Salvinorin A: Allosteric Interactions at the μ-Opioid Receptor

Richard B. Rothman, Daniel L. Murphy, Heng Xu, Jonathan A. Godin,
Christina M. Dersch, John S. Partilla, Kevin Tidgewell, Matthew Schmidt, and
Thomas E. Prisinzano

Clinical Psychopharmacology Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department and Health and Human Services, Baltimore, Maryland (R.B.R., D.L.M., H.X., J.A.G., C.M.D., J.S.P.); and Division of Medicinal & Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, Iowa (K.T., M.S., T.E.P.)

ABSTRACT

Salvinorin A [(2S,4aR,6aR,7R,9S,10aS,10bR)-9-(acetyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2h-naphtho[2,1-c]pyran-7-carboxylic acid methyl ester] is a hallucinogenic κ-opioid receptor agonist that lacks the usual basic nitrogen atom present in other known opioid ligands. Our first published studies indicated that Salvinorin A weakly inhibited μ-receptor binding, and subsequent experiments revealed that Salvinorin A partially inhibited μ-receptor binding. Therefore, we hypothesized that Salvinorin A allosterically modulates μ-receptor binding. To test this hypothesis, we used Chinese hamster ovary cells expressing the cloned human opioid receptor. Salvinorin A partially inhibited [³H]Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO) (0.5, 2.0, and 8.0 nM) binding with E_{MAX} values of 78.6, 72.1, and 45.7%, respectively, and EC$_{50}$ values of 955, 1124, and 4527 nM, respectively. Salvinorin A also partially inhibited [³H]diprenorphine (0.02, 0.1, and 0.5 nM) bin-

ding with E_{MAX} values of 86.2, 64, and 33.6%, respectively, and EC$_{50}$ values of 1231, 866, and 3078 nM, respectively. Saturation binding studies with [³H]DAMGO showed that Salvinorin A (10 and 30 μM) decreased the μ-receptor B_{max} and increased the K_d in a dose-dependent nonlinear manner. Saturation binding studies with [³H]diprenorphine showed that Salvinorin A (10 and 40 μM) decreased the μ-receptor B_{max} and increased the K_d in a dose-dependent nonlinear manner. Similar findings were observed in rat brain with [³H]DAMGO. Kinetic experiments demonstrated that Salvinorin A altered the dissociation kinetics of both [³H]DAMGO and [³H]diprenorphine binding to μ receptors. Furthermore, Salvinorin A acted as an uncompetitive inhibitor of DAMGO-stimulated guanosine 5′-O-(3-[³⁵S]thio)-triphosphate binding. Viewed collectively, these data support the hypothesis that Salvinorin A allosterically modulates the μ-opioid receptor.

Salvia divinorum is a plant from the sage family that has been used in the traditional spiritual practices by the Mazatec Indians of Oaxaca, Mexico to produce "mystical" or hallucinogenic experiences. The active ingredient isolated from the leaves of *S. divinorum* is Salvinorin A, a neoclerodane diterpene. Current evidence suggests that Salvinorin A-induced hallucinogenic effects are mediated by activation of κ-opioid receptors (for review, see Sheffler and Roth, 2003).

Salvinorin A, a κ-opioid receptor agonist (Roth et al., 2002), is a unique opioid receptor ligand. It bears little structural similarity to other structural classes of nonpeptidic opioid receptor ligands, including κ agonists, such as U50,488H and U69,593 (Harding et al., 2005). The common structural motif among all of these compounds is the presence of a basic amino group. Until recently, it had been assumed that the presence of a positively charged nitrogen atom in opioid compounds represented an absolute requirement for their interaction with opioid receptors (Rees and Hunter, 1990). The

ABBREVIATIONS: CHO, Chinese hamster ovary cells; hMOR-CHO, CHO cells expressing the cloned human μ-opioid receptor; DAMGO, Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol; herkinorin, (2S,4aR,6aR,7R,9S,10aS,10bR)-9-(benzoyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho-[2,1-c]pyran-7-carboxylic acid methyl ester; [³⁵S]GTPγS, guanosine 5′-O-(3-[³⁵S]thio)triphosphate; U50,488H, (±)-trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide; U69,593, 5α,7α,8β-(−)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-phenyl-benzeneacetamide; Salvinorin A, (2S,4aR,6aR,7R,9S,10aS,10bR)-9-(acetyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho[2,1-c]pyran-7-carboxylic acid methyl ester; SA, specific activity; MAPK, mitogen-activated kinase protein; KRBG, Krebs-Ringer bicarbonate buffer with glucose; [¹²⁵I]IOXY, 6β-iodo-3,14-dihydroxy-17-cyclopropylmethyl-4,5α-epoxymorphinon.

general assumption was that this cationic amino charge on the opioid ligand would interact with the side chain carboxyl group of an aspartate residue located in transmembrane III of the opioid receptor (Surratt et al., 1994; Eguchi, 2004). Given the lack of a basic nitrogen in Salvinorin A, this interaction is not an absolute requirement.

The pharmacology of Salvinorin A differs from that of other κ agonists (Wang et al., 2005). Although Salvinorin A and U50,488H stimulated [^{35}S]GTPγS binding with similar potency in Chinese Hamster ovary cells (CHO) expressing the cloned human κ receptor, salvinorin A was ~40-fold less potent than U50,488H in promoting receptor internalization. As observed with other κ agonists (Devine et al., 1993), Salvinorin A produces decreases in extracellular dopamine in both mouse caudate (Zhang et al., 2005) and rat nucleus accumbens (Carlezon et al., 2006).

Our initial binding studies showed that Salvinorin A weakly inhibited μ- and δ-opioid receptor binding (Roth et al., 2002), a finding replicated by others (Wang et al., 2005). In a subsequent report (Harding et al., 2005), using the radioligand [^{125}I]IOXY, we generated more detailed Salvinorin A inhibition curves and observed that Salvinorin A and certain other Salvinorin A analogs partially inhibited [^{125}I]IOXY binding to the cloned human μ receptor expressed in CHO cells (hMOR-CHO cells). In the present study, we characterized the interaction of Salvinorin A with μ-opioid receptors. We report evidence that Salvinorin A allosterically modulates μ-receptor binding.

Materials and Methods

Cell Culture and Membrane Preparation. The recombinant CHO cells (hMOR-CHO) were produced by stable transfection with the human opioid receptor cDNA and provided by Dr. Larry Toll (SRI International, Menlo Park, CA). The cells were grown on plastic flasks in Dulbecco's modified Eagle's medium/F-12 (50%/50%) medium (hMOR-CHO) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.20–0.25 mg/ml G-418 under 95% air/5% CO_2 at 37°C. Cell monolayers were harvested and homogenized using a Polytron grinding apparatus in 50 mM Tris-HCl, pH 7.4, containing 4 μg/ml leupeptin, 2 μg/ml chymostatin, 10 μg/ml bestatin, and 100 μg/ml bacitracin, The homogenate was centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant was discarded. The membrane pellets were resuspended in binding buffer and used for [^{35}S]GTPγS binding assays. For drug pretreatment experiments, the medium was changed, and then cells were incubated with various test drugs for 20 h. Cells were washed three times with phosphate-buffered saline (pH 7.4), and cell membranes were prepared as described above. This treatment produces tolerance to opioid drugs (Xu et al., 2003).

[^{35}S]GTPγS Binding Assays. [^{35}S]GTPγS binding was determined as described previously (Xu et al., 2001). In brief, test tubes received the following additions: 50 μl of buffer A (50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM MgCl$_2$, and 1 mM EDTA), 50 μl of GDP in buffer A (final concentration = 50 μM), 50 μl of drug in buffer A/0.1% bovine serum albumin, 50 μl of [^{35}S]GTPγS in buffer A (final concentration = 50 pM), and 300 μl of cell membranes (50 μg of protein) in buffer B. The final concentrations of reagents in the [^{35}S]GTPγS binding assays were: 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% bovine serum albumin. Incubations proceeded for 2 h at 25°C. Nonspecific binding was determined using GTPγS (40 μM). Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters. The filters were punched into 24-well plates, to which 0.6 ml of liquid scintillation cocktail (Cytoscint;

MP Biomedicals, Irvine, CA) was added. Samples were counted after an overnight extraction in a Trilux liquid scintillation counter at 60% efficiency.

Opioid Binding Assays. We used [^{3}H][D-Ala^2-MePhe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO) (SA = 46 Ci/mmol), [^{3}H]diprenorphine (SA = 54.9 Ci/mmol), and [^{125}I]IOXY (SA = 2200 Ci/mmol) to label μ-binding sites. All assays took place in 50 mM Tris-HCl, pH 7.4, with a protease inhibitor cocktail [bacitracin (100 μg/ml), bestatin (10 μg/ml), leupeptin (4 μg/ml), and chymostatin (2 μg/ml)] in a final assay volume of 0.5 ml. Nonspecific binding was determined using 20 μM levallorphan. Triplicate samples were filtered with Brandel cell harvesters (Biomedical Research and Development Inc., Gaithersburg, MD) over Whatman GF/B filters after a 2- to 3-h incubation at 25°C. For the [^{125}I]IOXY experiments, the filters were punched into 12 × 75-mm glass test tubes and counted in a Micromedic gamma counter at 80% efficiency. For the ^{3}H-ligand binding assays, the filters were punched into 24-well plates, to which was added 0.6 ml liquid scintillation cocktail (Cytoscint). Samples were counted, after an overnight extraction, in a Trilux liquid scintillation counter at 44% efficiency. Opioid binding assays using membranes prepared from hMOR-CHO cells had ~30-μg protein per assay tube.

Inhibition curves were generated by displacing a single concentration of radioligand by 10 concentrations of drug. For binding surface experiments (Rothman, 1986; Rothman et al., 1991), two different concentrations of radioligand were each displaced by ten concentrations of nonradioactive ligand agents in the absence or presence of various blockers.

Cyclic AMP Assays. Functional coupling of the cloned μ-opioid receptor to adenylate cyclase was determined by measuring changes in the levels of cellular cAMP. The assay procedures followed the protocol provided by CatchPoint cyclic-AMP fluorescent assay kit (a horseradish peroxidase-based competitive immunoassay kit). For acute studies, hMOR-CHO cells were grown to 80 to 90% confluence in 96-well black-walled, clear bottom plates that had been treated with poly-L-lysine. After aspirating the medium, cells were washed with 300-μl/well Krebs-Ringer bicarbonate buffer with glucose (KRBG, pH 7.4). KRBG containing 0.75 mM 3-isobutyl-1-methylxanthine and 1 mg/ml bovine serum albumin and appropriate agonists were added to each well (90 μl). After a 30-min incubation at 37°C, 100 μM forskolin in KRBG containing 0.75 mM 3-isobutyl-1-methylxanthine and 1 mg/ml bovine serum albumin was added to each well in a volume of 10 μl. Cyclic AMP production was terminated 40 min later by the addition of 50 μl of a cell-lysing solution (Molecular Devices, Sunnyvale, CA). For chronic studies, cells were grown to 80% confluence in 96-well black-walled, clear bottom plates (number 3603, Corning Inc., Corning, NY) that had been treated with poly-L-lysine. After treatment with medium or 10 μM drug for 20 h, cells were rinsed three times with 300-μl/well KRBG (pH 7.4) and assayed as described above. This assay was sensitive between 0.1 and 10 pmol of cAMP in a 40-μl sample volume. A FlexStation II (Molecular Devices) was used to read and quantitate fluorescence intensity of the plate. Data from three experiments were analyzed using the program Prism (version 3.0; GraphPad Software, Inc., San Diego, CA). Results are presented as the mean ± S.E.M.

Stimulation of p42/p44 MAPK Phosphorylation. The assay procedures followed the protocol provided by PhosphoPlus p44/42 MAPK (Thr202/Tyr204) antibody kit (Cell Signaling, Beverly, MA). In brief, cells were grown to 80 to 90% confluence in six-well plates. The assay started by the addition of any agonists and stopped after 5 min by rinsing the cells with ice-cold 1× phosphate-buffered saline. Cells were lysed by adding SDS sample buffer (100 μl) and immediately scraped to a microcentrifuge tube on ice, sonicated for 10 to 15 s, and boiled for 5 min. Samples (20 μl) were loaded onto SDS-polyacrylamide gel electrophoresis gel as described previously (Xu et al., 2005). Western blots were digitized and quantified using densitometric analysis (NIH Image software). Results from at least three experiments were analyzed using the program Prism.

Data Analysis and Statistics. For [^{35}S]GTPγS binding experiments, the percentage stimulation of [^{35}S]GTPγS binding was calculated according to the following formula: $(S - B)/B \times 100$, where B is the basal level of [^{35}S]GTPγS binding and S is the stimulated level of [^{35}S]GTPγS binding (Xu et al., 2004). EC_{50} values (the concentration that produces 50% maximal stimulation of [^{35}S]GTPγS binding) and E_{MAX} (percentage of maximal stimulation in the [^{35}S]GTPγS binding) were determined using the program MLAB-PC (Civilized Software, Bethesda, MD).

The amount of cAMP in the samples was measured using a cAMP standard curve. Forskolin (100 μM)-stimulated cAMP formation in the absence of agonist was defined as 100%. The EC_{50} (the concentration of agonist that produces 50% inhibition of forskolin-stimulated cAMP formation) and E_{MAX} (percentage of maximal inhibition of forskolin-stimulated cAMP) were calculated using the program Prism.

In receptor binding experiments, for drugs that produced inhibition curves without apparent plateaus, the data were fit to the two-parameter logistic equation for the best-fit estimates of the IC_{50} and n values (Nightingale et al., 2005). For curves with apparent plateaus, the data were transformed to "percentage inhibition" and fit to a two-parameter dose-response curve model: $Y = E_{MAX} \times ([D]/([D] + EC_{50}))$, for the best fit estimates of the E_{MAX} and EC_{50} using either KaleidaGraph version 3.6.4 or MLAB-PC (Nightingale et al., 2005). Radioligand binding surfaces generated with [^{3}H]DAMGO or [^{3}H]diprenorphine were fit to one-site binding models using MLAB-PC as described elsewhere (Rothman et al., 1991). Statistical significance among binding parameters was determined using the F-test (Rothman et al., 1991). Dissociation experiments were conducted with minor modification of published procedures, in which the data were fit to a two-component dissociation model (Rothman et al., 1991). Statistical significance among kinetic model parameters was determined using the Student's t test.

Sources. [^{35}S]GTPγS (SA = 1250 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Various opioid peptides were provided by Multiple Peptide System via the Research Technology Branch (National Institute on Drug Abuse, Baltimore, MD). [^{125}I]IOXY was prepared as described previously (de Costa et al., 1992; Ni et al., 1993). The sources of other agents are published elsewhere (Xu et al., 2004). Salvinorin A and herkinorin were synthesized as described previously (Harding et al., 2005). For experiments using Salvinorin A or herkinorin, drug dilution curves were made up from freshly prepared 10 mM stock solutions in dimethyl sulfoxide. As is our

standard operating procedure, all drug dilution curves used buffer with 1 mg/ml bovine serum albumin.

Results

Ligand Binding Experiments. Our initial experiments demonstrated that Salvinorin A, whose structure is shown in Fig. 1, partially inhibited [^{125}I]IOXY (0.9 nM) binding to membranes prepared from hMOR-CHO cells (Fig. 1), with an EC_{50} value of 1728 nM and an E_{MAX} value of 48%. Similar results were observed in rat brain membranes using [^{3}H]DAMGO to label μ receptors (Fig. 2). In this case, Salvinorin A partially inhibited μ-receptor binding with an EC_{50} value of 2322 nM and an E_{MAX} value of 60%.

To further characterize Salvinorin A-mediated partial inhibition of μ-receptor binding, we generated Salvinorin A inhibition curves using three concentrations of [^{3}H]DAMGO (0.5, 2.0, and 8.0 nM) designed to produce varying levels of μ-receptor occupation (the K_d is ~2 nM), using membranes prepared from hMOR-CHO cells. As reported in Fig. 3, Salvinorin A partially inhibited μ-receptor binding at all three [^{3}H]DAMGO concentrations. The EC_{50} and E_{MAX} values are reported in Table 1. These results show that the Salvinorin A inhibition curve observed with 8.0 nM [^{3}H]DAMGO resulted in a significantly lower E_{MAX} value and a higher EC_{50} value compared with the two lower [^{3}H]DAMGO concentrations.

Likewise, we generated Salvinorin A inhibition curves using three concentrations of [^{3}H]diprenorphine (0.02, 0.1, and 0.5 nM) designed to produce varying levels of μ-receptor occupation (the K_d is ~0.7 nM), using membranes prepared from hMOR-CHO cells. As reported in Fig. 4A, Salvinorin A partially inhibited μ-receptor binding at all three [^{3}H]diprenorphine concentrations. The EC_{50} and E_{MAX} values are reported in Table 1. These results show that the Salvinorin A inhibition curve observed with 0.5 nM [^{3}H]diprenorphine resulted in a significantly lower E_{MAX} value and a higher EC_{50} value compared with the two lower [^{3}H]diprenorphine concentrations. It is apparent from these inhibition curves that Salvinorin A is correctly

hMOR-CHO Cells: Partial Inhibition of [^{125}I]IOXY Binding
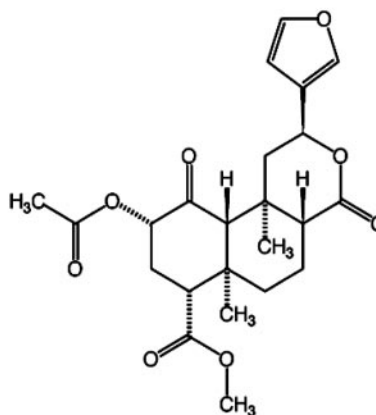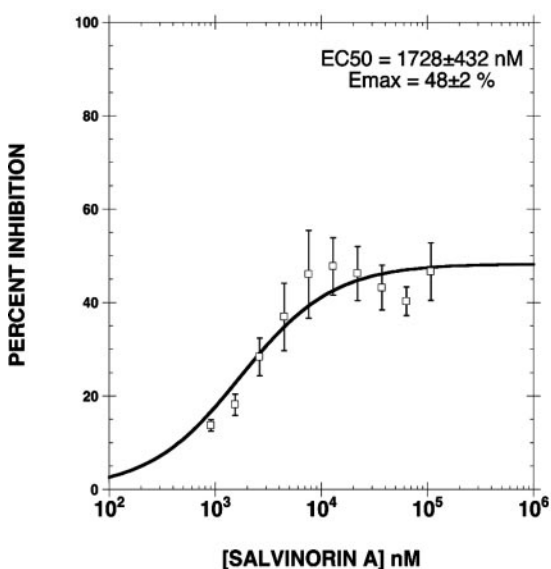
Fig. 1. Inhibition of [^{125}I]IOXY binding to hMOR-CHO cell membranes by Salvinorin A. [^{125}I]IOXY (0.9 nM) was displaced by 10 concentrations of Salvinorin A. The data of three experiments, expressed as percentage inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} ($\pm$ S.D.) as described under *Materials and Methods*. Each point is the mean $\pm$ S.D. ($n = 3$).
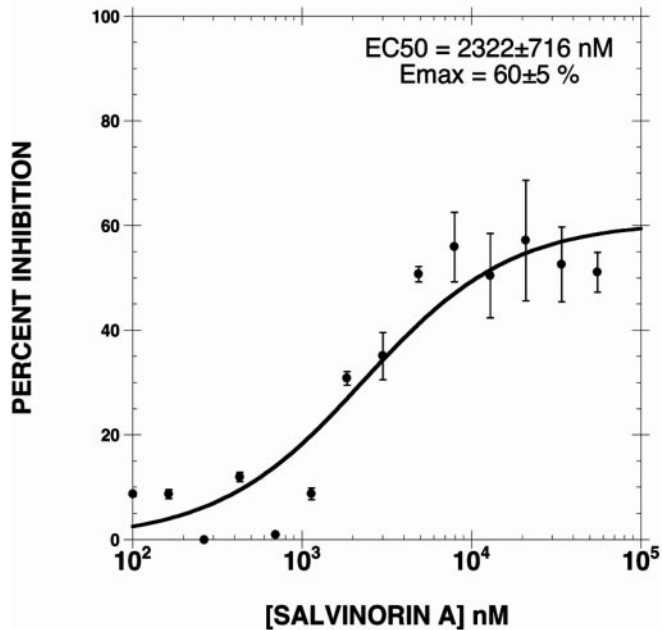
Salvinorin A

Rat Brain Memranes: Partial Inhibition of [³H]DAMGO Binding

Fig. 2. Inhibition of [³H]DAMGO binding to rat brain membranes by Salvinorin A. [³H]DAMGO (0.8 nM) was displaced by 14 concentrations of Salvinorin A. The data of two experiments, expressed as percentage inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} ($\pm$ S.D.) as described under *Materials and Methods.* Each point is the mean $\pm$ S.D. ($n = 2$).
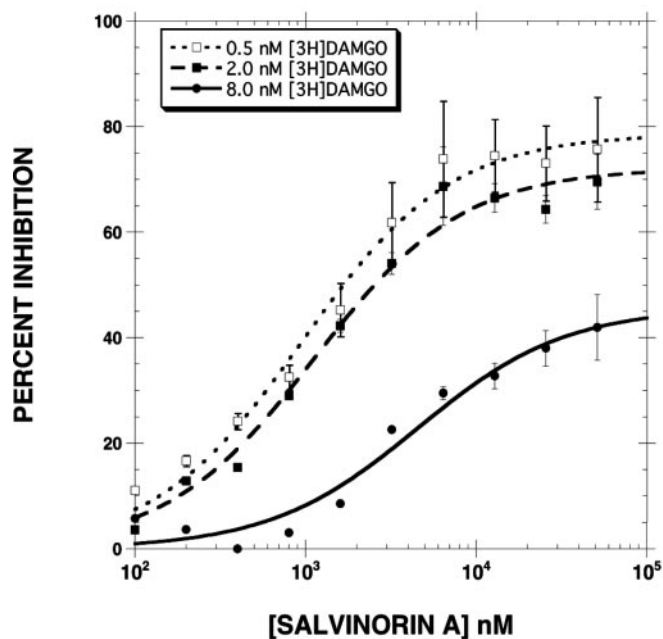
hMOR-CHO Cells: Partial Inhibition of [³H]DAMGO Binding

Fig. 3. Inhibition of [³H]DAMGO binding to hMOR-CHO membranes by Salvinorin A. Three concentrations of [³H]DAMGO were each displaced by 10 concentrations of Salvinorin A. The data, expressed as percentage inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} (See Table 1) as described under *Materials and Methods.* Each point is the mean $\pm$ S.E.M. ($n = 3$–7).

identified as being "inactive" or having less than 50% inhibition of μ-receptor binding at a concentration of 10 μM when higher [³H]ligand concentrations are used (Roth

TABLE 1
Summary of results for inhibition curves in hMOR-CHO cells
Drug inhibition curves were generated using membranes prepared from hMOR-CHO cells using the indication concentrations of either [³H]DAMGO or [³H]diprenorphine. The data of three to seven experiments were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} ($\pm$ S.D.) as described under *Materials and Methods.*

[³H]DAMGO	Plateau (E_{MAX}) Value	EC_{50} Value
nM	*% ± S.D.*	*nM ± S.D.*
Salvinorin A		
0.5 ($n = 7$)	78.6 ± 2.0	955 ± 112
2.0 ($n = 7$)	72.1 ± 2.2	1124 ± 152
8.0 ($n = 3$)	45.7 ± 3.3*	4527 ± 1108*
Salvinorin A		
[³H]Diprenorphine		
0.02 ($n = 4$)	68.2 ± 3.1	1231 ± 241
0.1 ($n = 4$)	64.0 ± 3.1	866 ± 192
0.5 ($n = 4$)	33.6 ± 4.6*	3078 ± 830*
(−)-U50,488		
0.02 ($n = 3$)	100 ± 5.2	1166 ± 138
0.1 ($n = 3$)	89.0 ± 5.6	729 ± 145*
0.5 ($n = 3$)	94.5 ± 3.1	2605 ± 228*
Naloxone		
0.02 ($n = 4$)	97.5 ± 2.7	2.15 ± 0.28
0.1 ($n = 4$)	97.3 ± 2.2	2.46 ± 0.25
0.5 ($n = 4$)	103 ± 4.3	11.7 ± 1.6*

* $P < 0.05$ compared with 0.02 nM [³H]DIP or 0.5 nM [³H]DAMGO (Student's *t* test).

et al., 2002). In contrast to the results observed for Salvinorin A, both (−)-U50,488, a κ agonist, and naloxone, a μ-receptor antagonist, fully inhibited [³H]diprenorphine binding, producing classic inhibition curves consistent with simple competitive inhibition (Fig. 4B; Table 1).

Using the method of binding surface analysis, we determined the effect of fixed concentrations of Salvinorin A on the K_d and B_{max} of [³H]DAMGO binding to membranes prepared from both MOR-CHO cells and rat brain. As reported in Table 2, Salvinorin A had complex actions on the K_d and B_{max} values in hMOR-CHO cells. Salvinorin A increased the K_d value in a dose-dependent manner, producing a maximal increase to ~8.9 nM. After increasing the B_{max} value at a concentration of 6400 nM, Salvinorin A proceeded to decrease the B_{max} value at higher concentrations. These data, normalized as percentage changes, are reported in Fig. 5, A and B. The data clearly show that Salvinorin A increased the K_d in dose-dependent nonlinear manner with an EC_{50} value of 1730 nM and an E_{MAX} value of 248%. In contrast, a competitive inhibitor increased the K_d in a strictly linear manner. In rat brain, 1000 nM Salvinorin A increased the K_d without changing the B_{max}. A higher concentration of Salvinorin A (5000 nM) substantially reduced the B_{max} by 48% while increasing the K_d to a smaller extent than 1000 nM Salvinorin A.

We also determined the effect of Salvinorin A on the K_d and B_{max} of [³H]diprenorphine binding to hMOR-CHO cells. As reported in Table 3, both 10,000 and 40,000 nM Salvinorin A decreased the B_{max} value by ~34% and increased the K_d by ~2-fold. Consistent with the plateau reported in Fig. 4, increasing the Salvinorin A concentration 4-fold from 10,000 to 40,000 nM had no additional effect on [³H]diprenorphine binding. On the other hand, naloxone (10 nM) acted as a competitive inhibitor of [³H]diprenorphine binding to hMOR-CHO cells (Table 3).

Kinetic Experiments. To determine whether Salvinorin A altered the rate of [³H]DAMGO dissociation from the μ-opioid receptor, membranes prepared from hMOR-CHO cells
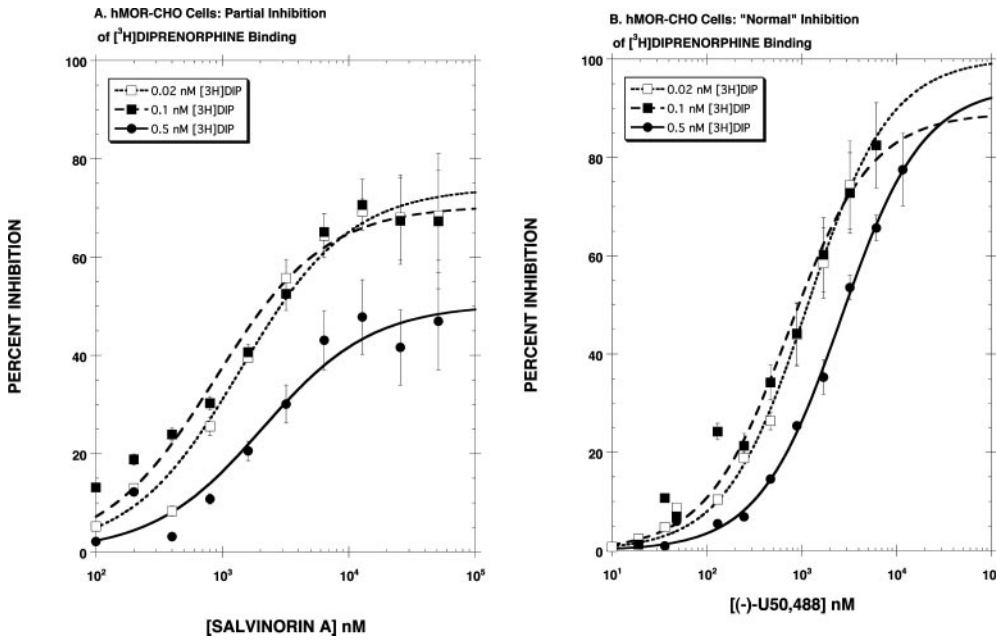
Fig. 4. Inhibition of [³H]diprenorphine binding to hMOR-CHO membranes by Salvinorin A. Three concentrations of [³H] diprenorphine were each displaced by 10 concentrations of Salvinorin A (A) or (−)-U50,488 (B). The data of three experiments, expressed as percentage inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} (see Table 1) as described under *Materials and Methods*. Each point is the mean ± S.E.M. ($n = 3$).

TABLE 2

[³H]DAMGO binding surfaces

DAMGO binding surfaces were generated by displacing two concentrations of [³H]DAMGO (0.5 and 2.5 nM) by nine concentrations of DAMGO in the absence and presence of the indicated concentrations of Salvinorin A. Experiment 1 was done four times, generating a total of 160 data points per experimental condition. Experiments 2 and 3 were done three times, generating 120 data points per experimental condition. The combined data of each condition was fit to the one-site binding model using MLAB-PC for the best-fit estimates (± S.D.) of the K_d and B_{max} values.

Salvinorin A	B_{max}	K_d
nM	*fmol/mg protein ± S.D.*	*nM ± S.D.*
Experiment 1 ($n = 4$) hMOR-CHO cells		
0	2275 ± 125	2.0 ± 0.1
1600	2225 ± 150	3.9 ± 0.3**
6400	2950 ± 225*	7.0 ± 0.5*
Experiment 2 ($n = 3$) hMOR-CHO cells		
0	2725 ± 125	2.8 ± 0.1
10,000	2025 ± 175*	8.3 ± 0.6**
30,000	1750 ± 200**	8.9 ± 1.0**
Experiment 3 ($n = 3$) rat brain		
0	111 ± 5	1.9 ± 0.1
5000	97 ± 11	6.4 ± 0.64**
10,000	53 ± 8**	3.2 ± 0.5*

* $P < 0.01$; ** $P < 0.001$ (*F*-test).

were incubated with 1 nM [³H]DAMGO for 120 min at 25°C. At this point, defined as time 0, baseline samples were filtered, and then drugs were added into paired samples to generate the following conditions: control (no addition), DAMGO (10 μM), Salvinorin A (30 μM), and DAMGO (10 μM) + SA (30 μM). Samples were then filtered at the indicated time points. As reported in Fig. 6, the addition of Salvinorin A to DAMGO seemed to slightly speed up the dissociation [³H]DAMGO binding, whereas the addition of Salvinorin A appeared to slow the dissociation [³H]DAMGO binding. Quantitative analysis of these data revealed that a two-component dissociation model fit the data much better than a one-component model ($P < 1E-10$) (Table 4) and that the addition of Salvinorin A to the DAMGO condition significantly increased the dissociation rate constant (K_2) of the faster-dissociating component. Salvinorin A significantly decreased (1.5-fold) the dissociation rate constant (K_1) of the

slower-dissociating component, accounting for the apparent slower dissociation rate observed in this condition.

Hoping to conduct a dissociation experiment under conditions of a one-component dissociation model, we repeated this experiment using the antagonist [³H]diprenorphine. These experiments were conducted at 37°C, because [³H]diprenorphine dissociation was too slow at 25°C. As reported in Fig. 7 and Table 5, the addition of 10 μM diprenorphine resulted in a fairly rapid dissociation that was best described by a two-component dissociation model. Salvinorin A alone (30 μM) resulted in a much slower dissociation of [³H]diprenorphine, an observation mainly accounted for by a decreased value of K_1 from 0.021 to 0.0025 min^{-1} (Table 5). Interestingly, the addition of an approximate IC_{50} concentration, (−)-U50,488 (1 μM), produced a dissociation curve not significantly different from Salvinorin A. It is noteworthy that the diprenorphine + Salvinorin A condition resulted in statistically significant changes in the kinetic parameters compared with the diprenorphine condition: decreased A_1, decreased K_1, and increased A_2.

Functional Experiments. Using the [³⁵S]GTPγS binding assay, we assessed the effect of Salvinorin A on measures of μ-receptor function. As reported in Fig. 8A and Table 6, Salvinorin A weakly stimulated [³⁵S]GTPγS binding with an EC_{50} of approximately 65,000 nM and an extrapolated E_{MAX} value (202%) approximately 40% lower than that of DAMGO. Interestingly, 10 nM naloxone reduced Salvinorin A-stimulated [³⁵S]GTPγS binding in a noncompetitive manner, significantly reducing the E_{MAX} value without changing the ED_{50} value.

Consistent with the partial agonist profile described above, Salvinorin A significantly reduced the E_{MAX} value of DAMGO-stimulated [³⁵S]GTPγS binding (Fig. 8B; Table 6) by 31 and 42% at 10 and 50 μM, respectively. Salvinorin A also increased the DAMGO ED_{50} values. At a concentration of 10 μM, Salvinorin A increased the ED_{50} for DAMGO from 39 to 192 nM, resulting in a calculated K_e (antagonist K_i value) of 2549 nM. If simple competitive antagonism of Salvinorin A at the μ receptor were responsible for this 4.9-fold increase in the DAMGO ED_{50}, one would predict that a 5-fold
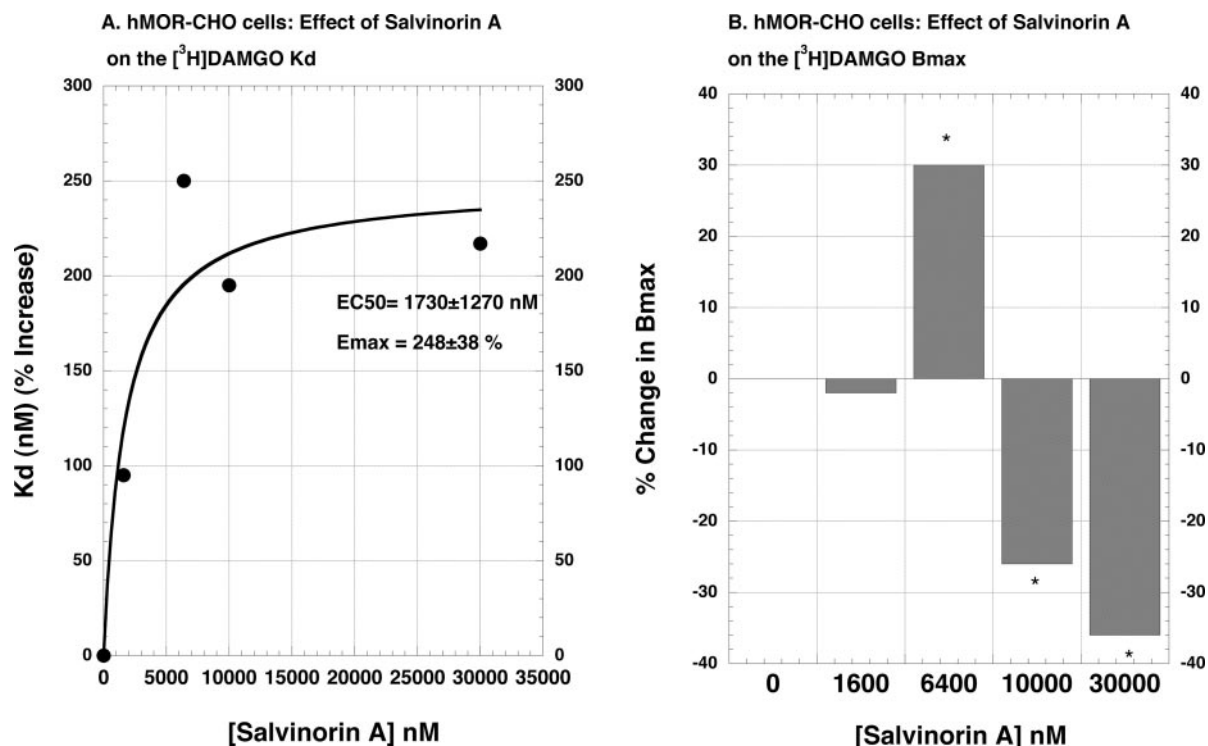
A. hMOR-CHO cells: Effect of Salvinorin A
on the [³H]DAMGO Kd

B. hMOR-CHO cells: Effect of Salvinorin A
on the [³H]DAMGO Bmax

Fig. 5. Salvinorin A has complex effects on the B_{max} and K_d of μ receptors labeled by [³H]DAMGO in hMOR-CHO cell membranes. As described in the legend to Table 2, DAMGO binding surfaces were generated by displacing two concentrations of [³H]DAMGO (0.5 and 2.5 nM) by nine concentrations of DAMGO in the absence and presence of the indicated concentrations of Salvinorin A. A, Salvinorin A increases the [³H]DAMGO K_d value in a dose-dependent manner. All Salvinorin A-induced K_d changes were statistically significant. Each value is the mean ± S.D. (n = 3–4). B, Salvinorin A decreases the [³H]DAMGO B_{max} value at 10 and 30 μM. Each value is the mean ± S.D. (n = 3–4). *, $P < 0.01$ compared with control (F-test).

TABLE 3

[³H]Diprenorphine binding surfaces in hMOR-CHO cells

[³H]Diprenorphine binding surfaces were generated by displacing two concentrations of [³H]diprenorphine (0.08 and 0.47 nM) by nine concentrations of diprenorphine in the absence and presence of the indicated concentrations of either Salvinorin A or naloxone, generating 20 data points per experimental condition. The combined data of each condition (160 points for the Salvinorin A experiments, 120 points for the naloxone experiments) were fit to the one-site binding model using MLAB-PC for the best-fit estimates (± S.D.) of the K_d and B_{max} values.

Test Drug	B_{max}	K_d
nM	*fmol/mg protein ± S.D.*	*nM ± S.D.*
Salvinorin A (n = 4)		
0	288 ± 18	0.73 ± 0.04
10,000	186 ± 18*	1.43 ± 0.11*
40,000	190 ± 14*	1.56 ± 0.12*
Naloxone		
0	515 ± 25	0.97 ± 0.05
10	445 ± 47	2.64 ± 0.25*

* $P < 0.001$ (F-test).

increase in the Salvinorin A concentration to 50 μM should further increase the DAMGO ED₅₀ to 804 nM, such that the same K_e value would result. However, 50 μM Salvinorin A increased the DAMGO ED₅₀ only an additional 1.13-fold to 218 nM. In contrast, 10 nM naloxone, a competitive antagonist, increased the DAMGO ED₅₀ more than predicted based on its K_e determined with a 2.5 nM dose.

We determined the effect of Salvinorin A (50 μM) and DAMGO (10 μM) on basal and forskolin-stimulated cAMP levels. As reported in Fig. 9A, DAMGO and Salvinorin A did not alter basal cAMP levels. As expected, DAMGO almost completely inhibited forskolin-stimulated cAMP accumulation. Salvinorin A inhibited forskolin-stimulated cAMP accumula-

tion by 44%. We compared the effect of naloxone (a competitive inhibitor) and Salvinorin A on DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation in the hMOR-CHO cells. As reported in Table 7, 50 μM Salvinorin A significantly increased the ED₅₀ value. The calculated apparent K_e was 27 μM. Salvinorin A also decreased the E_{MAX} value by ~9%. In contrast, naloxone increased the ED₅₀ without decreasing the E_{MAX}. Thus, in the cAMP assay, Salvinorin A demonstrated partial agonist activity.

The cellular adaptations produced by chronic opioids are generally accepted as signs of opioid dependence. We next determined the effect of Salvinorin A (50 μM) on the cellular adaptations produced by chronic treatment of cells with DAMGO (10 μM) and the novel μ-opioid agonist herkinorin (10 μM) (Harding et al., 2005). Cells were treated for 20 h with DAMGO or herkinorin in the absence and presence of Salvinorin A. We measured two endpoints: forskolin-stimulated cAMP, which detects cAMP superactivation, and naloxone-stimulated cAMP in the presence of forskolin, which detects the presence of constitutively active receptors. As reported in Fig. 9B, chronic DAMGO treatment produced cAMP superactivation without a naloxone overshoot. Chronic herkinorin treatment reduced forskolin-stimulated cAMP, but the addition of naloxone revealed the occurrence of cAMP superactivation and the presence of constitutively active receptors. Salvinorin A did not change the cellular response to either treatment. However, chronic Salvinorin A treatment produced signs of cAMP superactivation.

In contrast to the activity of Salvinorin A in functional assays that measured changes in the level of cellular cAMP, Salvinorin A was inactive in the MAPK assay, which is
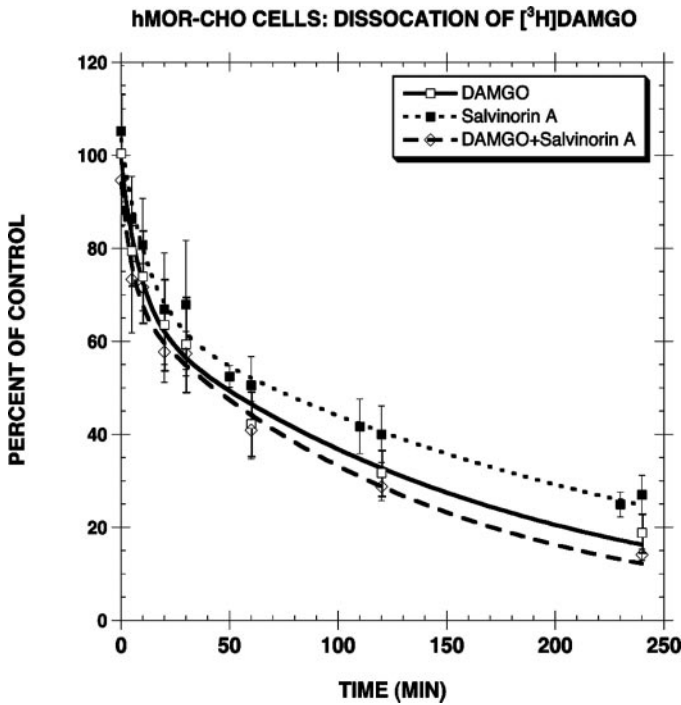
Fig. 6. Salvinorin A alters the dissociation of [³H]DAMGO binding from membranes prepared from hMOR-CHO cells. Membranes were incubated with 1 nM [³H]DAMGO for 120 min at 25°C. At this point, defined as time 0, baseline samples were filtered, and then drugs were added into paired samples to generate the following conditions: control (no addition), DAMGO (10 μM), Salvinorin A (30 μM), and DAMGO (10 μM) + Salvinorin A (30 μM). Samples were then filtered at the indicated time points. The percentage of control was the binding observed in the control condition. Each point is mean ± S.D. ($n = 8–12$).
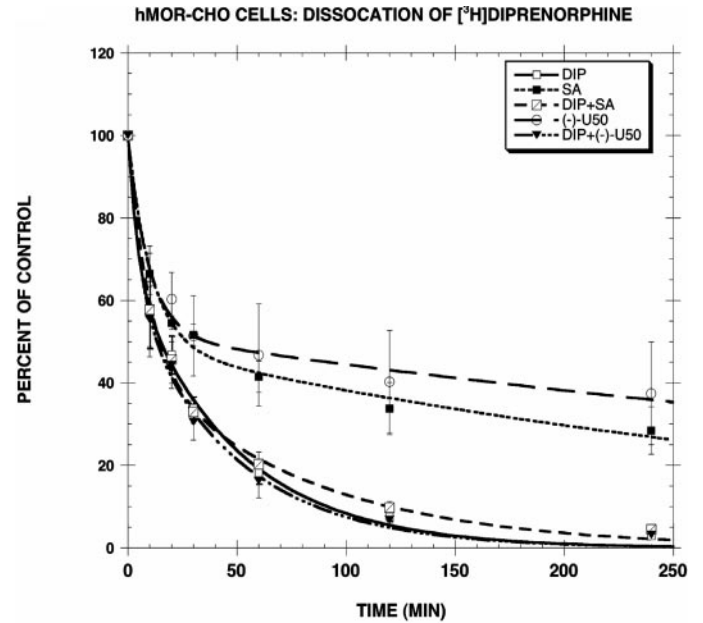

Fig. 7. Salvinorin A alters the dissociation of [³H]diprenorphine binding from membranes prepared from hMOR-CHO cells. Membranes were incubated with 0.1 nM [³H]diprenorphine for 120 min at 37°C. At this point, defined as time 0, baseline samples were filtered, and then drugs were added into paired samples to generate the following conditions: control (no addition), diprenorphine (10 μM), Salvinorin A (30 μM), diprenorphine (10 μM) + Salvinorin A (30 μM), (−)-U50,488 (1 μM), and (−)-U50,488 (1 μM) + diprenorphine (10 μM). Samples were then filtered at the indicated time points. The percentage of control was the binding observed in the control condition. Each point is mean ± S.D. ($n = 4$).

activated by the G$\beta\gamma$ subunit. As reported in Fig. 10, DAMGO stimulated the phosphorylation of MAPK. Salvinorin A alone had no effect and did not alter the effect of DAMGO in this assay.

Discussion

As described in a recent review (Christopoulos and Kenakin, 2002), allosteric modulators of G protein-coupled receptors may be of interest as potential targets for medication development. There are few reports of allosteric modulators of opioid receptors that we are aware of. In 1987, Vaysse et al. (1987) reported that cannabidiol noncompetitively inhibited radioligand binding to μ- and δ-opioid receptors, a finding consistent with allosteric modulation. Subsequent work by another laboratory provided additional evidence for this hypothesis by showing that cannabidiol accelerated the dissociation of [³H]DAMGO and [³H]naltrindole from rat brain μ and δ receptors, respectively (Kath-

mann et al., 2006). We reported in 1991 that pretreating rat brain membranes with (+)-cis-methylfentanyl increased the dissociation rate of μ receptors labeled with [³H]ohmfentanyl (Xu et al., 1991).

In our recent article (Roth et al., 2002), we briefly noted that Salvinorin A, a potent κ-opioid receptor agonist, partially inhibited [¹²⁵I]IOXY binding to μ-opioid receptors (see Table 1 in Harding et al., 2005). In the present study, we tested the hypothesis that Salvinorin A allosterically modulates μ-opioid receptors. Several lines of evidence support this hypothesis.

1) Salvinorin A partially inhibits μ-receptor binding using both the cloned human μ receptor expressed in CHO cells, as well as the native μ receptor present in rat brain membranes. We observed a partial inhibition pattern with three radioligands: [³H]DAMGO, [³H]diprenorphine, and [¹²⁵I]IOXY. As reported in Figs. 3 and 4, the presence of a plateau is most readily observed using radioligand concentrations sufficient to occupy a substantial fraction of μ receptors, such as 0.9 nM [¹²⁵I]IOXY (Fig. 1), 8 nM [³H]DAMGO (Fig. 3), or 0.5 nM [³H]diprenorphine (Fig. 4). The partial inhibition pattern we

TABLE 4

Best-fit parameter estimates for [³H]DAMGO dissociation

[³H]DAMGO (1.0 nM) dissociation curves were generated as described under *Materials and Methods* at 37° C. The data of each experimental condition, generated with hMOR-CHO cell membranes, were pooled and fit using MLAB-PC to the two-component exponential decay model for the best-fit estimates reported above. Each value is the mean ± S.D. ($n = 8–12$).

Condition	A_1	K_1	A_2	K_2	n (Data Points)
μM	% ± S.D.	min^{-1} ± S.D.	% ± S.D.	min^{-1} ± S.D.	
DAMGO (10)	64 ± 5	0.006 ± 0.0001	34 ± 5	0.10 ± 0.03	12 (88)
DAMGO (10) + Salvinorin A (30)	67 ± 3	0.007 ± 0.001	26 ± 4*	0.16 ± 0.06*	8 (61)
Salvinorin A (30)	67 ± 5	0.004 ± 0.001*	37 ± 5	0.08 ± 0.02	12 (84)

* $P < 0.05$ compared with the DAMGO condition (Student's *t*-test).

TABLE 5

Best-fit parameter estimates for [³H]diprenorphine dissociation

[³H]Diprenorphine (0.1 nM) dissociation curves were generated as described under *Materials and Methods* at 37° C. The data of each experimental condition, generated with hMOR-CHO cell membranes, were averaged and fit using KaleidaGraph 3.5 to the two-component exponential decay model for the best-fit estimates reported above. Each value is the mean ± S.D. ($n = 4$).

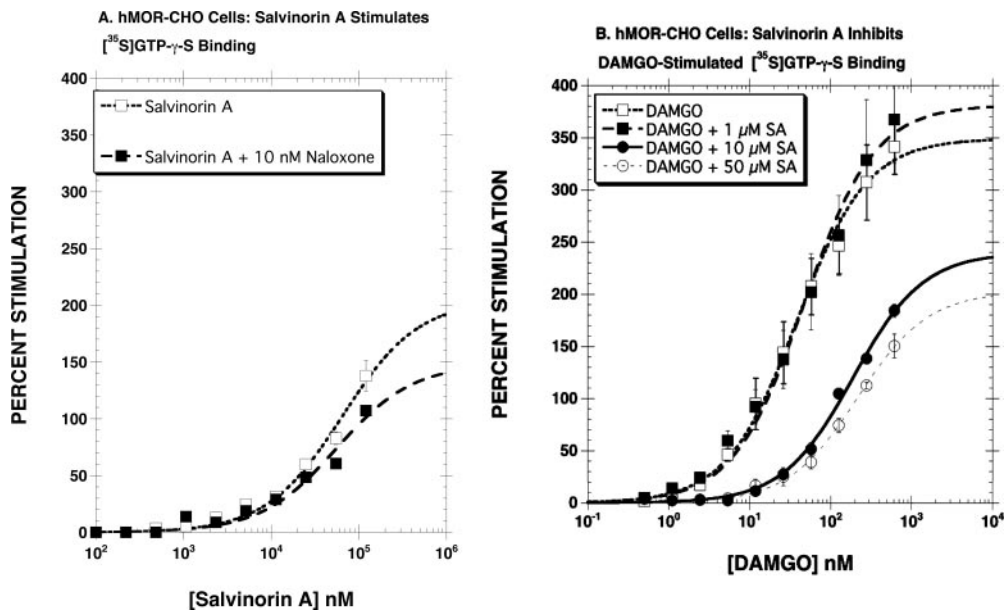| Condition	A_1	K_1	A_2	K_2	n (Data Points)
μM	$\% \pm S.D.$	$min^{-1} \pm S.D.$	$\% \pm S.D.$	$min^{-1} \pm S.D.$	
Diprenorphine (10)	66.8 ± 8.2	0.021 ± 0.003	33.1 ± 8.6	0.22 ± 0.16	4 (28)
Salvinorin A (30)	49.1 ± 3.7*	0.0025 ± 0.0006*	50.5 ± 4.3*	0.093 ± 0.017	4 (28)
Diprenorphine (10) + Salvinorin A (30)	46.0 ± 9.6*	0.012 ± 0.003*	53.6 ± 9.8*	0.10 ± 0.03	4 (28)
(−)-U50,488 (1)	51.8 ± 4.2*	0.0015 ± 0.0006*	47.7 ± 5.4*	0.10 ± 0.03	4 (28)
Diprenorphine (10) + (−)-U50,488 (1)	61.2 ± 11.3	0.021 ± 0.004	38.7 ± 11.6	0.18 ± 0.09	4 (28)

* $P < 0.05$ compared with the diprenorphine condition (Student's t-test).

A. hMOR-CHO Cells: Salvinorin A Stimulates [³⁵S]GTP-γ-S Binding

B. hMOR-CHO Cells: Salvinorin A Inhibits DAMGO-Stimulated [³⁵S]GTP-γ-S Binding

Fig. 8. A, Salvinorin A-stimulated [³⁵S]GTPγS binding in hMOR-CHO cells. Using membranes prepared from hMOR-CHO cells, Salvinorin A dose-response curves were generated in the absence and presence of 10 nM naloxone The data were pooled and analyzed for the best-fit estimates of the E_{MAX} and ED_{50} (see Table 6). Each value is mean ± S.D. ($n = 3$). B, Salvinorin A antagonizes DAMGO-stimulated [³⁵S]GTPγS binding. Using membranes prepared from hMOR-CHO cells, DAMGO dose-response curves were generated in the absence and presence of various concentrations of Salvinorin A and naloxone. The data were pooled and analyzed for the best-fit estimates of the E_{MAX} and ED_{50} (see Table 6). Each value is the mean ± S.D. ($n = 3$).

TABLE 6

Effect of Salvinorin A on [³⁵S]GTPγS binding

DAMGO-stimulated [³⁵S]GTPγS binding dose-response curves were generated using membranes prepared from hMOR-CHO cells as described under *Materials and Methods*. The data of three experiments were pooled, and the best-fit estimates of the ED_{50} and E_{MAX} were determined using MLAB-PC. Each value is mean ± S.D. ($n = 3$).

	E_{MAX}	ED_{50}	Apparent K_e
	$\% Increase \pm S.D.$	$nM \pm S.D.$	nM
A. Salvinorin A-stimulated [³⁵S]GTPγS binding			
Salvinorin A	202 ± 22	65,260 ± 17,060	
Salvinorin A + 10 nM naloxone	148 ± 22*	56,580 ± 19,920	
B. DAMGO-stimulated [³⁵S]GTPγS binding			
DAMGO	349 ± 11	39 ± 4	
DAMGO + 1 μM Salvinorin A	381 ± 16	47 ± 7	
DAMGO + 10 μM Salvinorin A	241 ± 7*	192 ± 14*	2549
DAMGO + 50 μM Salvinorin A	203 ± 9*	218 ± 23*	1089
DAMGO + 2.5 nM naloxone	274 ± 15*	57 ± 12	5.4
DAMGO + 10 nM naloxone	295 ± 10*	146 ± 20*	3.6

* $P < 0.05$ compared with control E_{MAX} (Student's t-test).

observed, where the inhibition curve shifts to the right with a lower E_{MAX} value as the radioligand concentration is increased, is consistent with the theoretical predictions made by Ehlert (1988) for negative allosteric modulators. This partial inhibition pattern is unique to Salvinorin A, because (−)-U50,488, a potent κ agonist, and naloxone, a competitive μ antagonist, produce "normal" inhibition curves without any evidence of a plateau.

2) Salvinorin A affects the K_d and B_{max} of the μ receptor in a manner inconsistent with competitive binding. Using [³H]DAMGO and hMOR-CHO cells, Salvinorin A first in-

creased the μ-receptor B_{max} followed by highly significant decreases in the B_{max} at higher concentrations. It is noteworthy that Salvinorin A increased the K_d of the μ receptor in a dose-dependent manner (Fig. 5A), rather than a linear manner, as would observed with a competitive inhibitor (Ehlert, 1988). Thus, the ability of Salvinorin A to increase the K_d reaches a ceiling at approximately 200% of control. Salvinorin A-mediated uncompetitive inhibition of μ-receptor binding was also observed in rat brain (Table 2) and in hMOR-CHO cells using [³H]diprenorphine (Table 3).

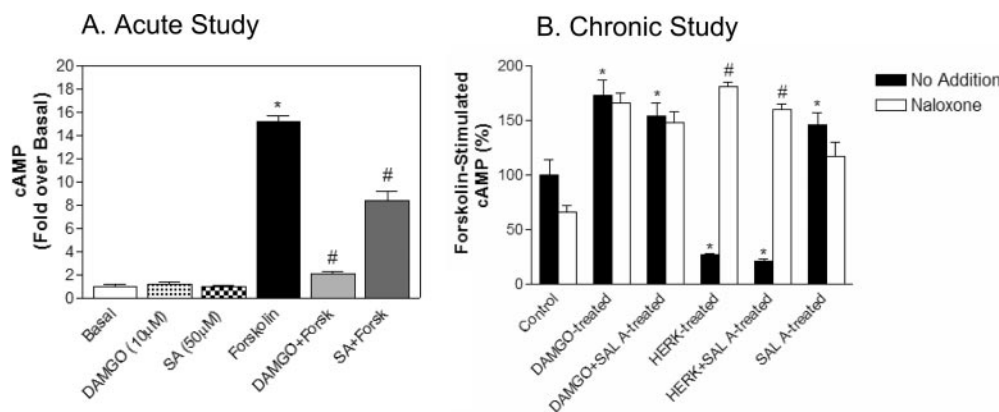3) Salvinorin A alters the kinetics of radioligand dissocia-

A. Acute Study

B. Chronic Study

Fig. 9. Effects of Salvinorin A on the cAMP. A, comparison of Salvinorin A (50 μM) and DAMGO (10 μM) on basal and forskolin-stimulated cAMP accumulation. Each value is mean ± S.E.M. ($n = 3$). ∗, $P < 0.01$ compared with basal; #, $P < 0.01$ compared with forskolin group. B, comparison of the effects of naloxone (10 μM) on forskolin (100 μM)-stimulated cAMP accumulation in the control or pretreated hMOR-CHO cells. Results are presented as mean ± S.E.M. ($n = 3$). ∗, $P < 0.05$ compared with control cells (two-tailed or one-tailed Student's t test); #, $P < 0.01$ compared with no addition group (two-tailed Student's t test).

TABLE 7

Effect of Salvinorin A on DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation

Dose-response curves for DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation were generated as described under *Materials and Methods*. EC$_{50}$ and E_{50} were determined using the program Prism. Each value is the mean ± S.E.M. ($n = 3$).

	ED$_{50}$	E_{MAX}	Apparent K_e
	$nM \pm S.D.$	*% maximal inhibition ± S.D.*	
DAMGO	4.0 ± 0.7	85.6 ± 1.0	
DAMGO + 10 μM Salvinorin A	8.7 ± 2.4	81.8 ± 4.0	8.6 μM
DAMGO + 50 μM Salvinorin A	11.6 ± 0.3**	78.7 ± 1.9*	27 μM
DAMGO + 10 nM naloxone	18.9 ± 3.0**	84.1 ± 2.0	2.7 nM

* $P < 0.05$; ** $P < 0.01$.

A. Phospho-MAP kinase (hMOR-CHO cells)
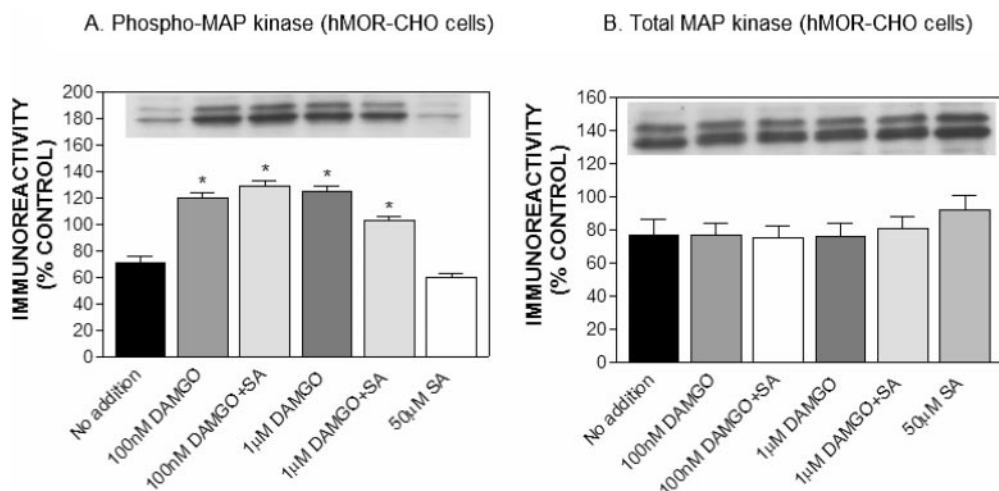
B. Total MAP kinase (hMOR-CHO cells)

Fig. 10. Agonist-stimulated p42/p44 MAPK phosphorylation in the hMOR-CHO cells. The assay was started by the addition of test agents and stopped after 5 min. Western blotting was performed as described under *Materials and Methods*. Results are presented as mean ± S.E.M. ($n = 4$). Representative blots of phosphorylated MAPK (A) and total MAPK (B) are shown. The concentration of SA was 50 μM. ∗, $P < 0.01$ compared with no addition group (two-tailed Student's t test).

tion. It is well known that allosteric modulators can alter the rate of radioligand dissociation (Kostenis and Mohr, 1996). As reported in Table 4, [³H]DAMGO dissociation from μ receptors expressed in hMOR-CHO cells was biexponential, with readily measurable slower ($K_1 = 0.006$ min^{-1}) and faster ($K_2 = 0.10$ min^{-1}) components. The addition of Salvinorin A to DAMGO increased the faster dissociation rate by 160% to 0.16 min^{-1} and decreased the A_2 value by 24 to 26%. Interestingly, the addition of Salvinorin A alone decreased K_1 by 33% to 0.004 min^{-1}. When the μ receptors were labeled with an antagonist ([³H]diprenorphine), the addition of Salvinorin A alone substantially slowed [³H]diprenorphine dissociation (Fig. 7), mainly by decreasing K_1 by more than 10-fold. Viewed in context with the other findings, such as the partial inhibition pattern, these data are consistent with an allosteric effect. However, the addition of a nonallosteric compound, (−)-U50,488, at an approximate IC$_{50}$ concentration resulted in a similarly slowed dissociation of [³H]di-

prenorphine, making it more difficult to interpret the slowed [³H]diprenorphine dissociation produced by Salvinorin A. However, the concurrent addition of Salvinorin A and diprenorphine decreased the A_1 value by 31%, decreased the K_1 value by 43%, and increased the K_2 value by 162%, providing direct evidence for an allosteric effect of Salvinorin A.

4) Salvinorin A acts as an uncompetitive inhibitor of DAMGO-stimulated [³⁵S]GTPγS binding (Fig. 8B). As reported in Table 6, Salvinorin A produced a dose-dependent decrease in the E_{MAX} and failed to increase the ED$_{50}$ value significantly when the Salvinorin A concentration was increased from 10 to 50 μM, as was observed for a competitive inhibitor, naloxone. We believe that these four lines of data support the hypothesis that Salvinorin A allosterically modulates μ-receptor binding and function.

The effects of Salvinorin A in the functional assays deserve additional study. For example, Salvinorin A stimulates [³⁵S]GTPγS binding with low potency and an extrapolated

E_{MAX} value of approximately 42% that of DAMGO. The simplest explanation of these data is that Salvinorin A is a weak partial μ agonist. This finding is supported by the ability of Salvinorin A to decrease forskolin-stimulated cAMP accumulation and to induce cAMP superactivation (Fig. 9). However, naloxone noncompetitively inhibits Salvinorin A-stimulated [^{35}S]GTPγS binding, suggesting that Salvinorin A acts at a site on the μ receptor distinct from that of typical μ ligands. This viewpoint is supported by point four in the previous paragraph and the fact that Salvinorin A, unlike DAMGO, had no activity in the MAPK assay (Fig. 10). Viewed collectively, the functional data indicate that Salvinorin A may have some partial agonist activity at μ receptors in addition to the allosteric effects described above. Assuming that Salvinorin A has partial agonist activity at μ receptors, it might be possible to detect μ-mediated antinociception following administration of Salvinorin A. However, Salvinorin A-induced antinociception was not observed in κ receptor knockout mice (Ansonoff et al., 2006), suggesting that the potency of Salvinorin A as a partial μ agonist is probably too low to produce detectable antinociception.

The ultimate significance of this work remains to be seen. However, the immediate significance of our findings is the clear demonstration that the μ-opioid receptor possesses an allosteric modulator site. A major challenge of subsequent work will be to identify more potent ligands for the allosteric site. Toward this end, we note that certain analogs of Salvinorin A also partially inhibit μ-opioid receptors (Tidgewell et al., 2006) as well as δ-opioid receptors (R. B. Rothman and T. E. Prisinzano, unpublished data). Thus, we anticipate that the Salvinorin A structural template will yield a number of allosteric modulators of opioid receptors. A more complete structure-activity profile of the allosteric site will be used to design more potent allosteric ligands. Once these are available, it will be possible to determine the in vivo effects and potential therapeutic application of allosteric modulators of μ-opioid receptors. It is possible, as observed with other classes of medications that work via allosteric mechanisms (benzodiazepines), that allosteric modulators of opioid receptors will have therapeutic value. Because a positive allosteric modulator will enhance the action of endogenous ligands acting via μ receptors, such a drug could produce analgesia with fewer adverse effects than that produced by direct μ-receptor agonists. Furthermore, it is unfortunate that we do not yet know the molecular basis of the allosteric actions of Salvinorin A at the μ-opioid receptor. Site-directed mutagenesis studies, such as those that delineated the interaction of Salvinorin A at the κ-opioid receptor (Yan et al., 2005), will be necessary to definitively prove that the Salvinorin A-induced allosteric effects reported here are mediated via a binding site on the μ receptor distinct from the binding site of other μ ligands.

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Address correspondence to: Dr. Richard B. Rothman, Clinical Psychopharmacology Section, 5500 Nathan Shock Drive, Baltimore, MD 21224. E-mail: rrothman@mail.nih.gov