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The antinociceptive effect of Salvinorin A in mice

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Abstract

Salvia divinorum is a hallucinogenic plant used by the Mazatec Indians of Mexico for traditional spiritual ceremonies. The active constituent, salvinorin A, induces profound hallucinations, however the biological mechanism for this action is not known. Affinity-binding studies suggest that the biologic activity of salvinorin A involves the κ-opioid receptor. The purpose of this study was to evaluate the antinociceptive effect of salvinorin A in mice. Salvinorin A and opioid receptor antagonists were administered intrathecally and the tail-flick latencies were used as a measure of antinociception. Salvinorin A increased tail-flick latencies in a dose-dependent manner (13.9–23.1 nmol) compared to control trials. Pretreatment with the κ-opioid receptor antagonist nor-binaltorphimine attenuated the salvinorin A induced increase in tail-flick latency. In contrast, neither the μ-opioid receptor antagonist β-funaltrexamine nor δ-opioid receptor antagonist naltrindole significantly affected the antinociceptive response of salvinorin A administration. These data support previous reports that salvinorin A represents a unique non-alkaloidal agonist for the κ-opioid receptor.

Keywords: Salvinorin A; Kappa opioid receptor; Antinociception

1. Introduction

Direct intracellular signaling by opioid receptors occurs through the activation of guanine-nucleotide-binding regulatory proteins (G_i- and G_o-proteins) (Birnbaumer, 1990). Agonist stimulation has been shown to increase K^+ conductance (North and Williams, 1985), block voltage-gated Ca^{+2} channels (Mudge et al., 1979), as well as inhibit the release of a variety of neurotransmitters including substance P (Jessell and Iversen, 1977), acetylcholine (Konishi et al., 1979; Mulder et al., 1984) Met-enkephalin (Xu et al., 1989), nor-epinephrine (Schoffelmeer and Mulder, 1984) and glutamate (Gannon and Terrian, 1991). The κ-opioid receptor is one of three well-described seven transmembrane G-protein coupled opioid receptors. The κ-opioid receptor is distributed throughout areas of the brain associated with pain perception and arousal including the nucleus accumbens, hypothalamus, periaqueductal gray, raphe magnus and rostral ventral lateral medulla (Ossipov et al., 2004). κ-opioid receptor agonists are potent analgesics (Millan, 1990), however they elicit many significant side effects including diuresis (Leander et al., 1987), sedation (Tang and Collins, 1985; Ukai and Kameyama, 1985; Dykstra et al., 1987) and psychotomimesis (Kumor et al., 1986; Pfeiffer et al., 1986).

Salvia divinorum, a hallucinogenic plant traditionally used by the Mazatec Indians of northeastern Mexico, is used for its psychoactive effects that aid in ritual divination and healing ceremonies (Wasson, 1962). Phytochemical investigations of this plant led to the isolation of a series of complex neoclerodane diterpenes including salvinorins A–F (Valdés et al., 1987, 1994; Munro and Rizzacasa, 2003). The active constituent of the plant, salvinorin A, has been reported to be the most potent, naturally occurring hallucinogen known, with effective doses of 200–1000 μg in humans (Valdés, 1994; Siebert, 1994). Traditionally, such psychotomimetic and dysphoric action has been attributed to activity at various 5-hydroxytryptamine (5-HT) and *N*-methyl-D-aspartate (NMDA) glutamate receptors, however recent studies have demonstrated the neuropharmacological profile of salvinorin A to be unique.

Receptor site screening by Siebert (1994) for interactions of salvinorin A with a plethora of biogenic amine, peptide, and neurotransmitter receptors and regulatory sites proved negative. Roth et al. (2002) examined the binding activity of salvinorin A at a variety of human G-protein-coupled receptors, σ_1 and σ_2-sigma

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receptors, cannabinoid CB-1 and CB-2 receptors, muscarinic and nicotinic cholinergic receptors, serotonin receptors (including 5-HT_{2A} receptors—the site of action of LSD) and various ionotropic and metabotropic glutamate receptors in HEK-293 cells and at native κ-opioid receptors expressed in guinea pig brain. Of fifty tested molecular targets screened in this study, none showed significant inhibition of radioligand binding by salvinorin A with the exception of the κ-opioid receptor (Roth et al., 2002). In *Xenopus* oocytes co-injected with inwardly rectifying K^+ channels and κ-opioid receptors salvinorin A was found to be a full agonist for inhibition of forskolin-stimulated cAMP release and stimulation of intracellular Ca^{2+} mobilization. Moreover, salvinorin A was significantly more efficacious than two standard κ-opioid receptor agonists, (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methane-sulfonate hydrate (U50488) and (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methane-sulfonate hydrate (U69593) (Chavkin et al., 2004). Salvinorin A's affinity for the κ-opioid receptor compares to dynorphin A (1–17), an endogenous κ-opioid receptor agonist (Chavkin et al., 1982) in the activation of human κ-opioid receptors (Chavkin et al., 2004) with Ki's ranging from 8–19 nM (Chavkin et al., 2004, Roth et al., 2002; Wang et al., 2005) and represents the first known specific, naturally occurring non-nitrogenous κ-opioid receptor full agonist.

Although the psychoactive effects of salvinorin A (Valdés, 1994; Siebert, 1994) and its receptor affinity for the κ-opioid receptor (Chavkin et al., 2004; Roth et al., 2002; Wang et al., 2005; Yan et al., 2005) have been well described in controlled experiments and anecdotally in the literature, its analgesic properties have not been thoroughly examined. Few studies have examined the effects of salvinorin A *in vivo*, and fewer still have assessed the agonist activity of this compound at μ-opioid, δ-opioid, and κ-opioid receptors centrally using selective antagonists. The purpose of this study was to examine the antinociceptive effect of salvinorin A *in vivo* and determine the biological mechanism underlying the effect.

2. Methods

2.1. Animals

Adult male CD–1 mice weighing 25–30 g (Charles River Laboratories, Wilmington, MA) were used for the study. All experiments conformed to the guidelines put forth by the National Institute of Health and were approved by the St. Lawrence University Animal Use and Care Committee. Animals were housed individually in a room maintained at 22±0.5 °C with an alternating 12–hour light/dark cycle. Food and water was available *ad libitum*. Animals were used only once in the experiments.

2.2. Drugs

Salvinorin A was obtained from the *Salvia divinorum* Research and Information Center (Malibu, CA) and assayed for purity by GC–MS and NMR Spectroscopy. GC–MS was performed on an Agilent 6890N/5973/N instrument on an Agilent HP-5MS capillary column (0.25 mm×30 m×0.25 μm) employing helium carrier gas at a constant flow of 1.2 mL/min. An injection port temperature of 260 °C was used. Oven temperature was set at 70 °C, held for 4 min and then increased at the rate of 20°/min to 190° and then at 10°/min to a final temperature of 300° which was held for 18 min. A 1.0 μl splitless injection of a dilute sample of salvinorin A in acetonitrile was carried out. Under these conditions, salvinorin A eluted with a retention time of 23.79 min and produced a mass spectrum (molecular ion m/e=432 amu) identical to that reported in the literature. (Giroud et al., 2000). ^1H and ^{13}C NMR spectra were recorded on a Jeol Eclipse spectrometer at 300 MHz and were consistent with the reported data (Valdés et al., 1984). The purity (uncorrected for detector response differences) of salvinorin A used was approximately 96%.

The μ-opioid receptor antagonist β-Funaltrexamine, κ-opioid receptor antagonist nor-binaltorphimine, and δ-opioid receptor antagonist naltrindole were obtained from Sigma–Aldrich Corp (St. Louis, MO).

Salvinorin A was prepared in 100% dimethyl sulfoxide (DMSO), and opioid receptor antagonists prepared in 0.9% NaCl. DMSO vehicle produced scratching behavior in mice that lasted approximately 2 min after the injection, but had no effect on subsequent tail-flick responses. Mice (n=10) were given a range of doses of salvinorin A (11.6 – 23.1 nmol). In separate experiments, mice (n=10) were pretreated i.t. with the selective opioid receptor antagonists nor–binaltorphimine or β-funaltrexamine 24 h prior, or naltrindole 10 min prior to i.t. challenge with salvinorin A, similar to other studies that have utilized successive i.t. injections (Wu et al., 2002). Appropriate blocking doses of the μ-antagonist β-funaltrexamine (5.0 nmol), δ-antagonist naltrindole (11.1 nmol) and κ-antagonist nor-binaltorphimine (6.8 nmol) were used based on previous work (Ward et al., 1982; Takemori and Portoghese, 1992; Portoghese et al., 1987).

2.3. Procedure for intrathecal injection

Intrathecal (i.t.) injections were made according to the procedure of Hylden and Wilcox (1980). Unanesthetized mice

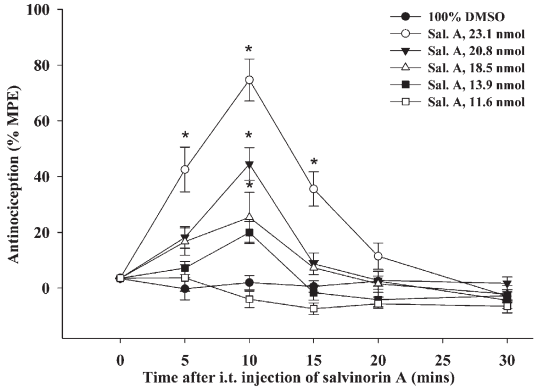

Fig. 1. Time course of changes of the tail_flick response to i.t. administered salvinorin A and DMSO vehicle. Groups of mice (10) were injected i.t. with salvinorin A (11.6 _ 23.1 nmol) or vehicle (5 μl) and the tail-flick responses were measured at 5, 10, 15, 20 and 30 min. Each value represents the mean, and the vertical bar represents the mean±S.E.M. * $P<0.05$ was considered statistically significant compared to vehicle.

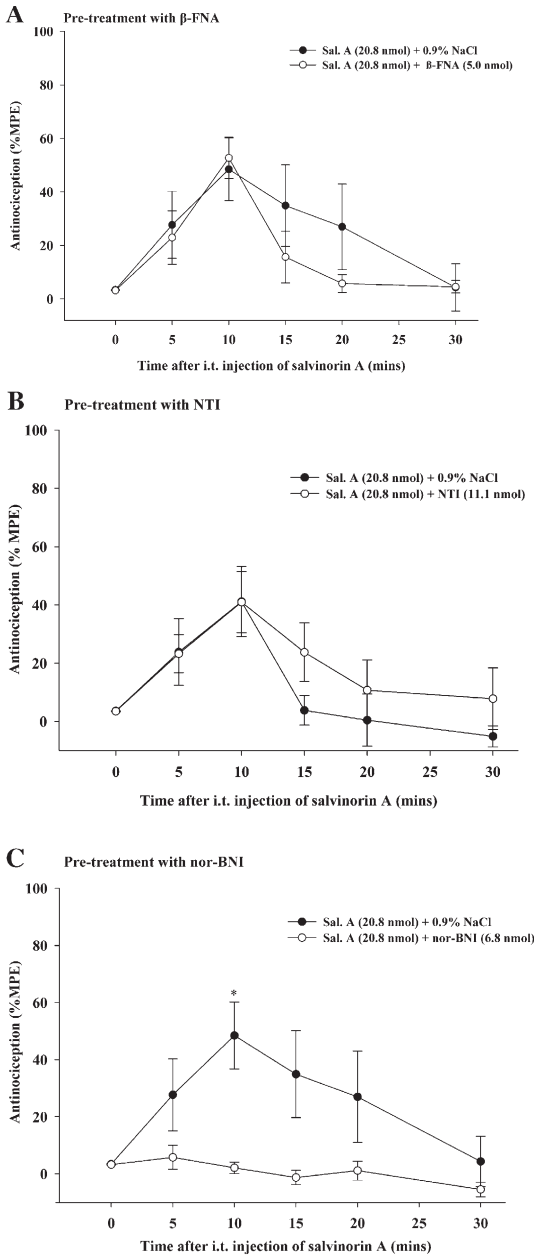

Fig. 2. Time course of changes of the tail-flick response to i.t. administered salvinorin A after pre-treatment with μ-, δ-, or κ-opioid receptor specific antagonists. (A) Groups of mice (10) were pre–treated (24–hrs) with β-funaltrexamine (5.0 nmol) or saline. (B) Groups of mice (10) were pre_treated (10–mins) with naltrindole (11.1 nmol) or saline. (C) Groups of mice (10) were pre–treated (24–hrs) with nor–binaltorphimine (6.8 nmol) or saline. Mice then received i.t. administered salvinorin A (20.8 nmol) and the tail-flick responses were measured at 5, 10, 15, 20 and 30 min. Each value represents the mean, and the vertical bar represents the mean±S.E.M. * $P<0.05$ was considered statistically significant compared to vehicle.

were injected between the L5 and L6 areas of the spinal cord with a 25-μl Hamilton syringe with a 30-gauge, ½ inch needle. Injection volumes of 5 μl were administered.

2.4. Assessment of antinociceptive response

Antinociceptive response was determined using the tail-flick test (D'Amour and Smith, 1941). The tail-flick response was elicited by applying radiant heat to the dorsal surface of the distal 4 cm of the tail. Mice were gently held with one hand with the tail positioned in the apparatus (Model TF6; EMDIE Instrument Co., Maidens, VA) for radiant heat stimulation using focused light from a projector lamp. In preliminary experiments, the intensity of the heat stimulus was adjusted so that the animal flicked its tail between 3 and 4 seconds. The latency of the tail-flick response was measured before (baseline=T_0) and 5, 10, 15, 20, and 30 min after i.t. injection. To minimize injury to the animal, the apparatus is designed to automatically shut the lamp after 10 seconds.

2.5. Data analysis and statistics

The inhibition of tail-flick response by salvinorin A is expressed as a percentage of the maximum possible effect, which was calculated as $[(T_1-T_0/\ T_2-T_0)]^{\ x}\ 100$, where the cut-off time, T_2, was set at 10 s for the tail-flick response. All data are expressed as the mean±S.E.M. A one-way analysis of variance was used for comparisons of treatment and time between groups followed by post-hoc Dunnett's *t*-test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Time courses of the tail-flick response to i.t. administration of Salvinorin A

Groups of mice (10) were injected i.t. with a range of doses of salvinorin A, and the tail-flick response was measured 5, 10, 15, 20, and 30 min after the injection. Intrathecal injection of salvinorin A resulted in a dose-dependent increase in tail-flick inhibition (Fig. 1). Significant peak inhibition was achieved 10 min following the injection (13.9 – 23.1 nmol), and the response returned to the pre–injection level approximately 20 min after injection (Fig. 1.). The ED_{50} value (95% confidence limit) was estimated to be 20.93 (18.49–23.69) nmol.

3.2. Effects of i.t. pretreatment with β–FNA, nor–BNI, or NTI on tail-flick inhibition induced by i.t. administered Salvinorin A

The tail-flick inhibition induced by i.t. administered salvinorin A (20.8 nmol) was not effected by i.t. pretreatment with β-funaltrxamine (5.0 nmol) or naltrindole (11.1 nmol) (Fig. 2A,B). In contrast, i.t. pretreatment with nor-binaltorphimine (6.8 nmol) completely abolished the tail-flick inhibition induced by salvinorin A (Fig. 2C).

4. Discussion

Previous studies have examined the myriad of behavioral effects of salvinorin A. Systemic administration of this compound has been shown to induce sedation, motor incoordination, decrease locomotor activity, and cause conditioned place aversion in mice. These effects are attenuated by the κ-opioid receptor antagonist, nor-binaltorphimine, but not naltrindole or naltrexone (Fantegrossi et al., 2005; Zhang et al., 2005). In

rhesus monkeys, salvinorin A demonstrated identical discriminative stimulus actions compared to the κ-opioid receptor agonist U69,593 (Butelman, et al., 2004). McCurdy et al. (2006) demonstrated that systemic administration of salvinorin A produces significant antinociception during the tail-flick, hot plate, and acetic acid abdominal constriction tests. Centrally, systemic administration of salvinorin A has been shown to decrease dopamine levels in the nucleus accumbens, which receives direct afferent input from the dorsal horn of the spinal cord (Burstein and Giesler, 1989), without affecting extracellular serotonin levels (Carlezon et al., 2005). These data are consistent with the extracellular effects of κ-opioid receptor agonists U50,488, bremazocine and tifluadom (Di Chiara and Imperato, 1988) and the endogenous κ-opioid receptor ligand dynorphin (Zhang et al., 2004), that decrease dopamine levels. Furthermore, salvinorin A induced pre-synaptic inhibition of electrically evoked potentials is inhibited by nor-binaltorphimine (Capasso et al., 2006).

While the behavioral effects of systemic administration of salvinorin A have been reasonably well described, no studies have examined the antinociceptive effect of salvinorin A at the spinal level. κ-opioid receptors are highly concentrated in outer laminae of the dorsal horn of the superficial layers of the lumbosacral spinal cord (Quirion, 1984), and spinally administered κ-opioid receptor agonists have been shown to produce significant antinociception (Ossipov et al., 2004). In the present study, i.t. injection of salvinorin A significantly attenuated the tail-flick response in a dose-dependant manner. Furthermore, μ-and δ-opioid receptor antagonists β-FNA and NTI did not effectively inhibit the activity of salvinorin A, however the κ-opioid receptor antagonist nor-binaltorphimine completely abolished the antinociceptive effect.

Our data support the mounting evidence that salvinorin A is a unique non-alkaloidal pure κ-opioid receptor agonist. Investigation of the compound's analgesic properties provides a unique approach to explaining its neurological activity. Salvinorin A, by virtue of its potency, efficacy, and selectivity as a κ-opioid receptor agonist will be an important tool for discovering the role that the κ-opioid receptor-dynorphinergic system has in health and disease.

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