

Kappa opioid mediation of cannabinoid effects of the potent hallucinogen, salvinorin A, in rodents

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Abstract

Rationale Salvinorin A, the primary psychoactive derivative of the hallucinogenic herb *Salvia divinorum*, is a potent and highly selective kappa-opioid receptor (KOR) agonist. Several recent studies, however, have suggested endocannabinoid system mediation of some of its effects.

Objectives This study represents a systematic examination of this hypothesis.

Methods Salvinorin A was isolated from *S. divinorum* and was evaluated in a battery of in vitro and in vivo procedures designed to detect cannabinoid activity, including CB₁ receptor radioligand and [³⁵S]GTPγS binding, calcium flux assay, in vivo cannabinoid screening tests, and drug discrimination.

Results Salvinorin A did not bind to nor activate CB₁ receptors. In vivo salvinorin A produced pronounced hypolocomotion and antinociception (and to a lesser extent, hypothermia). These effects were blocked by the selective KOR antagonist, JD₁c, but not by the CB₁ receptor antagonist rimonabant. Interestingly, however, rimonabant attenuated KOR activation stimulated by U69,593 in a [³⁵S]GTPγS assay. Salvinorin A

did not substitute for Δ⁹-tetrahydrocannabinol (THC) in mice trained to discriminate THC.

Conclusions These findings suggest that similarities in the pharmacological effects of salvinorin A and those of cannabinoids are mediated by its activation of KOR rather than by any direct action of salvinorin A on the endocannabinoid system. Further, the results suggest that rimonabant reversal of salvinorin A effects in previous studies may be explained in part by rimonabant attenuation of KOR activation.

Keywords Cannabinoid · JD₁c · Kappa-opioid agonist · Kappa-opioid antagonist · Rimonabant · *Salvia divinorum* · Salvinorin A

Salvia divinorum is a powerful psychoactive herb used in traditional spiritual and curative practices by the indigenous Mazatec people of southern Mexico (Wasson 1962). Despite its potent hallucinogenic properties, the Mazatecs have exploited *S. divinorum* for a variety of therapeutic purposes, including treatment of rheumatism, headache, and diarrhea (Valdes et al. 1983), conditions once treated with cannabis in India (Schultes 1979). The herb has also been smoked as a marijuana substitute by young Mexicans (Valdes 1994; Valdes et al. 1983) and recently has been gaining prominence as a drug of abuse in the United States and Europe (Giroud et al. 2000). The primary psychoactive constituent of *S. divinorum* is the *trans*-neoclerodane diterpenoid, salvinorin A (Fig. 1), a compound that is distinct in structure and mechanism of action from typical alkaloid hallucinogens such as lysergic acid diethylamide (LSD), *N,N*-dimethyltryptamine, and mescaline (Roth et al. 2002; Valdes et al. 1983). In addition, salvinorin A is one of the most potent naturally occurring hallucinogens known, similar in potency to LSD. Inhaled doses of 200–

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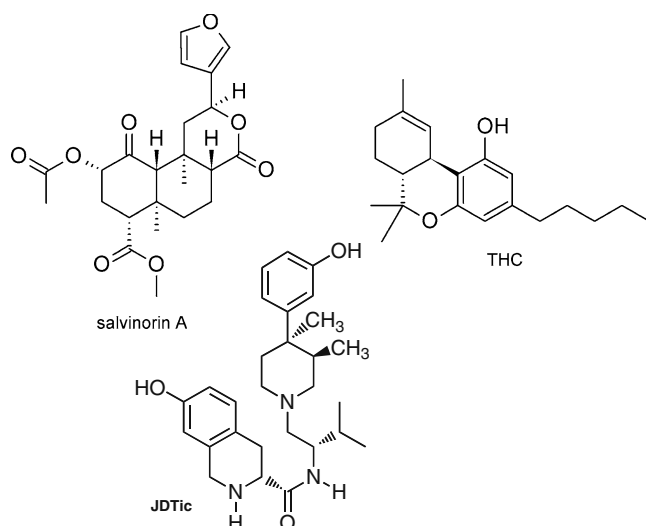


Fig. 1 Chemical structures of salvinorin A, JDtic, and Δ^9 -tetrahydrocannabinol (THC)

500 μg in humans produce profound hallucinations lasting up to 1 h, with themes including feelings of physical or mental displacement, exceptionally convincing illusions, and loss of identity (Siebert 1994).

Unlike classical hallucinogens, salvinorin A does not show affinity for any serotonin receptor subtype (Chavkin et al. 2004). Instead, radioligand displacement and [^{35}S] GTP γ S binding studies have revealed salvinorin A to be a full and selective kappa-opioid receptor (KOR) agonist with similar efficacy to dynorphin A (Roth et al. 2002). In contrast, salvinorin B, a compound that is found in much lower quantities in the *S. divinorum* plant and may be a metabolite of salvinorin A, is not active at this site. Like other KOR agonists, salvinorin A dose dependently decreased dopamine levels in the caudate putamen; however, unlike other KOR agonists, salvinorin A failed to decrease dopamine levels in the nucleus accumbens (Zhang et al. 2005). In addition, while KOR agonists generally produce conditioned place aversion and decreased locomotor activity, salvinorin A induced place preference (0.2–0.5 $\mu\text{g}/\text{kg}$) and enhanced locomotor activity (0.1–0.2 $\mu\text{g}/\text{kg}$) in zebrafish (Braidia et al. 2007). As expected, these effects were blocked by KOR antagonist nor-binaltorphimine (nor-BNI). Surprisingly, however, these effects, as well as salvinorin A self-administration in rats, were also blocked by the brain cannabinoid (CB $_1$) receptor antagonist rimona-bant (Braidia et al. 2008, 2007), suggesting that salvinorin A may also act on the endocannabinoid system. Given these recent studies that have suggested a role for the endocannabinoid system in some of the effects of salvinorin A, the objective of the present study was to examine the effects of salvinorin A using a comprehensive set of neurochemical and behavioral methods aimed at detecting cannabinoid activity.

Methods

Subjects

Adult male ICR mice (25–32 g) [Harlan, Dublin, VA, USA], housed in groups of five, served as subjects for the tetrad tests. The drug discrimination study used individually housed adult male C57B1/6J mice (20–25 g) [Jackson Laboratories, Bar Harbor, ME, USA]. All mice were housed in clear plastic cages (18 \times 29 \times 13 cm) with steel wire fitted tops and wood-chip bedding in a temperature-controlled (20–22 $^{\circ}\text{C}$) vivarium. Water and food were available ad libitum for mice in the tetrad test study. Mice in the drug discrimination study were maintained at 85–90% of free-feeding body weights by restricting daily ration of standard rodent chow and had ad libitum water availability except while in the operant conditioning chambers. All animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of Virginia Commonwealth University and the “Guide for the Care and Use of Laboratory Animals” (National Research Council 1996).

Apparatus

Assessment of spontaneous activity in mice occurred in standard plastic mouse cages (28 \times 16.5 cm) inserted into a frame containing 16 photocell beams and interfaced with a Digiscan Animal Activity Monitor (Omnitech Electronics, Inc., Columbus, OH, USA). Rectal temperature was measured by a thermistor probe (inserted 25 mm) and a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH, USA). Drug discrimination training and testing occurred in eight standard mouse operant conditioning chambers that were sound and light attenuated (MED Associates, St. Albans, VT, USA). Each operant conditioning chamber (18 \times 18 \times 18 cm) was equipped with a house light, two levers (left and right), and a recessed dipper receptacle centered between the levers. A dipper arm delivered sweetened milk in a 0.05-ml cup, which was available for 5 s. Fan motors provided ventilation and masking noise for each chamber. House lights were illuminated during training and testing sessions. A computer with Logic “1” interface (MED Associates, St. Albans, VT, USA) and MED-PC software (MED Associates) was used to control schedule contingencies and record data.

Procedures

Radioligand binding

The methods used for performing binding assays in transfected cells expressing human CB $_1$ (hCB $_1$) or CB $_2$

receptors are those described previously (Thomas et al. 1998). The CB₁ receptor involves a HEK-293 expression system whereas the CB₂ receptor is expressed in CHO-K1 cells. Binding is initiated with the addition of 40 pM of cell membrane protein to 96 well plates containing 7.2 nM [³H]CP55,940 or 20 nM [³H]SR141716, a test compound (for displacement studies), and a sufficient quantity of buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl₂, 5 mg/mL bovine serum albumin (BSA), pH 7.4) to bring the total incubation volume to 0.5 mL. After incubation (60 min), bound radioligand is separated from free via rapid vacuum filtration over GF-B filters using a 96-well harvester (Brandel Scientific; Gaithersburg, MD, USA). The plates are air-dried and 20 mL of Microscint20 (PerkinElmer, Waltham, MA, USA) is added to each well. Bound radioactivity is determined using a TopCount 12-detector instrument (Packard Instruments) using standard scintillation counting techniques. Data are analyzed using GraphPad Prism to calculate affinity (K₁) or activity/potency (PA₂), typically using global nonlinear regression analysis.

[³⁵S]GTPγS

Transfected cells expressing hCB₁ or human KOR were used for [³⁵S]GTPγS binding. The assays were conducted in a 96-well plate format as previously described (Carroll et al. 2005; Thomas et al. 2005). For measurement of [³⁵S]GTPγ binding activation, membranes were resuspended in 50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4. Concentration-effect curves were generated by incubating the appropriate concentration of membrane (5–10 μg) for 1 h in assay buffer with 1 g/L BSA containing increasing concentrations of CP55,940 or U69,593 (positive controls for CB₁ receptors and KOR, respectively), rimonabant, or salvinorin A (in duplicate), 30 mM GDP, and 100 pM [³⁵S]GTPγS. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The reaction was terminated by filtration under vacuum, followed by three washes with cold (4°C) Tris buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry. Duplicate samples were tested.

Calcium influx

The calcium flux assay (Navarro et al. 2009) was performed in hCB₁ receptors expressed in RD-HGA16 cells (Molecular Devices, Sunnyvale, CA, USA). These cells overexpress the promiscuous G protein, Ga16. Consequently, hCB₁ receptor activation is coupled to the mobilization of internal calcium instead of to G_i and the inhibition of adenylyl cyclase. The calcium 3 dye assays

(Molecular Devices) were run in wells of black clear-bottom 96-well tissue culture-treated plates that have been seeded with 20,000 cells. Cells were incubated with the calcium indicator dye for 1 h at 37°C. The plate was then placed into the FlexStation prewarmed to 37°C. Basal or unstimulated fluorescence intensity was recorded for 13 s followed by the addition of salvinorin A or CP55,940 (1 μM). Fluorescence intensity was recorded for an additional 47 s.

Mouse tetrad tests

Each mouse was tested in all of the tetrad assays: locomotor activity, tail flick, rectal temperature, and ring immobility (Martin et al. 1991). Prior to injection, rectal temperature and baseline latency in the tail flick test were measured in the mice. The latter procedure involved exposing the mouse's tail to an ambient heat source (i.e., bright light) and recording latency (in s) for tail removal. Typical control latencies were 2–4 s. A 10-s maximal latency was used in order to avoid damage to the mouse's tail. After measurement of temperature and baseline tail flick latency, mice were injected i.v. with vehicle or drug. Ten minutes later they were retested in the tail flick procedure. Immediately thereafter, the mice were placed into individual activity chambers for 10 min. Spontaneous activity was measured as the total number of beam interruptions during the entire session, which was expressed as percent inhibition of the control (vehicle) group's activity. Antinociception was expressed as the percent maximum possible effect (MPE) using a 10-s maximum test latency. Rectal temperature was expressed as the difference between pre- and postinjection rectal temperatures. Immediately after measurement of body temperature, the mice were placed on a 5.5-cm ring attached at a height of 16 cm to a ring stand, and the amount of time the animals remained motionless during a 5-min period was recorded. The time that each animal remained motionless on the ring was divided by 300 s and multiplied by 100 to obtain a percent immobility rating. Antagonism tests followed an identical time course, but included i.p. antagonist administration 1 h before drug for JDTic and 10 min before drug for rimonabant.

Drug discrimination

Each mouse was placed daily in a standard operant conditioning chamber for 15 min sessions and trained to press either of two levers according to a fixed ratio (FR) 1 schedule of reinforcement. Milk reinforcement was delivered after every lever press. The FR value was gradually increased to the final FR-10 schedule of reinforcement in which ten consecutive responses were required for delivery of milk reinforcement. After mice were trained on one

lever, the reinforcement criterion was switched to the other lever. Lever training at this second lever proceeded identically to training at the first. When a FR-10 schedule of reinforcement was met on the second lever, discrimination training began.

As described previously (Vann et al. 2009), mice were trained to press one lever following administration of THC and to press the other lever following vehicle injection, according to a FR-10 schedule of milk reinforcement. Responses on the incorrect lever reset the ratio requirement on the correct lever. Daily injections were administered on a double alternation sequence of THC and vehicle (e.g., drug, drug, vehicle, vehicle). Daily 15-min training sessions were held Monday–Friday until the mice had met two criteria during seven of eight consecutive sessions: (1) the first completed FR-10 was on the correct lever and (2) $\geq 80\%$ of the total responding occurred on the correct lever. When the two criteria were met, discrimination training was established and substitution testing began.

Following successful training, stimulus substitution tests were conducted on Tuesdays and Fridays during 15-min test sessions. Training continued on Mondays, Wednesdays, and Thursdays. During test sessions, responses on either lever delivered reinforcement according to an FR-10 schedule. To be tested, mice must have completed the first FR-10 on the correct lever and $\geq 80\%$ of the total responding must have occurred on the correct lever. In addition, the mouse must have met these same criteria during previous training sessions with the alternate training compound (training drug or vehicle). Prior to substitution tests, a generalization curve for THC was generated in all mice. Then, substitution tests were conducted with salvinorin A. Control tests with vehicle and THC were redetermined prior to conducting substitution tests.

Drugs

Salvinorin A was isolated from *S. divinorum* leaves according to modification of a procedure developed by Gartz (2001) and described on the website at <http://designer-drugs.com/pte/12.162.180.114/dcd/chemistry/salvinorin.extraction.html>. A sample of dried leaves (200 g) and a 4-L beaker with acetone (3,500 mL) were separately and cooled (-20 – 0°C). Subsequently, they were combined in a 4-L beaker and stirred with a large spatula for 1 min. The acetone extract was decanted through a large, cooled porcelain Büchner funnel (without filter paper) into a 4-L Erlenmeyer flask. A second extraction was conducted on the leaves with another 3,500 mL of cold acetone. The combined extracts were then filtered through a Celite bed on a Nylon membrane ($0.45\ \mu\text{m}$). This process was repeated in four batches for an additional 800 g of leaves. The combined extracts were rotary evaporated under vacuum and the

residue was dried on a vacuum pump to yield 10 g of dark green solid. The solid was dissolved in hot MeOH (190 mL) and recrystallized at room temperature overnight. The solution was placed in the freezer for 2 h, filtered, and the solid was washed $3 \times 15\ \text{mL}$ cold MeOH and $3 \times 15\ \text{mL}$ hexanes. The solid was dried to yield three crops of off-white crystals totaling 3.00–3.4 g of impure salvinorin A per kilogram of leaves from repeated runs. A second recrystallization from methanol afforded an average of 2.6 g of $>99\%$ pure crystals (HPLC) from a kilogram of leaves. ^1H NMR, CMR, and mass spectra (APCI M-1 431) agree with those reported previously for salvinorin A (Valdes et al. 1984).

For the in vivo studies, THC (National Institute on Drug Abuse; NIDA, Rockville, MD, USA), rimonabant (NIDA) and salvinorin A were dissolved in a vehicle of 7.8% Tween 80 and 92.2% physiological saline. (3*R*)-7-Hydroxy-*N*-[(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide (JDTic; Cai et al. 2008), synthesized in RTI laboratories, was dissolved in a vehicle of 1:1:18 emulphor/ethanol/saline. All drugs were administered at a volume of 0.1 ml/10 g.

Data analysis

Radioligand binding

Competitive radioligand displacement data were analyzed by unweighted nonlinear regression analysis. Curve fitting and IC_{50} calculation were done with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA), which fits the data to one and two-site models and compares the two fits statistically. IC_{50} values were then converted to K_i values by reported methods (Cheng and Prusoff 1973).

[^{35}S]GTP γS binding

The specific binding of [^{35}S]GTP γS in the absence of test compound was termed “basal” activity. Test compounds were characterized as agonist, antagonist, or inverse agonist based on their effect on basal activity. Effects of the test compounds were reported as the percent change from basal. Curves were analyzed and EC_{50}s were calculated using nonlinear curve fitting (Prism, GraphPad).

Calcium influx

Drug effects in the calcium flux assay were expressed as relative fluorescence units, calculated as the difference between minimum and maximum fluorescence during the 47-s recording period. Values are reported as means \pm SEM from three independent experiments.

Tetrad

Antinociception was calculated as percent of maximum possible effect $\{\%MPE = [(test - control\ latency)/(10 - control)] \times 100\}$. Rectal temperature values were expressed as the difference between control temperature (before injection) and temperatures following drug administration ($\Delta^\circ C$). Spontaneous activity was expressed as percentage of inhibition of activity of the vehicle group. The total amount of time that the mouse remained motionless was divided by 300 s and multiplied by 100 to obtain a percent immobility rating. In order to determine whether THC and salvinorin A produced agonist effects, separate one-way ANOVAs were conducted for each of the tetrad measures and the effects of each drug was compared to vehicle only (V/V) condition. In order to determine whether the JDtic or rimonabant antagonized the effects of THC and/or salvinorin A, separate two-way ANOVAs [first injection (vehicle, JDtic or rimonabant) \times second injection (THC or salvinorin A)] were performed for each measure. Tukey post-hoc tests ($\alpha < 0.05$) were used for post-hoc comparisons when appropriate.

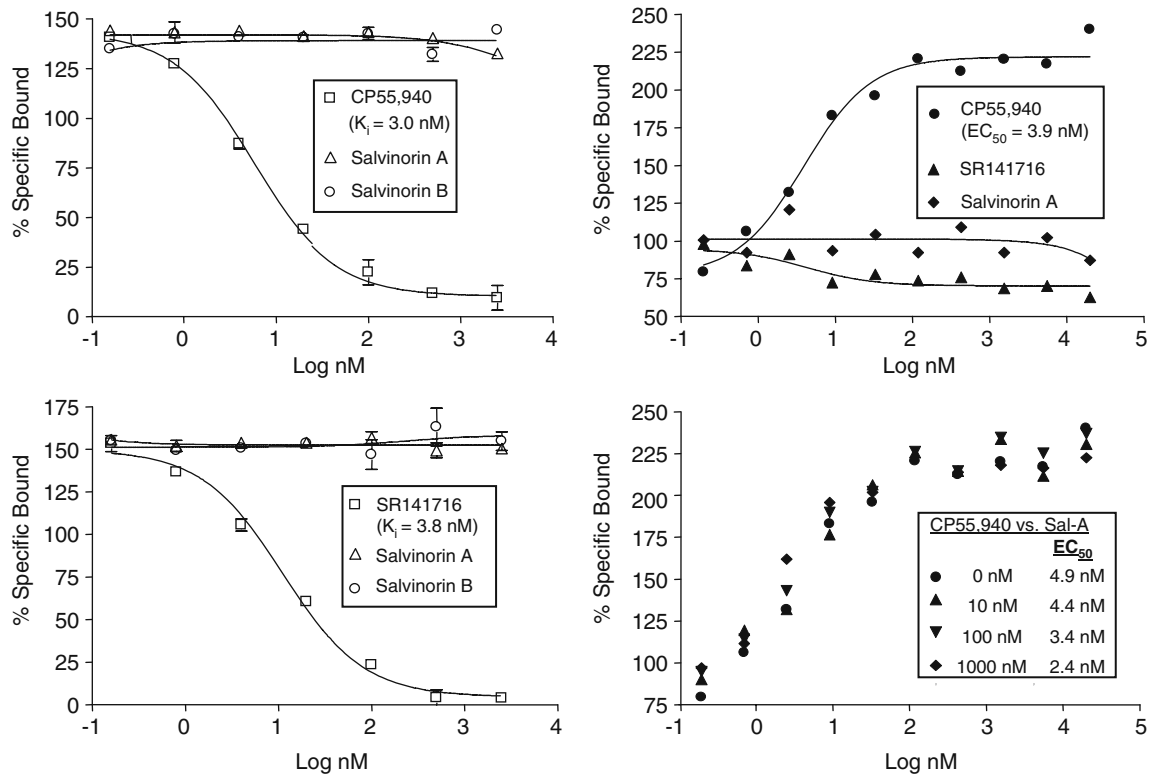


Fig. 2 Binding affinity of salvinorin A and salvinorin B at hCB₁ receptors in radioligand competition binding assays using [³H]CP55,940 (top left panel) and [³H]SR141716 (bottom left panel) in transfected CHO cells. Control curves obtained with CP55,940 and SR141716 (rimonabant), respectively, are shown for comparison. Effects of salvinorin A on [³⁵S]GTP γ S binding and CP55,940-

Drug discrimination

For each test session, percentage of responses on the drug lever and response rate (responses/s) were calculated. Full substitution was defined as $\geq 80\%$ drug-lever responding. Since mice that responded less than ten times during a test session did not press either lever a sufficient number of times to earn a reinforcer, their data were excluded from analysis of drug lever selection, but response rate data were included.

Results

Salvinorin A and B failed to show affinity or efficacy at CB₁ receptors in any of the in vitro evaluations. Neither compound displaced a radiolabeled CB₁ receptor agonist, [³H]-CP55,940, or CB₁ receptor antagonist, [³H]-SR141716, from hCB₁ receptors expressed in transfected CHO cells (Fig. 2, left top and bottom panels, respectively). In [³⁵S]GTP γ S assays, salvinorin A did not activate hCB₁ receptors nor did it block their activation by CP55,940

stimulated [³⁵S]GTP γ S binding in hCB₁ receptors (top and bottom right panels, respectively). For comparison purposes, the effects of a CB₁ receptor agonist (CP55,940) and/or a CB₁ receptor antagonist/inverse agonist (SR141716) are also shown. Values represent the mean (\pm SEM) of three independent experiments

(Fig. 2, right top and bottom panels, respectively). Salvinorin A and B also did not significantly affect calcium ion flux in hCB₁ receptors (Fig. 3, top panel). Hence, in vitro results show that salvinorin A does not bind to nor activate CB₁ receptors. In contrast, rimonabant antagonized the effects produced by dose-dependent stimulation of the KOR agonist U69,593 in the receptors as measured in the [³⁵S]GTPγS assay (Fig. 3, bottom panel).

As expected, THC produced a profile of in vivo activity in mice that is characteristic of psychoactive cannabinoids (Fig. 4). THC dose dependently suppressed spontaneous activity, decreased body temperature, and produced antinociception and catalepsy. Salvinorin A also showed dose-dependent activity in each of the four assays, although its efficacy for inducing hypothermia and catalepsy was decreased as compared to THC. Antagonist tests showed that the in vivo effects of salvinorin A (10 mg/kg) were reversed by coadministration of the KOR antagonist JD₁Tic (30 mg/kg), but not by coadministration of the CB₁ receptor antagonist rimonabant (10 mg/kg) [Fig. 5]. In contrast,

THC (10 mg/kg) induced locomotor suppression, antinociception, and catalepsy effects in the cannabinoid tetrad tests and these effects were attenuated by rimonabant (10 mg/kg), but not by JD₁Tic (30 mg/kg), whereas its hypothermic effects were attenuated by both antagonists (Fig. 5). Neither antagonist produced effects in the cannabinoid tetrad tests when administered alone (data not shown).

Figure 6 shows the effects of salvinorin A in mice trained to discriminate 10 mg/kg THC from vehicle. Whereas THC fully and dose dependently substituted for the training dose, salvinorin A failed to substitute at any dose, including doses that suppressed spontaneous activity in the tetrad assessment.

Discussion

Subsequent to isolation of salvinorin A as the primary psychoactive substituent of *S. divinorum* (Ortega et al. 1982), a number of studies have shown that the pharmacology of this substituent is mediated by activation of KOR (Chavkin et al. 2004; Roth et al. 2002). Recently, however, the results of several studies have suggested that the endocannabinoid system also may play a role in the pharmacological effects of salvinorin A (Braida et al. 2009, 2008, 2007). While lower doses of salvinorin A produced conditioned place preference in both rats and zebrafish and were self-administered via intracerebroventricular infusion in rats, higher doses either had no effect or produced aversion in these procedures (Braida et al. 2008, 2007). A more recent study by the same lab group reported that salvinorin A also produced anxiolytic- and antidepressant-like behavior in rats (Braida et al. 2009). Effects in each procedure were reversed by the KOR antagonist nor-BNI, but also by the CB₁ receptor antagonists, rimonabant, and AM251. While the authors suggest that these results demonstrate mediation of the behavioral effects of salvinorin A by both KOR and endocannabinoid systems, direct agonist activity at CB₁ receptors is unlikely, as several studies have shown that salvinorin A does not bind to these receptors (Chavkin et al. 2004; Roth et al. 2002) or has only weak affinity (Braida et al. 2009; Capasso et al. 2008). The purpose of the present study was to evaluate salvinorin A in a comprehensive battery of in vivo and in vitro assays that have traditionally been used to screen for cannabinoid activity (Martin et al. 1991). In parallel experiments, mediation of the in vivo pharmacological effects of salvinorin A via KOR activity was assessed.

Since binding affinity of cannabinoids may depend upon the radioligand (Thomas et al. 1998), each series of displacement experiments with salvinorin A was conducted with both [³H]CP55,940 and [³H]SR141716. Consistent

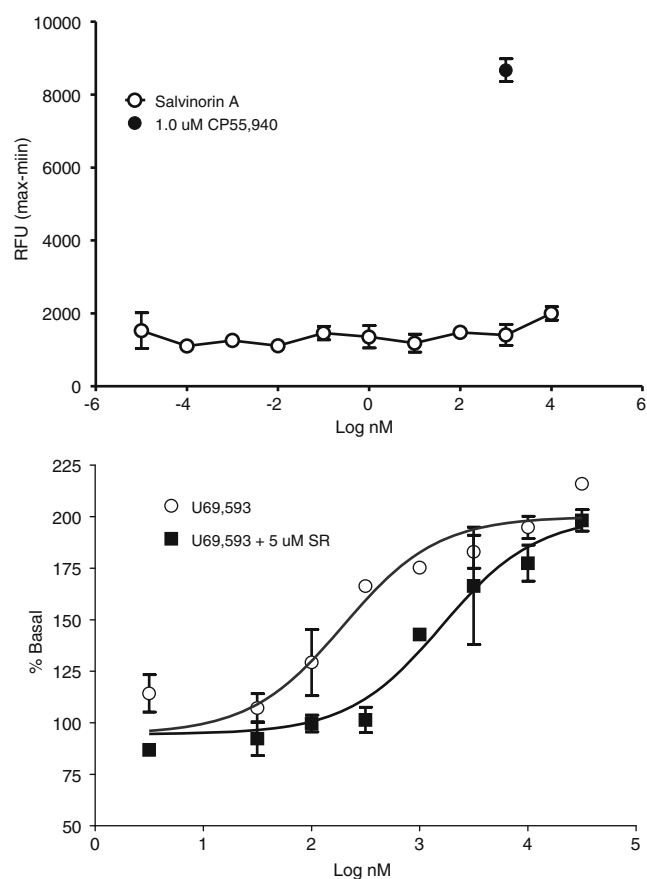
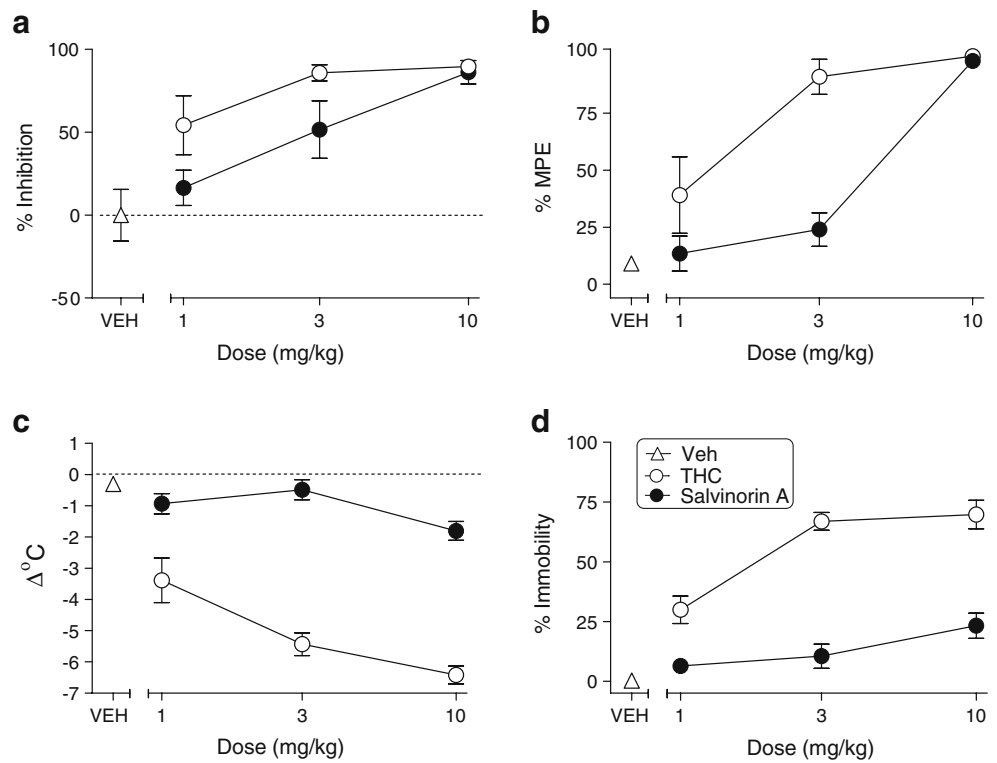


Fig. 3 Effects of salvinorin A, salvinorin B, and CP55,940 on calcium ion flux in hCB₁ transfected CHO cells (*top panel*). The *bottom panel* shows SR141716 (rimonabant) antagonism of KOR activation by agonist U69,593 in a [³⁵S]GTPγS binding assay. Values represent the mean (±SEM) of three independent experiments

Fig. 4 Effects of THC (10 mg/kg) and salvinorin A (10 mg/kg) on percentage of inhibition of spontaneous activity [panel A], percentage of maximum possible antinociceptive effect (%MPE) [panel B], change in rectal temperature ($\Delta^{\circ}\text{C}$) [panel C], and catalepsy (measured as percentage of time immobile on an elevated ring apparatus) [panel D]. Values represent the mean (\pm SEM) of five to 12 mice per group



with previous reports (Chavkin et al. 2004; Roth et al. 2002), results of the present study showed that salvinorin A did not have affinity for CB₁ receptors expressed in CHO cells, regardless of whether radiolabeled agonist or antagonist was used for displacement. It also did not activate CB₁ receptors in functional assays of G protein and calcium channel signaling, as do known cannabinoid agonists (Childers 2006; Turu and Hunyady 2010), nor did it block

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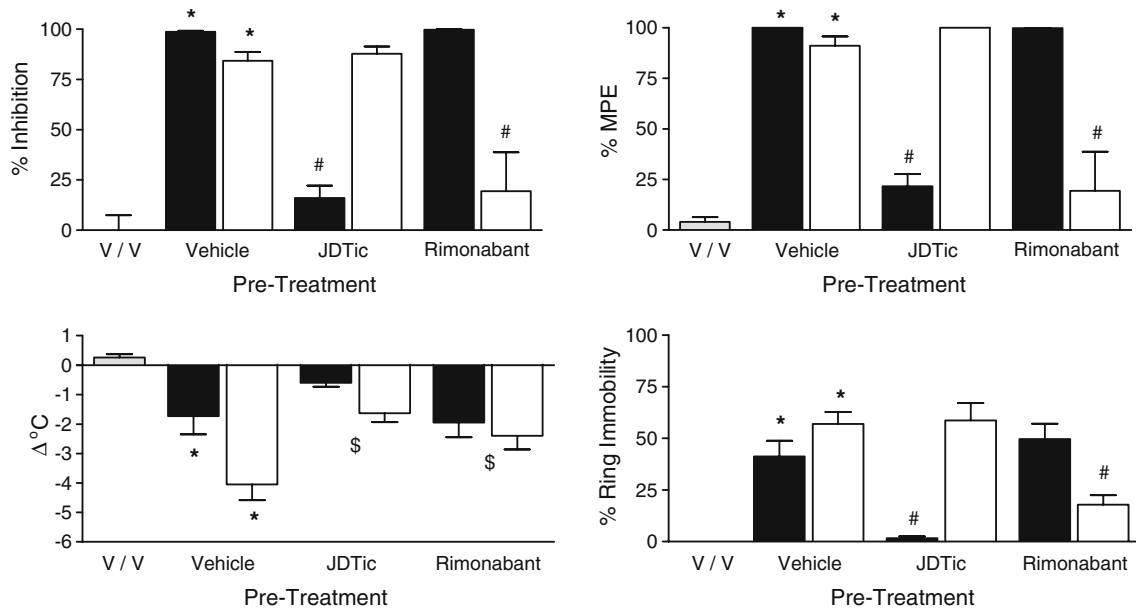


Fig. 5 Effects of pretreatment with vehicle, 30 mg/kg JDtic (KOR antagonist) or 10 mg/kg rimonabant followed by an injection of 10 mg/kg salvinorin A (filled bars) or 10 mg/kg THC (unfilled bars) on percentage of inhibition of spontaneous activity [top left panel], percentage of maximum possible antinociceptive effect (%MPE) [top right panel], change in rectal temperature ($\Delta^{\circ}\text{C}$) [bottom left panel], and catalepsy (percentage of time immobile on an elevated ring

apparatus) [bottom right panel]. Mice in a control group received two injections of vehicle (V/V). Values represent the mean (\pm SEM) of five to 12 mice per group. Asterisk indicates significant effect (vs. V/V) for THC or salvinorin A. Number sign indicates significant interaction and post-hoc difference from corresponding vehicle pretreatment. Dollar sign indicates main effect of pretreatment (vs. vehicle)

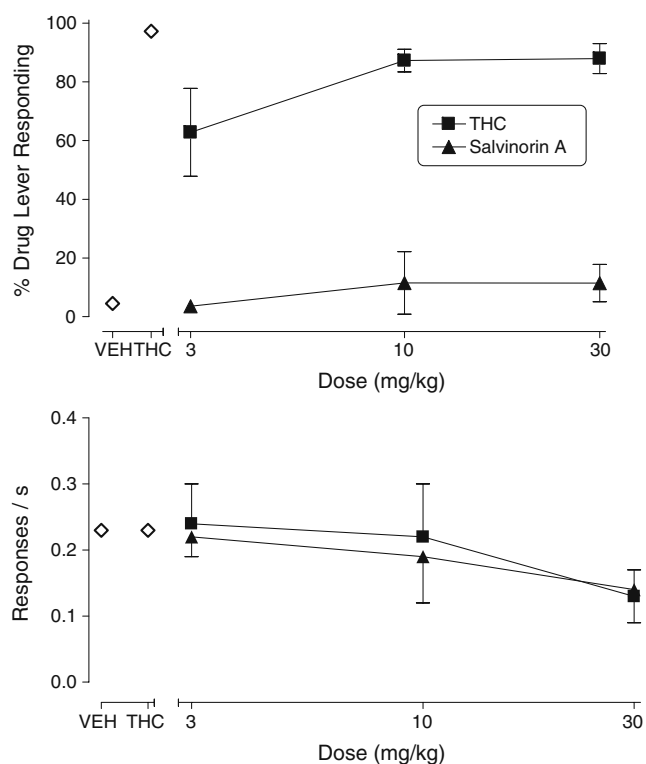


Fig. 6 Effects of THC and salvinorin A on percentage of THC-lever responding (*top panel*) and response rates (*bottom panel*) in mice trained to discriminate 10 mg/kg THC from vehicle. Points above VEH and THC represent the results of control tests with vehicle and 10 mg/kg THC conducted before each dose–effect determination. For each dose–effect curve determination, values represent the mean (\pm SEM) of five to seven mice

the stimulatory effects of CP55,940 in the [35 S]GTP γ S assay, as do cannabinoid antagonists (Selley et al. 1996). Hence, empirical evidence that salvinorin A acts directly on CB $_1$ receptors or affects signaling at these receptors is absent.

In vivo, salvinorin A shared some, but not all, of the profile of effects observed with psychoactive cannabinoids. Similar to THC, salvinorin A dose dependently decreased spontaneous activity and produced antinociception; however, it induced only mild hypothermia and catalepsy. Further, whereas THC increased food consumption in food-restricted mice (Wiley et al. 2005), doses of salvinorin A that did not reduce overall activity failed to alter food consumption under identical conditions (unpublished data). The effects of salvinorin A in the tetrad were reversed by the KOR antagonist JD Tic (Carroll et al. 2004), but not by rimonabant. In contrast, rimonabant attenuated THC's tetrad effects whereas JD Tic attenuated only its hypothermic effects. These findings suggest that the pharmacological effects of salvinorin A in the tetrad tests are mediated by the KOR and not by the CB $_1$ receptor. While psychoactive cannabinoids generally produce all four

effects in a dose-dependent manner, other classes of drugs also may produce activity in some or all of the tetrad tests (Wiley and Martin 2003). Thus, the tetrad test battery is more useful for precluding, rather than establishing, cannabinoid receptor activity, as was the case in the present study.

By contrast, the discriminative stimulus effects of THC are CB $_1$ receptor mediated and substitution for THC is pharmacologically selective for other psychoactive cannabinoids (Barrett et al. 1995; Wiley 1999). In addition, the procedure has been used as an animal model of marijuana intoxication (Balster and Prescott 1992). In our mouse THC discrimination model, salvinorin A did not elicit THC-like responding. Indeed, a number of studies have demonstrated that salvinorin A possesses discriminative stimulus effects similar to other KOR agonists (e.g., U69,593) in both rhesus monkeys (Butelman et al. 2004) and rats (Willmore-Fordham et al. 2007). Additionally, cross substitution has been observed between salvinorin A and KOR agonists U69,593 and U50,488 in rats trained to discriminate salvinorin A from vehicle (Baker et al. 2009). Taken together, these results would strongly predict that salvinorin A does not produce a state of intoxication comparable to THC. Existing demographic data also support this claim, as only a small percentage of *S. divinorum* users likened its effects to those elicited by marijuana (Gonzalez et al. 2006).

Not surprisingly, given its KOR activity, the most robust tetrad effect produced by salvinorin A was antinociception. While results of antagonism experiments presented here demonstrate that the effects of salvinorin A in the tetrad tests were mediated by KOR and not by CB $_1$ receptors, previous work has suggested that cross talk between endocannabinoid and KOR systems may occur, perhaps through actions on a common signaling pathway (Hampson et al. 2000). For example, Smith et al. (1993, 1994) reported that THC's acute antinociceptive effects, but not its other tetrad effects, were attenuated by nor-BNI. Further, mice that were made tolerant to these effects exhibited cross tolerance with the KOR agonist U50,488. A more recent report extends these findings with exogenous cannabinoids to the endocannabinoid anandamide and suggests that opioid antinociception may be mediated through an indirect interaction between anandamide and KOR systems (Haller et al. 2008). Cross talk between endocannabinoid and KOR systems has also been noted outside of the realm of antinociception in such diverse areas as inflammation of the gut (Capasso et al. 2008) and motivational properties of drugs of abuse (Ghozland et al. 2002). In addition, data presented here show that rimonabant attenuated activation of KOR by U69,593, suggesting that rimonabant reversal of salvinorin A effects in some previous studies may have been related to rimonabant's antagonistic effects on KOR

rather than through activation of CB₁ receptors by salvinorin A.

In summary, the results of this intensive investigation of the effects of salvinorin A in assays used for screening of psychoactive cannabinoid agonists demonstrate that salvinorin A does not have direct effects on CB₁ cannabinoid receptors. While some of its pharmacological effects in the tetrad resemble those seen following cannabinoid administration, these effects are blocked by the selective KOR antagonist JDITic, but not by rimonabant, suggesting that the mechanism responsible for these effects is activation of KOR rather than CB₁ cannabinoid receptors. These findings are consistent with previous reports that salvinorin A binds to and is an *in vivo* agonist at KOR (Chavkin et al. 2004; Roth et al. 2002). Still open for discussion is the issue of whether cross-talk between endocannabinoid and KOR and/or rimonabant action on KOR may provide an indirect mechanism for some of the centrally mediated pharmacological effects of salvinorin A that prior reports have suggested are mediated through its action on the endocannabinoid system.

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