al., 1998), to our knowledge the potential modulatory role of membrane fluidity on agonist efficacy has never been evaluated. The results presented suggest that membrane microviscosity can modulate specific agonist-sensitive conformational changes in the absence of any effect on antagonist binding. Membrane microviscosity also modulates the efficacy of opioid agonists, further supporting a regulatory role for the lipid bilayer in opioid agonist binding and signal transduction. A preliminary report of this study was presented previously (Emmerson et al., 1994).

MATERIALS AND METHODS

Materials

[^{3}H]Sufentanil was purchased from Research Diagnostics (Flanders, NJ, U.S.A.). The μ-opioid antagonist [^{3}H]D-Phe-Cys-Tyr-D-Tyr-Orn-Thr-Pen-Thr-NH_2 ([^{3}H]CTOP) and the agonist [D-Ala^2,*N*-MePhe^4,Gly-ol^5][*Tyr*-3,5-^{3}H]enkephalin ([^{3}H]DAMGO) were from Amersham Co. (Arlington Heights, IL, U.S.A.), and [^{3}H]naltrexone and [^{3}H]diprenorphine were obtained from the National Institute on Drug Abuse. [γ-^{32}P]GTP and guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) were obtained from Du Pont-NEN (Wilmington, DE, U.S.A.). DAMGO, cholesteryl hemisuccinate (CHS), Dulbecco's modified Eagle's medium, GTPγS, and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other unlabeled opioids were supplied by the Narcotic Drug and Opiate Peptide Basic Research Center at the University of Michigan. Pertussis toxin (PTX) was purchased from List Biochemicals (Campbell, CA, U.S.A.). Fetal bovine serum was from GIBCO Life Sciences (Gaithersburg, MD, U.S.A.). *cis*-Vaccenic acid was purchased from Serdary Research Laboratories (London, Ontario, Canada). Diphenylhexatriene (DPH) and trimethylammonium-DPH (TMA-DPH) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Human neuroblastoma SH-SY5Y cells, originating from Dr. June L. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.), were a generous gift from Dr. S. K. Fisher (Department of Pharmacology, University of Michigan).

Cell culture

SH-SY5Y cells (passages 76–100) were grown for 8–10 days in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in tissue culture flasks under a 10% CO_2 atmosphere at 37°C. Medium was partially replaced every third day. PTX treatment was carried out by incubating the cells with 50 ng/ml for 24 h at 37°C in culture flasks. C6μ cells (Emmerson et al., 1996) were grown under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 mg/ml geneticin.

Membrane preparation

Cells were washed three times with ice cold phosphate-buffered saline (0.9% NaCl, 0.61 m*M* Na_2HPO_4, and 0.38 m*M* KH_2PO_4, pH 7.4) and incubated with modified Ca^{2+}/Mg^{2+}-free Pucks buffer to dissociate the cells (Carter and Medzihradsky, 1992). The cell suspension was pelleted by centrifugation for 3 min at 300 *g*. Nuclei were removed by resuspending the cells in 10 volumes of ice-cold hypotonic phosphate buffer (0.61 m*M* Na_2HPO_4, 0.38 m*M* KH_2PO_4, and 0.2 m*M* $MgSO_4$, pH 7.4 at 4°C) using a Dounce all-glass tissue homogenizer followed by centrifugation for 10 min at 500 *g* and 4°C. Membranes were collected by centrifuging the supernatant for 20 min at 20,000 *g* and 4°C and resuspended in Tris buffer (50 m*M*, pH 7.4). Membranes from C6μ cells were prepared by lysis in 0.32 *M* sucrose as previously described (Emmerson et al., 1996). All membranes were suspended in Tris buffer, and aliquots at 2 mg/ml were frozen at −80°C until used.

NEM treatment of membranes

Alkylation of G protein by NEM was accomplished under conditions that protect the activity of the μ-opioid receptor as described earlier (Tocque et al., 1987). Membranes (1–2 mg of protein/ml) were incubated in Tris buffer for 30 min at 25°C with 50 n*M* naltrexone and 3 m*M* NaCl. Subsequently, 5 m*M* NEM was added, and the solution was incubated for an additional 2 h. The reaction was terminated by addition of an equal volume of ice-cold Tris buffer and centrifugation for 15 min at 20,000 *g*. Membranes were washed by resuspension in Tris buffer and centrifugation. To remove residual naltrexone and NaCl, the pellet was resuspended in Tris buffer and incubated for 30 min at 25°C followed by two additional washes of the membranes. The final pellet was resuspended in Tris buffer, and individual aliquots were frozen at −80°C. Inactivation of the μ-opioid receptor was determined by comparison of the loss in specific opioid agonist and antagonist binding.

CHS and *cis*-vaccenic modification of membranes

CHS and *cis*-vaccenic acid were added as a solution in ethanol to membranes suspended in Tris buffer (25°C, 50 m*M*, pH 7.4) with vigorous mixing. Control membranes were incubated with a corresponding volume of ethanol. After incubation for 30 min at 25°C, the mixture was centrifuged for 15 min at 20,000 *g* and 4°C, and the pellet was resuspended in Tris buffer using a Dounce homogenizer. CHS incorporation was determined following lysis of the succinate ester by incubation of cell membranes and standards with 2.5 *M* KOH for 30 min at 25°C. After neutralization with 2.5 *M* HCl, total cholesterol content was determined by an enzymatic-fluorometric procedure (Heider and Boyett, 1978). Recovery of CHS was >95%.

Measurement of membrane microviscosity

The fluorescent probe DPH or its cationic derivative TMA-DPH (0.3 μM) was incubated for 45 min at 25°C with membranes (50–100 μg of protein). Fluorescence anisotropy was measured with a Spex FluoroMax spectrofluorometer using excitation and emission wavelengths of 340 and 450 nm, respectively. The results were expressed as anisotropy values (r), where $r = (I_0 - I_{90})/(I_0 - 2I_{90})$ and I_0 and I_{90} represent the intensities of light when light polarizing filters were in a parallel and perpendicular orientation, respectively.

Quantification of protein

Membranes were initially digested with 1 *M* NaOH for 45 min at 25°C. Protein was then analyzed by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Stimulation of low-K_m GTPase activity

Basal and opioid stimulated low-K_m GTPase activities were determined as previously described (Clark et al., 1989) with the following modifications. Membranes were preincubated in the same buffer used for the [^{35}S]GTPγS binding assay [50 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, and 1 m*M* dithiothreitol] for 10 min with 10 μM GDP in the absence or presence of agonist, followed by addition of 1 n*M* [γ-^{32}P]GTP. Following a 10-min incubation at 37°C, the reaction was terminated by addition of a slurry of charcoal and phosphoric acid followed by centrifugation. The free ^{32}P remaining in the supernatant was quantified by liquid scintillation counting.

Stimulation of [^{35}S]GTPγS binding

Agonist stimulation of [^{35}S]GTPγS binding was measured as described previously (Emmerson et al., 1996). C6μ cell membranes (5 μg per tube) were preincubated with ligand for 10 min at 25°C. The experiment was initiated by addition of [^{35}S]GTPγS binding assay buffer to yield a final concentration in 100 μl of 50 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* dithiothreitol, 50 μM GDP, and 50 p*M* [^{35}S]GTPγS. Tubes were incubated for 30 min at 25°C, and the reaction was terminated by diluting the sample with 2 ml of ice-cold Tris buffer containing 5 m*M* $MgCl_2$ and 100 m*M* NaCl and rapidly filtering the tube contents through glass fiber filters (no. 32; Schleicher and Schuell, Keene, NH, U.S.A.). The filters were then washed an additional three times with 2 ml of buffer. Filters were placed in vials containing 0.4 ml of ethanol and 4 ml of scintillation cocktail for liquid scintillation counting. Basal activity was defined by the difference between the [^{35}S]GTPγS binding in the absence or presence of 50 μM GTPγS.

Ligand binding

Equilibrium ligand binding to SH-SY5Y and C6μ membranes was determined as previously described (Carter and Medzihradsky, 1992; Emmerson et al., 1996). In brief, assay tubes containing 15–50 μg of membrane protein in Tris buffer were incubated with NaCl (150 m*M*) or NaCl (150 m*M*) plus GTPγS (1 μM) for 15 min at 25°C. Subsequently, radioligand was added, and the tubes were incubated at 25°C to equilibrium (60 min for [^{3}H]naltrexone and [^{3}H]diprenorphine, 80 min for [^{3}H]DAMGO, and 120 min for [^{3}H]sufentanil). Specific binding of the radioligand was determined in the absence and presence of excess (1 μM) unlabeled ligand. Binding of [^{3}H]-naltrexone and [^{3}H]diprenorphine to δ-opioid receptors in SH-SY5Y membranes was suppressed by inclusion of 50 n*M* oxymorphindole to the assay (Remmers and Medzihradsky, 1991*b*). Samples were filtered through Schleicher and Schuell no. 32 glass fiber filters presoaked for 30 min in 0.05% polyethylenimine (pH 7.4), mounted in a Brandel 24-well cell harvester, and washed four times with ice-cold Tris buffer. Ethanol (0.4 ml) and scintillation fluid (4 ml) were added to the filters, and the samples were subjected to liquid scintillation counting.

Data analysis

Saturation binding data were pooled from three or four experiments and compared with one-site, one-site plus a nonsaturable linear component, and two-site saturation models by nonlinear regression analysis using the NONLIN module of the computer program SYSTAT (Wilkenson, 1988) as described earlier (Carter and Medzihradsky, 1992). The best-fit model was determined using the *F*-ratio test to compare the weighted residual sum of squares with $p < 0.05$. [^{35}S]GTPγS binding curves were fit to sigmoidal curves, and radioligand displacement curves were fit using one- versus two-site competition models using Graph Pad Prism (GraphPad, San Diego, CA, U.S.A.). All *t* tests performed were two-tailed and unpaired.

RESULTS

Modulation of SH-SY5Y membrane microviscosity

The initial results presented here (Fig. 1) confirm the effects of added cholesterol on agonist binding observed in rat brain membranes (Heron et al., 1981; Lazar and Medzihradsky, 1993). CHS rigidification of SH-SY5Y membranes was examined using both nonpolar DPH and cationic TMA-DPH fluorescent membrane probes. The polarized fluorescence of these probes reflects membrane microviscosity at the core and surface of the membrane, respectively. Addition of CHS (0.5–2.75 μmol/mg of membrane protein) increased both DPH and TMA-DPH fluorescence anisotropy (Fig. 1A). CHS addition dramatically increased the microviscosity at the core of the membrane (45%), whereas the increase at the surface was limited (11%). Quantification of the levels of CHS incorporated into the membrane showed that >90% of added CHS was retained in the membrane at 0.45 μmol of added CHS/mg of protein and 80% at 2.4 μmol of added CHS/mg of protein. Addition of CHS increased the total membrane cholesterol content by ~60 and 590% at 0.45 and 2.4 μmol of CHS added/mg of membrane protein, respectively. No change in membrane protein was found on CHS addition (data not shown). To distinguish between the effects of CHS-mediated changes in membrane microviscosity versus specific sterol–protein interactions, the effects of CHS on membrane microviscosity were reversed by addition of a potent fluidizing fatty acid, *cis*-vaccenic acid (Remmers et al., 1990). Following CHS addition (2 μmol/mg of protein), addition of *cis*-vaccenic acid reversed the change in TMA-DPH anisotropy by 95% and that of DPH by 61%, indicating a reversal of the CHS rigidification of the surface and core of the membrane (Fig. 1A, inset).

Opioid binding in control and CHS-modified membranes

Following modification with CHS, the binding of opioid agonists and antagonists was examined. CHS addition selectively modulated the inhibitory effect of so-

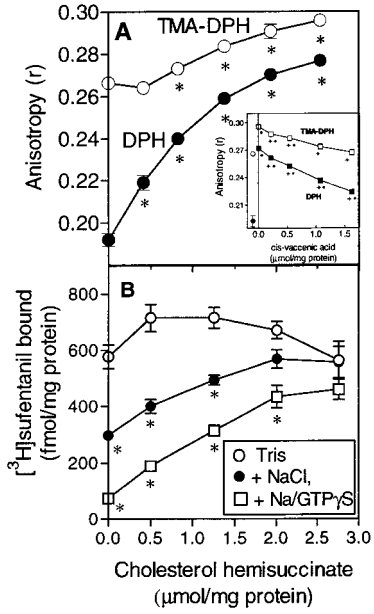

FIG. 1. Modulation of SH-SY5Y membrane microviscosity. **A:** Membranes from SH-SY5Y cells were incubated with increasing concentrations of CHS (circles) or (**inset**) with CHS (2.45 μmol/mg of protein) followed by increasing concentrations of *cis*-vaccenic acid (squares). Following centrifugation, the membranes were labeled with TMA-DPH (open symbols) or DPH (solid symbols) and the anisotropy (*r*) of the samples was determined as described in Materials and Methods. Data are mean ± SEM (bars) values of three experiments each carried out in duplicate. $^*p < 0.01$, significantly different from control membranes. Inset: $^*p < 0.01$, significantly different from + CHS control membranes with no *cis*-vaccenic acid added; $^+p < 0.01$, significantly different from − CHS control membranes indicated in the main portion (circles). **B:** [^{3}H]Sufentanil (0.1 n*M*) binding was evaluated in CHS-modified membranes in Tris-magnesium buffer (○) or in the presence of 150 m*M* NaCl (●) or in the presence of 150 m*M* NaCl and 1 μM GTPγS (□). $^*p < 0.01$, significantly different from control membranes with no CHS added.

dium and guanine nucleotides on agonist binding to the opioid receptor in SH-SY5Y membranes. Addition of CHS did not significantly alter [^{3}H]sufentanil binding in the absence of sodium and GTPγS (Fig. 1B). The most striking effect of CHS was the reduced inhibition of sufentanil binding by sodium and guanine nucleotide. In unmodified membranes, 0.1 n*M* [^{3}H]sufentanil binding was reduced by addition of sodium and to a greater extent by both sodium and GTPγS. Increasing concentrations of CHS strongly reduced the inhibitory effect of sodium and GTPγS. At high concentrations of CHS (2.76 μmol/mg of protein), the reduction of agonist binding by sodium was completely abolished, and that by the combination of sodium and GTPγS was greatly reduced.

In contrast to sufentanil binding, CHS addition slightly decreased DAMGO (1 n*M*) binding in the absence of sodium and also reduced the modulatory effect of sodium (Fig. 2A), although to a lesser extent than for sufentanil binding. This suggests that the effects of CHS on agonist binding may be determined, in part, by some characteristic of the ligand. In contrast to the effect of sodium on antagonist binding in rat brain membranes (Lazar and Medzihradsky, 1993), the binding of the antagonist [^{3}H]naltrexone was insensitive to addition of NaCl in membranes from SH-SY5Y cells (Fig. 2A). Also, addition of CHS had no effect on the binding of naltrexone in the presence of 150 m*M* NaCl and only slightly decreased naltrexone binding in the absence of NaCl (Fig. 2A). Also, at high concentrations of CHS, no loss in antagonist binding or membrane protein content was found. These findings suggest that the effects of membrane rigidification were mediated through agonist-sensitive conformational changes in the receptor and not

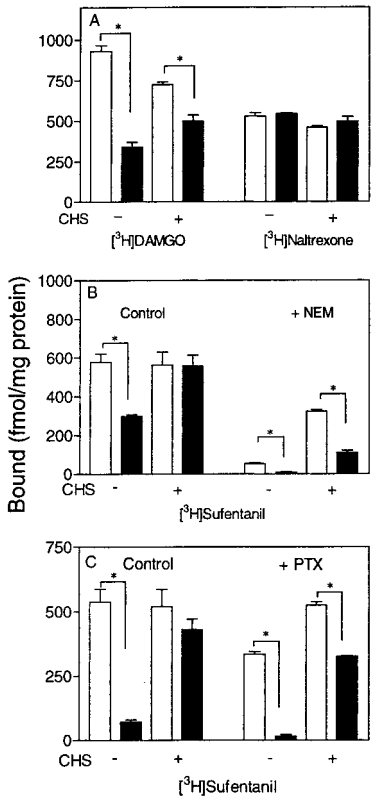

FIG. 2. Opioid agonist and antagonist binding in control and CHS-modified SH-SY5Y membranes. SH-SH5Y membranes were modified with 2.5 μmol of CHS/mg of protein as described in Materials and Methods. **A:** The specific binding of [^{3}H]DAMGO (1.0 n*M*) and [^{3}H]naltrexone (2.0 n*M*) in the absence (open columns) and presence (solid columns) of NaCl (150 m*M*) was measured. **B:** Membranes were pretreated with NEM as described in Materials and Methods followed by CHS addition. [^{3}H]Sufentanil binding (0.1 n*M*) was measured in control (**left**) and alkylated (**right**; + NEM) membranes in the absence (open columns) and presence (solid columns) of 150 m*M* NaCl. **C:** Cells in culture were incubated with PTX (50 ng/ml) for 24 h, membranes were prepared, and CHS was added. [^{3}H]Sufentanil binding (0.1 n*M*) was measured in control (**left**) and toxin-treated (**right**; + PTX) membranes in the absence (open columns) and presence (solid columns) of 150 m*M* NaCl and 10 μM GTPγS. Data are mean ± SEM (bars) values from three experiments, each carried out in duplicate. $^*p < 0.01$, significant differences between the pairwise comparisons indicated by the bars.

through altered accessibility of ligand for the μ receptor ("vertical displacement").

To determine whether CHS rigidification of the membrane alters the conformation of the receptor or alters its interaction with G protein, the effects of CHS addition on agonist binding were examined in membranes where G proteins had been inactivated by the sulfhydryl reactive agent NEM or by PTX pretreatment of cells. Because the μ-opioid receptor contains an NEM-sensitive sulfhydryl, the alkylation reactions were done in the presence of protecting concentrations of sodium and naltrexone (Simon and Groth, 1975; Tocque et al., 1987). Under these conditions, 2 h of NEM treatment reduced antagonist ([^{3}H]naltrexone) binding by $<$20% but decreased agonist ([^{3}H]DAMGO) binding by $>$80%, indicating that NEM treatment uncoupled the μ receptor from G protein (data not shown). In addition, low-K_m GTPase basal activity was reduced by 93%, and this activity was not increased by incubation with agonist (data not shown). Addition of CHS to NEM-treated membranes resulted in a fivefold increase in [^{3}H]sufentanil binding (Fig. 2B). That this increase in binding was the result of a conversion of the receptor to a high-affinity conformation was confirmed by comparison of the dissociation of [^{3}H]sufentanil in NEM-treated membranes and those modified with CHS. Bound [^{3}H]sufentanil in NEM-treated membranes displayed both fast and slow components of dissociation (site 1, $k_{-1} = 0.28 \pm 0.03$ min^{-1}, 52%; site 2, $k_{-1} = 0.016 \pm 0.002$ min^{-1}, 44%), whereas in NEM-treated membranes modified with CHS, [^{3}H]sufentanil dissociation was best fit to a single slow dissociation rate (0.0047 ± 0.0014 min^{-1}). In PTX-treated membranes, CHS addition (2.6 μmol/mg of protein) resulted in increased [^{3}H]sufentanil (58 ± 5%; Fig. 2C) and [^{3}H]DAMGO (61 ± 2%) binding in the absence of sodium and greatly attenuated the inhibitory effects of sodium on agonist binding (data not shown). The greater sensitivity to CHS in NEM-treated membranes when compared with PTX-treated membranes likely results from a lower level of functional G protein in the NEM-treated membranes. This is supported by the greater residual high-affinity [^{3}H]sufentanil binding in the PTX-treated membranes (334 ± 11 fmol/mg of protein) compared with the NEM-treated membranes (54 ± 6 fmol/mg of protein).

Although the binding of opioid agonists was increased in membranes with reduced amounts of functional G protein following CHS addition, the reduced effect of sodium on agonist binding following CHS modification appears to require the presence of a functional G protein. At 2.5 μmol of CHS/mg of protein, a concentration that completely suppressed the sodium effect in control membranes (Figs. 1B and 2B), [^{3}H]sufentanil binding was still strongly reduced by sodium (-65 ± 5%) in NEM-treated membranes (Fig. 2B). Even at levels of CHS two times (4.6 μmol/mg of protein) that used in control membranes, which resulted in a maximal increase of the anisotropy at both the membrane surface and core, a strong inhibition of agonist binding by sodium was still

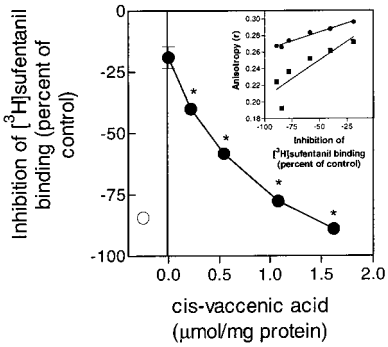

FIG. 3. *cis*-Vaccenic acid reversal of the CHS effects on the modulation of opioid agonist binding. In control membranes (○) or those modified with CHS (2.45 μmol of CHS/mg of protein) and increasing concentrations of *cis*-vaccenic acid (●), the binding of [^{3}H]sufentanil (0.1 n*M*) in the absence and presence of NaCl (150 m*M*) and GTPγS (10 μ*M*) was measured. Data are mean $\pm$ SEM (bars) values of percent inhibition of [^{3}H]sufentanil binding by NaCl and GTPγS from three experiments, each carried out in duplicate. $^*p < 0.05$, significantly different from the absence of *cis*-vaccenic acid. **Inset:** Correlation of membrane microviscosity in the membrane surface (●) and core (■) with the inhibition of sufentanil binding caused by sodium and GTPγS.

observed (-54 ± 4%; data not shown). Similarly, PTX pretreatment also reduced the effectiveness of CHS. In control membranes, [^{3}H]sufentanil binding was strongly depressed by sodium and guanine nucleotide, and this inhibition was reversed by CHS addition (Fig. 2C). Following PTX treatment, the strong inhibition of [^{3}H]sufentanil binding by sodium and guanine nucleotide was partially reversed but remained statistically significant (Fig. 2C). As indicated above, the observation that CHS modification of PTX-pretreated membranes reduced the effectiveness of sodium and guanine nucleotide to some degree may relate to the higher level of residual G protein in these membranes as compared with the NEM-treated membranes. No effect of CHS was observed on [^{3}H]naltrexone binding in either the absence or presence of NaCl in PTX-treated membranes (data not shown). These data suggest that a functional G protein is necessary for the reduced effect of sodium on agonist binding that is observed following CHS addition to the membranes.

To determine if the effect of cholesterol was due to its alteration of the membrane microviscosity or due to the presence of a specific sterol–protein interaction, the ability of sodium and guanine nucleotide to inhibit sufentanil binding was addressed following the reversal of membrane fluidity. In membranes rigidified with CHS, addition of *cis*-vaccenic acid was found to reverse the rigidification of the membrane (Fig. 3). Reversal of the CHS-induced increase in membrane microviscosity by *cis*-vaccenic acid addition also restored the sensitivity of agonist binding to ions and nucleotides (Fig. 3). NaCl and GTPγS inhibited sufentanil (0.1 n*M*) binding in control and CHS-modified membranes by 85 and 20%, respectively. Subsequent addition of *cis*-vaccenic acid to the CHS-modified membranes increased the inhibition

TABLE 1. *Parameters of opioid binding in control and CHS-modified membranes*

Radioligand	Modification	Assay buffer	K_D (n*M*)	B_{max} (fmol/mg of protein)	K_{lin} (fmol/mg/n*M*)
[^{3}H]Sufentanil	Control	Tris	0.028 ± 0.001	673 ± 9	
		+ NaCl	0.035 ± 0.005	333 ± 28	198 ± 81
		+ NaCl/GTPγS	0.08 ± 0.02	78 ± 18	387 ± 123
	CHS (2.19 ± 0.17)	Tris	0.039 ± 0.002	864 ± 17	
		+ NaCl	0.035 ± 0.001	685 ± 16	141 ± 43
		+ NaCl/GTPγS	0.049 ± 0.002	647 ± 23	251 ± 58
[^{3}H]CTOP[a]	Control	Tris	0.257 ± 0.015	857 ± 24	
[^{3}H]Naltrexone[a]	Control	+ NaCl	0.345 ± 0.007	693 ± 7	
	CHS (2.05 ± 0.12)	+ NaCl	0.327 ± 0.01	699 ± 11	
[^{3}H]Diprenorphine[a]	Control	+ NaCl	0.095 ± 0.004	529 ± 16	
	CHS (2.06 ± 0.11)	+ NaCl	0.101 ± 0.005	515 ± 12	

Ligand binding data were determined in control membranes and membranes modified with the concentration (μmol/mg of protein) of CHS indicated as described in Materials and Methods. Where indicated, NaCl (150 m*M*) and GTPγS (10 μ*M*) were added to the Tris buffer (50 m*M*, pH 7.4). The parameters shown were obtained from nonlinear regression analysis using the program SYSTAT using binding models with a single saturable binding site, one site plus a nonsaturable linear component (K_{lin}), or two saturable binding sites. The best fit was determined by the *F*-ratio test. Data are mean ± SEM values from three experiments, each carried out in duplicate.

[a] [^{3}H]CTOP parameters were determined in the same preparation of membranes used for the determination of [^{3}H]sufentanil parameters. [^{3}H]Naltrexone and [^{3}H]diprenorphine binding parameters were determined in a separate preparation of membranes.

by sodium to that observed in control membranes (89%). The respective decrease and restoration of the inhibition of agonist binding most closely correlated with the changes in TMA-DPH anisotropy (Fig. 3, inset). In contrast, addition of *cis*-vaccenic acid to CHS-modified membranes had little effect on antagonist ([^{3}H]diprenorphine) binding. At a concentration of *cis*-vaccenic acid (0.542 μmol/mg of protein) that restored 60% of the inhibition of [^{3}H]sufentanil binding by sodium and GTPγS, the binding of the antagonist was not significantly altered ($p = 0.168$, data not shown). At higher concentrations of *cis*-vaccenic acid, the binding of the antagonist was reduced as previously reported (Remmers et al., 1990). These data support the conclusion that alterations in membrane fluidity and not a specific sterol–protein interaction underlie the effects of CHS on opioid binding.

Equilibrium saturation binding of antagonist and agonist in CHS-modified membranes was performed to determine if the changes in agonist binding seen in Figs. 1 and 2 were due to alterations in ligand binding affinity or capacity. In the presence of NaCl, saturation binding of the antagonists [^{3}H]naltrexone and [^{3}H]diprenorphine in control membranes was characterized by a single saturable site (Table 1). Addition of CHS (2.0 μmol/mg of protein) did not alter either the affinity or binding capacity of [^{3}H]naltrexone or [^{3}H]diprenorphine (Table 1). In addition, the apparent rate of dissociation of [^{3}H]diprenorphine binding was similar in control (k_{-1} = 0.0107 ± 0.0002 min^{-1}) and CHS (2.2 μmol/mg of protein)-modified (k_{-1} = 0.0115 ± 0.0002 min^{-1}; data not shown) membranes. In contrast, addition of CHS to membranes increased the number of high-affinity [^{3}H]sufentanil binding sites and strongly reduced the inhibition by sodium and guanine nucleotide of agonist high-affinity binding. Saturation binding of the agonist [^{3}H]sufentanil to SH-SY5Y cell membranes was characterized by high binding affinity (28 p*M*) and capacity (Table 1). We were unable to characterize accurately the low-affinity [^{3}H]sufentanil binding site. This component was best described as a nonsaturating linear component of the binding, K_{lin}. Addition of NaCl reduced the high-affinity binding capacity by 50% with no significant change in K_D. Further addition of both NaCl plus GTPγS reduced the number of high-affinity binding sites by 88% and decreased the affinity to 80 p*M* (Table 1), suggesting a strong uncoupling of the receptor from G protein. Addition of CHS to membranes increased the high-affinity binding capacity of [^{3}H]sufentanil by 28% with little change in agonist affinity. The number of [^{3}H]sufentanil high-affinity binding sites in the presence of CHS was similar to the total number of μ-opioid receptors in the membrane preparation as determined by saturation binding of the μ-opioid antagonist [^{3}H]CTOP (Table 1) (Hawkins et al., 1989). Also, in control membranes, two [^{3}H]sufentanil affinity states were indicated by a biexponential dissociation of radioligand (site 1, k_{-1} = 0.04 ± 0.01 min^{-1}, 34%; site 2, k_{-1} = 0.0027 ± 0.0007 min^{-1}, 64%), whereas in CHS-modified membranes, only the slowly dissociating component was observed (0.0018 ± 0.0001 min^{-1}; data not shown). Addition of NaCl to CHS-modified membranes reduced the high-affinity binding (−20.7%) to a lesser degree than observed in control membranes (−50.5%), and again no change in affinity was observed. The further addition of sodium plus guanine nucleotide to CHS-modified membranes produced an additional small inhibition of the high-affinity binding (−25.1%) and also reduced the affinity of sufentanil for the receptor, although not to the extent observed in control membranes. Thus, addition of CHS affected only agonist binding by reducing the sodium and GTPγS-induced uncoupling of the receptor from G protein.

TABLE 2. *Receptor binding and stimulation of [^{35}S]GTPγS binding in control and CHS-modified C6μ cell membranes*

	[^{3}H]Naltrexone displacement				[^{35}S]GTPγS binding		
	TM buffer		Assay buffer				
Ligand addition	Log(EC_{50})	EC_{50} (n*M*)	Log(EC_{50})	EC_{50} (n*M*)	EC_{50} (n*M*)	Stimulation (fmol/mg of protein) (% control)[a]	Intrinsic activity[b]
DAMGO							
Control							
K_1	−8.98 ± 0.02	1.0	−7.28 ± 0.18	53	21.2 ± 0.9	240 ± 19 (391%)	1.0
F_1			0.46 ± 0.10				
K_2			−5.74 ± 0.18	1,820			
+ CHS							
K_1	−8.80 ± 0.01	1.6	−8.10 ± 0.06	7.9	4.9 ± 0.2	133 ± 5 (252%)	1.0
F_1			0.66 ± 0.11				
K_2			−7.06 ± 0.05	88			
Nalbuphine							
Control K_1	−9.06 ± 0.01	0.9	−8.21 ± 0.01	6.2	1.70 ± 0.04	14 ± 3 (23%)	0.06
+ CHS K_1	−8.95 ± 0.02	1.1	−8.34 ± 0.01	4.6	1.50 ± 0.20	68 ± 4 (129%)	0.51

Membranes were modified with CHS (4 μmol/mg of protein) as described in Materials and Methods, and experiments were performed as described in the legends to Figs. 4 and 5. The data were fit to one- and two-site competition curves, and an *F*-test (Graph Pad Prism) was used to determine if the added site provided a significantly better fit of the data. The one-site fit was the best fit for all of the nalbuphine data sets and for the DAMGO in Tris/magnesium (TM) buffer data. The DAMGO binding data in control and CHS membranes in GTPγS assay buffer (Assay buffer) were best described by a two-site fit. F_1 represents the fraction of high-affinity (K_1) binding sites. Data are mean ± SEM (values) of three experiments, each carried out in duplicate, presented as the increase in [^{35}S]GTPγS binding relative to that in the absence of added ligand (61.3 ± 1.6 and 52.7 ± 0.5 fmol/mg of protein) in the absence and presence of CHS, respectively.

[a] Numbers in parentheses indicate the percent stimulation over basal [^{35}S]GTPγS binding.

[b] Intrinsic activity is expressed as the fraction of stimulation relative to DAMGO.

Opioid efficacy in control and CHS-modified membranes

The effect of reduced membrane fluidity on μ-opioid receptor agonist efficacy was studied in membranes from C6μ cells. This cell line has previously been shown to provide an excellent model system for the study of opioid agonists of both high, e.g., DAMGO, and low, e.g., nalbuphine, efficacy due to the high level of opioid receptors (10–20 pmol of receptor/mg of membrane protein) and opioid-stimulated [^{35}S]GTPγS binding (Emmerson et al., 1996). Agonist-sensitive conformational changes in the μ-opioid receptor were studied by displacement of [^{3}H]naltrexone binding in both Tris-magnesium buffer (TM buffer; high-affinity agonist binding) and [^{35}S]GTPγS binding "assay buffer" (with 100 m*M* NaCl and 50 μ*M* GDP; low-affinity agonist binding). Similar to SH-SY5Y cell membranes, agonist binding affinity in C6μ cell membranes was reduced by addition of NaCl and GTPγS in a manner that correlated with agonist efficacy (Table 2). Because sodium and guanine nucleotides reduce DAMGO binding affinity to a greater extent than that observed for sufentanil (Emmerson et al., 1996), DAMGO was chosen to examine the effects of CHS on binding and activation of G protein. Although DAMGO binding affinity was strongly reduced, the affinity of nalbuphine was only weakly reduced by addition of sodium and GDP in the assay buffer (Fig. 4B and Table 2). CHS (4 μmol/mg of protein) addition to C6μ cell membranes had no effect on [^{3}H]naltrexone binding in either the absence or presence of sodium (data not shown). Also, CHS addition did not alter the affinity of either the full agonist DAMGO or the low-efficacy agonist nalbuphine when assayed in TM buffer (Fig. 4 and Table 2). As observed in SH-SY5Y cell membranes, CHS addition to C6μ cell membranes dramatically reduced the sensitivity of DAMGO binding to sodium and GDP addition (Fig. 4A and Table 2). DAMGO binding in the presence of sodium and GDP was best fit by a two-site model. Addition of CHS was found to increase slightly the fraction of high-affinity sites; however, the affinity of both of the sites was increased by CHS addition. This is consistent with the results observed for [^{3}H]sufentanil (Table 1). The apparent effect of CHS was to decrease the high-affinity B_{max} for [^{3}H]sufentanil. Presumably the decrease in B_{max} for [^{3}H]sufentanil described in Table 1 reflects a shift of receptors from high- to low-affinity conformation, which is not well characterized in a [^{3}H]sufentanil saturation curve because the affinity is so low. It is described by the linear component K_{lin}. Table 1 shows that the low-affinity [^{3}H]sufentanil K_{lin} component (+ Na/GTPγS) is smaller following CHS addition and that the number of high-affinity sites is approaching that for control membranes in Tris buffer. Thus, the trend is similar in that both DAMGO and sufentanil binding characteristics (+ CHS in sodium/guanine nucleotide) approach those of control (Tris buffer). It is interesting that CHS had little effect on the binding of nalbuphine in the presence of sodium and GDP (Fig. 4B and Table 2), suggesting that the binding of low-efficacy agonists to receptor are differentially affected by lipid modifications.

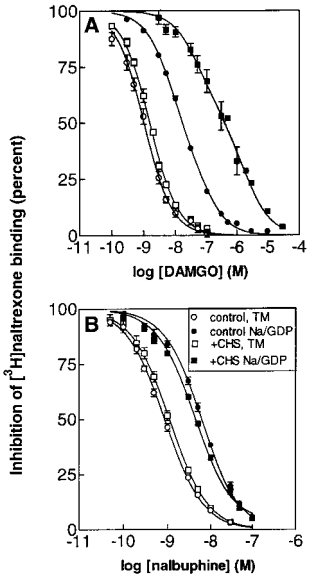

FIG. 4. Agonist displacement of [^{3}H]naltrexone in control and CHS-modified membranes. Control membranes (circles) or those modified with CHS (squares; 4 μmol/mg of protein) were incubated with [^{3}H]naltrexone (0.5 n*M*) and various concentrations of (**A**) DAMGO or (**B**) nalbuphine in Tris-magnesium buffer (control; open symbols) or assay buffer (Na/GDP; solid symbols) for 2 h at 25°C. The curves are a result of nonlinear least-squares fit to a model of either one or two binding sites as described in Materials and Methods. Data are mean ± SEM (bars) values from three experiments, each carried out in duplicate. The IC_{50} values and SEM are shown in Table 2.

To evaluate the functional consequences of membrane rigidification, agonist-mediated activation of G proteins was quantified by measuring the agonist-induced increase in the binding of [^{35}S]GTPγS as well as by GTPase activity. Although these assays measure G protein activation and G protein deactivation, respectively, the relative agonist efficacy correlated with the direction and degree of CHS modification in both assays. Both assays indirectly reflect the ability of agonist-liganded receptor to stimulate GDP dissociation from G protein. DAMGO increased [^{35}S]GTPγS binding by 240 fmol/mg of protein (391%), and that for nalbuphine was increased by 14 fmol/mg of protein (23%). In CHS-modified membranes, the potency of DAMGO to stimulate nucleotide binding was strongly increased; however, the maximal stimulation was reduced by 45% (Fig. 5 and Table 2), suggesting a decrease in full agonist efficacy. However, increased partial agonist efficacy was observed in C6μ cell membranes modified with CHS. Following CHS addition, the maximal stimulation of guanine nucleotide binding by nalbuphine was increased almost fourfold (Fig. 5 and Table 2). Furthermore, stimulation of low-K_m GTPase activity by an additional series of partial μ-opioid agonists was examined in control and CHS-modified membranes (Fig. 5 and Table 3). CHS addition increased the potency of the full agonist DAMGO (7.3-fold) and the partial agonists meperidine (6.4-fold) and profadol (3.8-fold) while leaving the potency of the very weak partial agonist butorphanol unaffected. Similar to the effect observed in the [^{35}S]GTPγS binding assay, the maximal stimulation for DAMGO was reduced, whereas the efficacies of all three partial agonists were significantly increased compared with DAMGO (Fig. 6 and Table 3). These data suggest that the effect of CHS on opioid receptor–G protein coupling was dependent on the efficacy of the opioid tested.

DISCUSSION

The results described here show that membrane rigidification (in both SH-SY5Y and C6μ cell membranes) results in agonist-specific alterations in ligand binding as well as a significant increase in partial agonist efficacy. These findings suggest that membrane rigidification stabilizes the G protein-coupled form of the μ-opioid receptor. These findings further extend those by our laboratory (Lazar and Medzihradsky, 1993) and others (Heron et al., 1981) to show that modulation of opioid receptor function by the lipid bilayer involves effects not only on the binding of ligands to the receptor but also on the coupling of the receptor to G protein. The following equation is a general model of agonist binding and subsequent G protein activation on which we base our discussion of the potential modulatory mechanisms of membrane microviscosity:

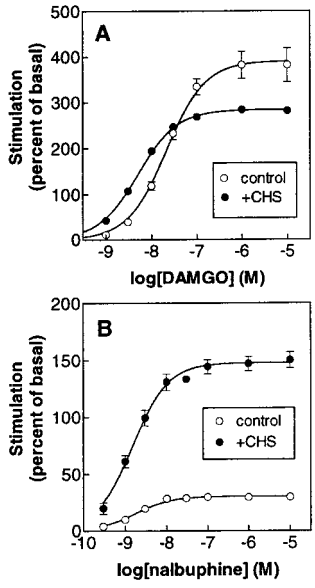

FIG. 5. Agonist stimulation of [^{35}S]GTPγS binding in control and CHS-modified membranes. Stimulation of [^{35}S]GTPγS binding by (**A**) DAMGO and (**B**) nalbuphine was examined in control membranes (○) or those modified with CHS (●; 4 μmol/mg of protein) as described in Materials and Methods. [^{35}S]GTPγS binding in the absence of ligand was 61.3 ± 1.6 and 52.7 ± 0.5 fmol/mg of protein in control and CHS-modified membranes, respectively. Data are mean ± SEM (bars) values of percent stimulation of [^{35}S]GTPγS binding over that in the absence of added ligand from three experiments, each carried out in duplicate. Results of sigmoidal dose–response curve fitting are listed in Table 2.

TABLE 3. *Stimulation of GTPase activity in control and CHS-modified C6μ cell membranes*

	EC_{50} (n*M*) (95% CI)		Efficacy[a]	
Ligand	Control	+ CHS	Control	+ CHS
DAMGO	80 (29–212)	11 (4–26)[b]	1.0	1.0
Meperidine	14,120 (9,984–19,960)	2,200 (1,369–3,535)[b]	0.43 ± 0.02	0.74 ± 0.04[b]
Profadol	295 (135–648)	78 (60–102)[b]	0.29 ± 0.01	0.74 ± 0.04[b]
Butorphanol	0.8 (0.2–3)	2 (1–5)	0.08 ± 0.01	0.46 ± 0.08[b]

Membranes were prepared from C6μ cells, control or modified with CHS, and agonist-stimulated GTPase activity was measured as described in Materials and Methods. Data from two experiments were combined and fit to a sigmoidal dose–response equation. EC_{50} values are mean values (95% confidence interval), and efficacy values are mean ± SEM values. Unpaired, two-tailed *t* tests were performed using Graph Pad Prism, comparing the log(EC_{50}) or efficacy, SE, and the number of points for control and treated membranes.

[a] The efficacy for each ligand is relative to that of DAMGO, where DAMGO efficacy in both control and CHS-modified membranes is defined as 1.0. DAMGO efficacy in CHS-modified membranes is 0.57 ± 0.04 relative to that of DAMGO in control membranes.

[b] $p < 0.05$, significant difference between EC_{50} or efficacy values from control and CHS-treated membranes.

$$L + R \underset{\text{Step 1}}{\rightleftharpoons} LR^* + G \underset{\text{Step 2}}{\rightleftharpoons} LR^*G \underset{\text{Step 3}}{\rightarrow} LR^*G^* \quad (1)$$

In step 1, ligand (L) binds to receptor (R) to form activated ligand–receptor complex LR*. Step 2 indicates the binding of LR* to heterotrimeric G protein (G). Step 3 indicates G protein activation (G*), which includes the exchange of GDP for GTP. Because basal GTPγS binding does not change significantly in CHS-modified membranes, activated unliganded receptor (R*) was not included in the model. These data suggest that all three steps are affected by decreased membrane fluidity.

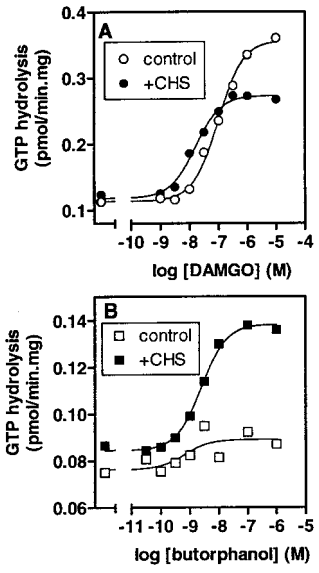

FIG. 6. Agonist-stimulated GTPase activity in control and CHS-modified membranes. GTPase activity stimulated by (**A**) DAMGO and (**B**) butorphanol in control and CHS-modified membranes was measured as described in Materials and Methods. Shown is the best fit of a sigmoidal dose–response curve for representative experiments performed in duplicate that were repeated. Average EC_{50} values and relative efficacies for these and two additional agonists are shown in Table 3.

Ligand binding in the presence of functional G protein (steps 1 and 2)

Addition of CHS to SH-SY5Y and C6μ cell membranes was found to alter selectively the regulation of opioid agonist binding by sodium and guanine nucleotides. CHS incorporation strongly and maximally increased the microviscosity of SH-SY5Y membranes without affecting the binding of two opioid antagonists, [^{3}H]naltrexone and [^{3}H]diprenorphine, or the protein content of these membranes even at very high levels of CHS incorporation. However, the modulation of opioid agonist binding by sodium and guanine nucleotide was strongly reduced by the increase in membrane microviscosity. In contrast to the findings of Heron et al. (1981), the effects of CHS were agonist-specific and did not result in a loss in receptor binding ("shedding") or membrane protein content even at high CHS concentrations, indicating that CHS does not modulate the accessibility of ligand for the receptor as suggested by a vertical displacement model of CHS action. These specific effects on agonist binding may result from (a) direct lipid–receptor interaction, (b) alteration in conformational flexibility of the receptor through increased microviscosity (step 1), or (c) microviscosity effects on receptor–G protein interactions (step 2). The reversibility of the effects of CHS by membrane fluidizers suggests that a direct lipid–receptor interaction is not likely. Second, as observed in TM buffer, the ability of CHS to increase agonist binding in the absence of functional G protein (step 1 in Eq. 1) indicates that microviscosity, in addition to G protein, can modulate the agonist-bound receptor conformation. Third, there appears to be a complex interaction among changes in membrane microviscosity, the ability of G protein to stabilize high-affinity agonist binding (step 2), and the observed increase in agonist efficacy.

The molecular consequence of sodium binding to the opioid receptor was suggested to be a conformational change from an "agonist-favoring" to an "antagonist-favoring" state (Simon and Groth, 1975; Simon and Hiller, 1981), although in the present study this simple conformational model does not fully explain the observed changes in receptor binding because agonist binding was strongly modulated by sodium, whereas antagonist binding was unaffected. The presence of a G protein has been shown to be required for the stabilization of the high-affinity opioid agonist binding (Remmers and Medzihradsky, 1991*a*). In the absence of G protein, the receptor exists in a conformation with low affinity for ligand. Based on the suggestion that membrane microviscosity can modulate the conversion of integral membrane proteins between conformational states (Swann, 1984; Yeagle, 1991; Mitchell et al., 1992), we postulated that the interaction of the μ-opioid receptor with G protein and the conformational change resulting in the conversion of receptor between agonist high- and low-affinity states may be modulated by addition of CHS. In the absence of sodium, addition of CHS rigidified the cell membrane and increased agonist binding, resulting in the formation of a single agonist high-affinity conformation of the receptor as indicated by a single slowly dissociating binding component. The conversion of the receptor to a single affinity state is also supported by the close agreement between the number of high-affinity agonist binding sites in the presence of CHS and the total number of μ-opioid binding sites in this preparation determined by the antagonist [^{3}H]CTOP. These findings suggest that under some conditions, the conformation of the μ receptor may be directly modulated by its lipid environment.

Ligand binding in the absence of functional G protein (step 1)

In membranes where G protein had been inactivated, addition of CHS greatly enhanced the binding of agonist to the free receptor (Fig. 2B), indicating a conversion of the receptor to a higher-affinity conformation. Similarly, low temperature-induced conversion of the α_1-adrenergic receptor to a single agonist high-affinity state was not dependent on the presence of a G protein (Lynch et al., 1988). Rigidification of the membrane bilayer or low temperature may create a condition in the lipid surrounding the receptor where increased lateral pressure (Yeagle, 1991) directly alters the equilibrium between low- and high-affinity receptor conformations, leading to the stabilization of a G protein-independent agonist high-affinity state. However, in G protein-free membranes after CHS modification, the rate of [^{3}H]sufentanil dissociation remained faster ($t_{1/2}$ = 147 min) than observed in control membranes after CHS addition ($t_{1/2}$ = 384 min), indicating that these receptor conformations may not be identical. Increased membrane microviscosity may incompletely alter receptor conformation either owing to its indirect effects on the receptor or owing to the stabilization of an intermediate conformation of the receptor, which may then be converted by G protein to the high-affinity state. In fact, mutation of the β-adrenergic receptor stabilizes a receptor conformation with intermediate agonist binding affinity (Samama et al., 1993), suggesting that G protein-linked receptors may exist in multiple conformations with agonist, G protein, and possibly the membrane environment, affecting the equilibrium between these states.

In membranes where G protein was inactivated, CHS no longer completely reduced the inhibitory effect of sodium and guanine nucleotide on agonist binding. These data suggest that increased microviscosity alone is not sufficient to stabilize the receptor in a high-affinity sodium and guanine nucleotide-insensitive conformational state. Therefore, the effects of CHS and reduced membrane fluidity likely require the presence of a functional G protein to attenuate the effects of sodium on agonist binding. In these experiments, membranes were modified with CHS before addition of sodium and guanine nucleotides. Under these conditions, receptor and G protein are known to be precoupled (Graeser and Neubig, 1993; Tian et al., 1994). Thus, rigidification of the membrane may stabilize the receptor–G protein complex by either inhibiting its dissociation or enhancing its formation. Examination of the affinity states of the purified and reconstituted μ-opioid receptor may be required to define unequivocally the role of membrane microviscosity on opioid receptor affinity states and G protein coupling.

Agonist efficacy (steps 2 and 3)

Agonist efficacy has been characterized by the affinity of ligand-bound receptor LR for G protein (step 2) (Tota and Schimerlik, 1990) as well as the ability to dissociate GDP from the G protein leading to subsequent GTP binding (step 3) (Lorenzen et al., 1996). In addition to the observed effects of membrane rigidification on the affinity of agonist for receptor, CHS appears to stabilize the LRG complex and thus increase agonist potency. Clarke and Bond (1998) suggested that intrinsic efficacy may be dependent on the biological system in which the assay is performed. They proposed that the probability of a certain receptor conformation might be influenced by the microenvironment of the protein. The data showing increased partial agonist efficacy in rigidified membranes support this hypothesis. However, in these experiments, we did not evaluate the possibility that CHS may also decrease GDP affinity for G protein, thus allowing for the more rapid binding of GTPγS (step 3). The observed decrease in maximal DAMGO-stimulated GTPγS binding in CHS-modified membranes may be due to reduced membrane diffusion of receptor to additional G protein, reduced agonist switching (Stickle and Barber, 1991; Mahama and Linderman, 1994) due to slower agonist dissociation rates, or a decrease in the number of G proteins able to couple functionally to receptors due to potential alterations in membrane domains.

The possibility exists that CHS exerts its effects not only through alterations of membrane fluidity but also through alteration of membrane lipid domains. Due to

the lack of correlation between changes in microviscosity and the function of integral membrane proteins, reorganization of membrane lipid domains has also been suggested as a mechanism for alterations induced by lipid addition (Carruthers and Melchior, 1986). In addition to previously described caveolae and clathrin-coated pits, membrane subdomains enriched in sphingolipid and cholesterol have been shown to form detergent-insoluble ordered domains (Schroeder et al., 1998). Recent evidence (Feder et al., 1996; Holowka and Baird, 1996; Fielding and Fielding, 1997) suggests that the antigen-mediated immunoglobulin receptor activation process involves the interaction of aggregated high-affinity IgE receptor with specialized plasma membrane domains that may localize important signaling molecules in the vicinity of aggregated receptors. Although these various studies were aimed toward understanding the operation of one cell surface receptor, they provide new insights into plasma membrane structure and dynamics. Except for the immunoglobulin complex formation and the requirement for receptor internalization, the significance of membrane subdomains on receptor–effector interaction is largely unknown. The stabilization of membrane subdomains by CHS addition could potentially account for the increased efficacy of partial agonists observed here. Our experiments do not distinguish between gross or subdomain changes in membrane microviscosity, and therefore we can only hypothesize on the molecular mechanisms of increased G protein activation.

The possibility of membrane subdomains does not diminish the observation that changes in membrane biophysical properties correlate with changes in opioid receptor binding. The actions of CHS (Lazar and Medzihradsky, 1993) and fatty acid isomers (Remmers et al., 1990; Lazar and Medzihradsky, 1992) were suggested to modulate opioid receptor binding in a manner correlated with changes in microviscosity. In SH-SY5Y membranes, a close correlation between CHS rigidification of membranes and reversal by *cis*-vaccenic acid and the reduction and restoration of opioid agonist modulation by sodium and guanine nucleotide supports the conclusion that the observed effects are mediated by changes in the membrane biophysical properties. The fact that *cis*-vaccenic acid could restore both surface membrane fluidity and agonist binding to control levels in CHS-modified membranes and the close correlation between membrane surface fluidity and agonist binding suggest that the phosphate head group region of the membrane may be most influential in the regulation of agonist binding in the presence of sodium and guanine nucleotide.

These effects indicate that the lipid bilayer is a regulator of opioid receptor binding and signal transduction. The changes in membrane composition and biophysical properties resulting from diet (McMurchie, 1988), age (Gurdal et al., 1995), and pathophysiological states (Raber and Bast, 1989; Ma et al., 1994; Roth et al., 1995) suggest a significant regulatory role for the membrane bilayer in cellular signal transduction. The use of newly developed fluorescently labeled opioids (Emmerson et al., 1997) will allow the further examination of the interaction between the lipid bilayer and the opioid receptor.

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