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Effects of Salvinorin A, a κ-Opioid Hallucinogen, on a Neuroendocrine Biomarker Assay in Nonhuman Primates with High κ-Receptor Homology to Humans

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ABSTRACT

This study focused on the in vivo effects of the κ-opioid hallucinogen salvinorin A, derived from the plant *Salvia divinorum*. The effects of salvinorin A (0.0032–0.056 mg/kg i.v.) were studied in a neuroendocrine biomarker assay of the anterior pituitary hormone prolactin in gonadally intact, adult male and female rhesus monkeys ($n = 4$ each). Salvinorin A produced dose- and time-dependent neuroendocrine effects, similar to the synthetic high-efficacy κ-agonist U69,593 ((+)-(5α,7 α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidiniyl)-1-oxaspiro[4.5]dec-8yl]-benzeneacetamide), but of shorter duration than the latter. Salvinorin A was approximately equipotent to U69,593 in this endpoint (salvinorin A ED_{50}, 0.015 mg/kg; U69,593 ED_{50}, 0.0098 mg/kg). The effects of i.v. salvinorin A were not prevented by a small dose of the opioid antagonist nalmefene (0.01 mg/kg s.c.) but were prevented by a larger dose of nalmefene (0.1 mg/kg); the latter nalmefene dose is sufficient to produce κ-antagonist effects in this species. In contrast, the 5HT2 receptor antagonist ketanserin (0.1 mg/kg i.m.) did not prevent the effects of salvinorin A. As expected, the neuroendocrine effects of salvinorin A (0.0032 mg/kg i.v.) were more robust in female than in male subjects. Related studies focused on full-length cloning of the coding region of the rhesus monkey κ-opioid receptor (*OPRK1*) gene and revealed a high homology of the nonhuman primate *OPRK1* gene compared with the human *OPRK1* gene, including particular C-terminal residues thought to be involved in receptor desensitization and internalization. The present studies indicate that the hallucinogen salvinorin A acts as a high-efficacy κ-agonist in nonhuman primates in a translationally viable neuroendocrine biomarker assay.

Salvinorin A, a diterpenoid, is the main active compound from the leaves of the hallucinogenic plant, *Salvia divinorum* (Valdes, 1994). *S. divinorum* preparations were originally used in ethnomedical practice by the Mazatec people of Oaxaca, Mexico, but have recently become widely commercially available. There are emerging reports of use of such salvinorin A-containing products as hallucinogens, mainly by the smoking route (http://biopsych.com/cpdd/CPDD04_PDFs/CPDD04_981339497336.pdf; Gonzales et al., 2006).

A recent study determined that salvinorin A was a highly selective agonist at κ-opioid receptors (Roth et al., 2002). Salvinorin A was approximately equipotent and equieffective in vitro to arylacetamide κ-agonists such as U69,593, in the stimulation of guanosine 5′-*O*-(3-thio)triphosphate binding, or the inhibition of adenylate cyclase (Roth et al., 2002). In another signal transduction system (potentiation of G protein-coupled inwardly rectifying potassium channel currents), salvinorin A appeared to be an "ultrahigh" efficacy agonist (Chavkin et al., 2004). Salvinorin A was also found to have a lesser propensity to cause κ-receptor desensitization and internalization in vitro, compared with arylacetamide κ-agonists (Wang et al., 2004). It is unknown whether this in vitro profile confers salvinorin A with unique properties as a κ-agonist in vivo, possibly underlying its hallucinogenic effects.

There are some studies of the effects of salvinorin A in vivo, mostly in rodents. Salvinorin A caused κ-receptor

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ABBREVIATIONS: U69,593, (+)-(5α,7 α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidiniyl)-1-oxaspiro[4.5]dec-8yl]-benzeneacetamide; ANOVA, analysis of variance; *OPRK1*, κ-opioid receptor gene; PCR, polymerase chain reaction.

mediated place aversion and decreases in striatal dopamine dialysates in mice, similarly to synthetic κ-agonists (Zhang et al., 2004, 2005). Salvinorin A also caused depressive-like behavioral effects and reduced dopamine dialysate levels in nucleus accumbens in rats (Carlezon et al., 2006). Salvinorin A produced κ-receptor-mediated sedation/motor incoordination in mice (Fantegrossi et al., 2005). Salvinorin A may produce brief antinociceptive effects under certain conditions in rodents but is devoid of antipruritic effects, typically observed with κ-agonists (Ko et al., 2003; Wang et al., 2004; Ansonoff et al., 2006; McCurdy et al., 2006). Salvinorin A was generalized by nonhuman primates trained to discriminate U69,593 in an operant assay (Butelman et al., 2004). Few studies have addressed in vivo the apparent efficacy of salvinorin A or that have endpoints that may be easily adapted to humans, thus having translational value.

Serum prolactin levels have been used in nonhuman primates to study the potency, receptor selectivity, and apparent efficacy of κ-agonists in vivo (other compounds, including μ-opioid agonists, also cause prolactin release) (Bowen et al., 2002; Butelman et al., 2002). This neuroendocrine biomarker assay has also been used in clinical populations in the study of κ-opioid effects of the neuropeptide dynorphin A (Kreek et al., 1999; Bart et al., 2003). These studies document the effects of salvinorin A in this biomarker assay and are consistent with the high efficacy ascribed to salvinorin A at κ-receptors, based on in vitro studies.

Nonhuman primates, such as *Macaca mulatta* (used herein) may be valuable models for translational studies of κ-opioid function. Studies suggest that there are differences in rodent versus human or nonhuman primate κ-receptor populations, in terms of neuroanatomical localization, relative B_{max}, and neurobiological interactions (Mansour et al., 1988; Rothman et al., 1992; Berger et al., 2006). In addition, comparative studies in cloned human and rat κ-receptors have detected differences in agonist-induced desensitization and internalization, and these could be ascribed to interspecies differences in protein structure at the C terminus of the receptor (e.g., at the 358-amino acid residue position; Li et al., 2002; Liu-Chen, 2004). To determine whether this nonhuman primate species shares these critical amino acid residues with human κ-receptors, we present information on full-length cloning of the coding region of the *M. mulatta* κ-receptor.

Materials and Methods

Experimental Subjects in Neuroendocrine Studies. Captive-bred, gonadally intact rhesus monkeys (*M. mulatta*; four male and four female; age range, 8–11 years old approximately; weight range, 5.8–12.5 kg) were used. Monkeys were singly housed in a room maintained at 20 to 22°C with controlled humidity, and a 12-/12-h light/dark cycle (lights on at 7:00 AM). Monkeys were fed approximately 11 jumbo primate chow biscuits (PMI Feeds, Richmond, VA) daily, supplemented by appetitive treats, and multivitamins plus iron. An environmental enrichment plan was in place in the colony rooms. Water was freely available in home cages, via an automatic waterspout.

Procedure for Neuroendocrine Experiments. Chair-trained monkeys were tested after extensive prior exposure to the experimental situation. Monkeys were chaired and transferred to the experimental room between 10:00 AM and 11:00 AM on each test day. An in-dwelling catheter (24 gauge; Angiocath; Becton Dickinson, Sandy, UT) was placed in a superficial leg vein and secured with elastic tape. An injection port (Terumo, Elkton, MD) was attached to the hub of the catheter; the port and catheter were flushed (0.3 ml of 50 U/ml heparinized saline) before use and after each blood sampling or i.v. injection. Approximately 15 min following catheter placement, two baseline blood samples of approximately 2 ml were collected, 5 min apart from each other (defined as −10 and −5 min, relative to the onset of dosing), and kept at room temperature until the time of spinning (3000 rpm at 4°C) and serum separation. Serum samples were then kept at −40°C until the time of analysis, typically within 2 weeks of collection. The samples were analyzed in duplicate with a standard human prolactin immunoradiometric kit (DPC, Los Angeles CA), following manufacturer's instructions. There is high protein homology between human and rhesus monkey prolactin, and antibody cross-reactivity between human and rhesus monkey prolactin has also been reported (Brown and Bethea, 1994; Pecins-Thompson et al., 1996; Ordog et al., 1998). The reported sensitivity limit of the present assay was 0.1 ng/ml; each individual kit was calibrated with known standards, in the range 2 to 200 ng/ml. The intra- and interassay coefficients of variation with this kit in the laboratory were 2 and 9%, respectively.

Monkeys were tested in a time course procedure. Following baseline sample collection, a single agonist (salvinorin A or U69,593) injection was administered, followed by sampling at 5, 15, 30, 60, 90, and 120 min after administration. Unless otherwise stated, agonists were injected by the i.v. route. In antagonism experiments, a single dose of antagonist (s.c. nalmefene or i.m. ketanserin) was administered 30 min before salvinorin A, followed by testing as above. Each experiment was typically carried out in four males; selected experiments were carried out in four females in follicular phase (days 2–12 of each cycle of approximately 28 days, as defined by the onset of visible bleeding). Consecutive experiments in the same subject were separated by at least 96 h.

Design of Neuroendocrine Studies. Time course studies were carried out with salvinorin A and U69,593 (0.0032–0.056 mg/kg i.v.; typically $n = 4$) and vehicle. For salvinorin A and U69,593, the largest dose was only studied in three of four subjects. The fourth subject was not administered the largest dose for safety reasons, based on greater sensitivity to untoward effects of the compounds (e.g., tremors). In other studies, the opioid antagonist nalmefene (0.01 or 0.1 mg/kg s.c.) was administered as a pretreatment before the largest salvinorin A dose at which all subjects were studied (0.032 mg/kg). A similar pretreatment study was completed with the 5HT2 antagonist ketanserin (0.1 mg/kg i.m.), before salvinorin A (0.032 mg/kg). Female subjects were studied at the 0.0032 mg/kg i.v. dose, a dose that results in robust prolactin elevation in females but not in males. Female subjects were also studied after s.c. administration of salvinorin A (0.032 mg/kg), with and without nalmefene (0.1 mg/kg s.c.) pretreatment.

Data Analysis. Prolactin values are presented as mean ± S.E.M., after subtraction of individual mean preinjection baselines for each session (Δ nanograms per milliliter). Dose-effect curves are also presented, as collated from a time of peak prolactin release caused by salvinorin A or U69,593 (15 min post-i.v. injection). Linear regression was used to calculate ED_{50} values from individual data points above and below the 50% level of effect.

Significant differences in a parameter (e.g., log ED_{50} values) were considered to occur if there was a lack of overlap in their 95% confidence limits. Unless otherwise stated, experiments were carried out with $n = 4$. Repeated measures ANOVA was followed by post hoc tests [using either GraphPad Prism (GraphPad Software Inc., San Diego, CA) or SPSS-Sigmastat (SPSS Inc., Chicago, IL)]; the level of significance (α) was set at $p = 0.05$.

Drugs. Salvinorin A was extracted from commercially obtained *S. divinorum* leaves (Ethnogens.com, Berkeley, CA) in the laboratory of Dr. T.E. Prisinzano, as described previously (Tidgewell

et al., 2004; Harding et al., 2006). In brief, dried *S. divinorum* leaves (1.5 kg) were ground to a fine powder and percolated with acetone. The acetone extract was concentrated under reduced pressure to afford a crude green gum, which was subjected to repeated column chromatography on silica gel with elution, using a mixture of EtOAc/hexanes to afford salvinorin A (thin-layer chromatography) and other minor diterpenes. The melting point, ^{1}H NMR, and ^{13}C spectra of salvinorin A were in agreement with previous reports (Ortega et al., 1982; Valdes et al., 1984). Salvinorin A solutions for injection were prepared daily in ethanol/Tween 80/sterile water (1:1:8, v/v; maximum concentration in this vehicle was 0.2 mg/ml).

Nalmefene HCl (Baker Norton, Miami, FL) was dissolved in sterile water; U69,593 (Pharmacia-Upjohn, Kalamazoo, MI) was dissolved in sterile water with the addition of 1 drop of lactic acid. All of the above drug doses are expressed as milligrams per kilogram of the forms indicated above. Ketanserin tartrate (Sigma, St. Louis, MO) was dissolved in 5% dimethyl sulfoxide in sterile water (v/v) and was injected i.m. The ketanserin dose is expressed as the base, for consistency with prior publications (Fantegrossi et al., 2002). All drugs were injected in volumes of 0.05 to 0.1 ml/kg whenever possible.

Cloning and Sequencing of *M. mulatta* κ-Opioid Receptor (*OPRK1*) cDNA. The coding region of the *OPRK1* gene was obtained by PCR amplification of *M. mulatta* brain cDNA (obtained from BioChain, Hayward, CA) with the forward primer 5′-TC-CTCGCC TT CCTGCTGCA-3′, located 30 nucleotides upstream of ATG codon, and the reverse primer 5′-TCAGACTGC AGTAG-TATC-3′, located 69 nucleotides downstream of the termination codon. The primer design was based on the human *OPRK1* sequence (GenBank accession no. NM_000912). The final product, approximately 1260 bp in size, was purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and cloned in pCR II plasmid (Invitrogen, Carlsbad, CA). The clones were sequenced in both directions using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3700 capillary sequencer.

Single Nucleotide Polymorphism Analysis. Genomic DNA was isolated from peripheral white blood cells, obtained by venipuncture from 14 subjects in the colony, including all eight subjects used in the present neuroendocrine studies (Versagene kit; Gentrasystems, Minneapolis, MN). The C terminus of the *M. mulatta OPRK1* was amplified using forward primer 5′-ATTCTCTACGCCTTTCTT-GAT-3′, located 160 bp upstream of the termination codon, and reverse primer 5′-TCAGACTGCAGTAG TATC-3′, located 69 bp downstream of the termination codon. PCR products, 257 bp in size, were sequenced to identify single nucleotide polymorphisms, as described above.

Results

Baseline Prolactin Values and Effects of Vehicle Administration

Preinjection prolactin values in males were relatively consistent and exhibited small decreases over time, after i.v. vehicle administration. Thus, mean preinjection values were 15.5 ng/ml (S.E.M. = 4.2); these values decreased gradually over a 120-min session, following i.v. administration of vehicle (1:1:8 ethanol/Tween 80/sterile water v/v; 0.16 ml/kg; see Fig. 1). Female subjects in follicular phase had similar preinjection baselines (mean = 15.1 ng/ml; S.E.M. = 3.0), and also exhibited a gradual decrease in prolactin levels over the 120-min experiment following i.v. vehicle.

Effects of Salvinorin A or U69,593

Male Subjects. Intravenous salvinorin A and U69,593 (0.0032–0.056 mg/kg) caused robust dose- and time-dependent increases in prolactin levels (Fig. 1). Salvinorin A effects were observable by 5 min after i.v. administration, peaked at 15 min after administration, and declined gradually by 120 min. A two-way (time × dose) repeated measures ANOVA for i.v. salvinorin A (5–120 min and 0.0032–0.032 mg/kg vehicle) revealed a main effect of time [$F(5,15) = 13.00$], dose [$F(3,9) = 12.48$], and their interaction [$F(15,45) = 9.70$]. The largest salvinorin A dose (0.056 mg/kg) was not included in this analysis because one of the subjects could not be studied at this dose, for safety reasons. Newman-Keuls comparisons at different times post-salvinorin A revealed significant differences for all salvinorin A doses (except the smallest dose, 0.0032 mg/kg) versus vehicle at 5, 15, and 30 min. At 60 min, only the largest salvinorin A (0.032 mg/kg) was different from vehicle. By 90 and 120 min after salvinorin A, no significant differences were detected with Newman-Keuls comparisons.

U69,593 effects were similar to those of salvinorin A, with longer duration of action, as suggested by prolactin elevations persisting at the end of the 120-min study period (Fig. 1). A two-way (time × dose) repeated measures ANOVA for i.v. U69,593 (5–120 min and 0.0032–0.032 mg/kg and vehicle) revealed a main effect of time [$F(5,15) = 15.73$], dose [$F(3,9) = 32.99$], and their interaction [$F(15,45) = 16.10$]. The largest U69,593 dose (0.056 mg/kg) was not included in the analysis because one of the subjects could not be tested at this dose (the same subject as in the salvinorin A studies).

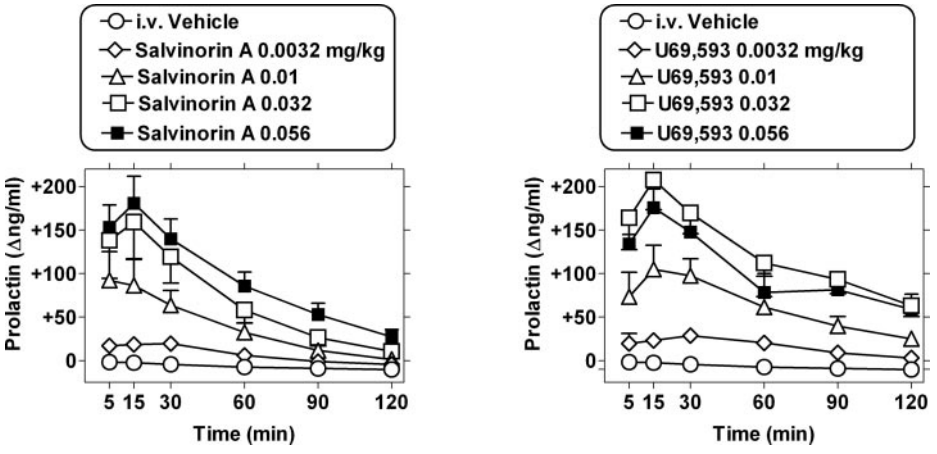

Fig. 1. Time course effects of i.v. salvinorin A (left) and i.v. U69,593 (right) on serum prolactin levels in male subjects (0.0032–0.056 mg/kg; $n = 4$, except at the largest dose, which was $n = 3$). Abscissae, time in minutes from i.v. injection. Ordinates, serum prolactin levels, expressed as change from individual preinjection baseline (Δ nanograms per milliliter). Data are mean ± S.E.M.; in cases where no error bars are visible, these fall within the symbol for each data point.

Newman-Keuls comparisons at different times post-U69,593 revealed significant differences for all salvinorin A doses (except the smallest dose, 0.0032 mg/kg) versus vehicle at 5, 15, 30, 60, and 90 min. At 120 min after U69,593, only the largest U69,593 dose (0.032 mg/kg) was significantly different from vehicle.

Dose-effect curves for salvinorin A and U69,593 were plotted at 15 min after i.v. administration (a time of peak effect) and exhibit approximately equal maximum effect and potency (Fig. 2). Clear maximum "plateau" effects were not observed at the largest doses studied in each subject, and this limited the quantitative determination of maximum plateau by nonlinear regression. Larger doses than those used herein (i.e., 0.032 for one subject and 0.056 for the other three) were not probed, due primarily to solubility limitations. Intravenous potency was quantified by linear regression and did not differ significantly between salvinorin A and U69,593 [ED_{50} for salvinorin A = 0.015 mg/kg (95% confidence limit = 0.0048–0.050); ED_{50} for U69,593 = 0.0098 mg/kg (95% confidence limit = 0.0041–0.020)].

Subcutaneous Administration of Salvinorin A. The effects of a probe dose of salvinorin A (0.032 mg/kg) were also studied by the s.c. route in male subjects and resulted in much smaller prolactin release than that observed by the i.v. route and a slower onset. For example, the peak effect after s.c. salvinorin A (0.032 mg/kg) was observed at 60 min postinjection and reached a maximum mean of 31.4 Δng/ml (S.E.M. = 11.6) (Fig. 3; note *y*-axis break added for illustration). A one-way repeated measures ANOVA for time (including mean preinjection baseline and 5–120 min after salvinorin A administration) was significant [$F(6,18) = 7.27$]. Newman-Keuls comparisons revealed that 0.032 mg/kg s.c. salvinorin A produced a prolactin increase compared with preinjection baseline only at 60, 90, and 120 min.

Antagonism Experiments. In separate studies, nalmefene (0.01 or 0.1 mg/kg) was administered as a pretreatment to salvinorin A (0.032 mg/kg i.v.), a dose that produced maximal or near-maximal prolactin release in all subjects. The smaller nalmefene pretreatment dose did not cause significant antagonism of salvinorin A, whereas the larger nalmefene dose (0.1 mg/kg) robustly antagonized the effects of salvinorin A (Fig. 4). A two-way repeated measures ANOVA [time × pretreatment condition (no pretreatment versus nalmefene 0.01 or 0.1 mg/kg)] revealed significant

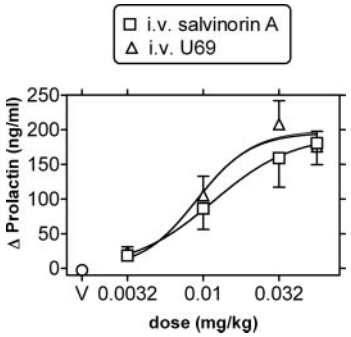

Fig. 2. Dose-effect curve for the effects of i.v. salvinorin A and i.v. U69,593 on serum prolactin levels in male subjects (data are obtained from 15 min after administration of each dose; see Fig. 1). Abscissa, dose of salvinorin A or U69,593. Ordinate, serum prolactin levels, expressed as change from individual preinjection baseline (Δ nanograms per milliliter). Other details as in Fig. 1.

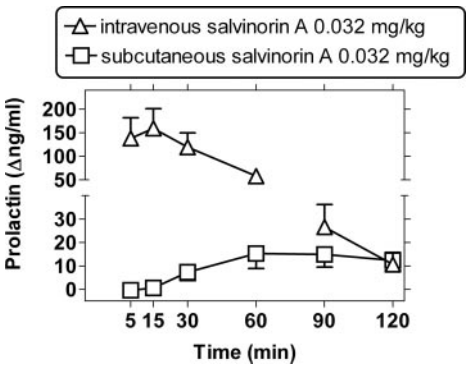

Fig. 3. Time course effects of salvinorin A (0.0032 mg/kg) administered by the i.v. or s.c. route on serum prolactin levels in male subjects ($n = 4$ each). Abscissa, time in minutes from injection. Ordinate, serum prolactin levels, expressed as change from individual preinjection baseline (Δ nanograms per milliliter; note axis break). Other details as in Fig. 1.

effects of time [$F(5,15) = 11.78$] and pretreatment condition [$F(2,6) = 11.49$] and their interaction [$F(10,30) = 8.54$]. Newman-Keuls comparisons revealed that only the larger nalmefene pretreatment dose (0.1 mg/kg) was significantly different from the "no pretreatment" condition. Antagonism surmountability experiments were not attempted for practical reasons, primarily solubility limitations for salvinorin A. In a separate pretreatment study with the 5-HT2 antagonist ketanserin (0.1 mg/kg i.m.), no antagonism of the same probe dose of salvinorin A (0.032 mg/kg i.v.) was observed (Fig. 4).

Female Subjects. Salvinorin A (0.0032 mg/kg i.v.) was studied in follicular phase females ($n = 4$). This salvinorin A dose, which produced only slight effects in males (above), produced larger prolactin elevations for this assay in the female subjects (see Fig. 5; with comparison to male subjects). Pilot studies with larger salvinorin A i.v. doses (0.032 mg/kg) revealed even greater neuroendocrine effects. To probe the effects of salvinorin A route of administration, a larger salvinorin A dose (0.032 mg/kg) was studied by the s.c. route. In females, s.c. salvinorin A produced robust prolactin release from 15 min after administration, and this effect persisted for at least 120 min (see Fig. 6). This effect of salvinorin A was prevented by nalmefene (0.1 mg/kg s.c. 30 min pretreatment). In this case also, the effects of salvinorin A were more robust in females than in males (compare Figs. 3 and 6).

Cloning of *OPRK1* and Genotyping of C-Terminal Sequence. The cloned cDNA contained 30 bp of the 5′-untranslated region, 1140 bp of the coding region, and 87 bp of the 3′-untranslated region. The obtained full-length coding sequence for rhesus monkey *OPRK1* was compared with the published sequence for the human *OPRK1*. There were 21 nucleotides and 6 amino acid residues that differed between the rhesus monkey and human *OPRK1* (see Fig. 7). Two predicted amino acid residue changes are located in the N terminus, and two others are in the C terminus of the rhesus monkey *OPRK1*, compared with the human *OPRK1* (Fig. 7). It is noteworthy that a proposed phosphorylation site serine residue (S358) in the C terminus in the human *OPRK1* is conserved in the rhesus monkey *OPRK1* (Liu-Chen, 2004). Sequence analysis of the C terminus of genomic DNA from 14 rhesus monkeys in the colony (including all eight subjects used in the present neuroendocrine studies, four male and four female) confirms the presence of this S358 residue in all

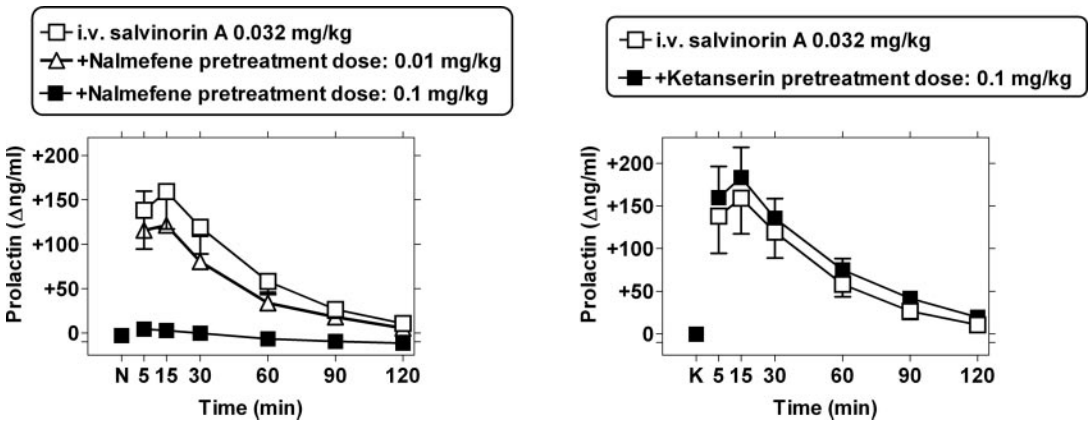

Fig. 4. Effects of nalmefene (0.01 or 0.1 mg/kg s.c.) or ketanserin (0.1 mg/kg i.m.) pretreatment to the effects of salvinorin A (0.032 mg/kg i.v.) in male subjects (n = 4 each). Abscissae, time in minutes from i.v. injection (point above N or K are samples obtained 20 min after administration of nalmefene or ketanserin alone, respectively). Ordinates, serum prolactin levels, expressed as change from individual preinjection baseline (Δ nanograms per milliliter). Other details as in Fig. 1.

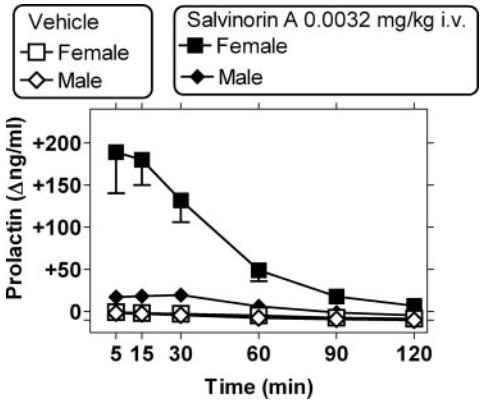

Fig. 5. Effects of i.v. salvinorin A (0.0032 mg/kg) or vehicle in male or female subjects (n = 4 each). Other details as in Fig. 1.

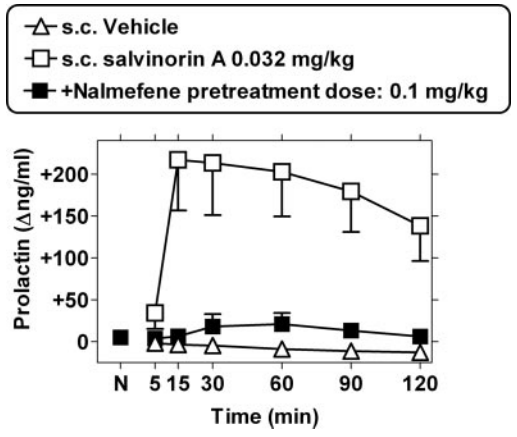

Fig. 6. Effects of s.c. salvinorin A (0.032 mg/kg) alone or after pretreatment with nalmefene (0.1 mg/kg s.c.) in female subjects. Point above N represents sample obtained 20 min after nalmefene alone. Other details as in Fig. 1.

subjects; no polymorphisms were detected in this region overall.

Discussion

The main aim of these studies was to examine the neuroendocrine effects of the widely available hallucinogen salvinorin A in an assay shown to be a useful biomarker for κ-opioid agonist effects in rhesus monkeys. A related aim of these studies was to determine the similarity of the rhesus monkey κ-receptor sequence, given reports of relevant interspecies differences between human κ-receptors and common experimental rodent species such as rat (Liu-Chen, 2004).

Salvinorin A was reported to be a selective κ-agonist, with potentially unique pharmacodynamic effects (Roth et al., 2002; Chavkin et al., 2004; Wang et al., 2004). In the present studies, i.v. salvinorin A caused robust dose-dependent prolactin release in male rhesus monkeys. Salvinorin A was approximately equipotent and equieffective to the synthetic high-efficacy κ-agonist U69,593, similar to initial in vitro reports (Roth et al., 2002). As expected from prior studies in humans, probe experiments with salvinorin A in gonadally intact female monkeys revealed quantitatively greater effects (Kreek et al., 1999). A probe experiment revealed that the neuroendocrine effects of a probe salvinorin A dose (0.032 mg/kg) were significantly greater by the i.v. than the s.c. route in males. Reasons for this are unclear but may be related to pharmacokinetic factors, possibly limiting bioavailability by the s.c. route (Schmidt et al., 2005).

As mentioned above, salvinorin A is a highly selective agonist at κ-receptors; however, it is known that other compounds, including μ-agonists, can also cause prolactin release in mammals. We therefore carried out antagonism studies with the clinically available compound nalmefene, which acts as a μ-opioid antagonist in rhesus monkeys at small doses (e.g., 0.01 mg/kg), and acts as both a μ- and κ-antagonist at relatively larger doses (e.g., 0.1 mg/kg) (France and Gerak, 1994; Butelman et al., 2002). In these studies, the smaller dose of nalmefene mentioned above did not prevent the effects of salvinorin A, whereas the larger dose of nalmefene fully prevented such effects. Taken together with previous data (France and Gerak, 1994; Butelman et al., 2002), these studies are consistent with mediation by κ-receptors in the neuroendocrine effects of salvinorin A.

Salvinorin A is distinct from classic hallucinogens such as *d*-lysergic acid diethylamide, in that it does not bind to the 5-HT2A receptor (Roth et al., 2002). We wanted to determine whether the present neuroendocrine effects of salvinorin A could be indirectly mediated by 5-HT2 receptors. In a probe experiment, the 5-HT2 antagonist ketanserin (0.1 mg/kg) did not block the neuroendocrine effects of salvinorin A under the

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Rhesus OPRK1     1 MDSPVQIFRGEPGPTCAPSACLPPNSSAWFPGWAELDSNGSAGSEDAQLEPAHISPAIPV
Human OPRK1      1 MDSPIQIFRGEPGPTCAPSACLPPNSSAWFPGWAEPDSNGSAGSEDAQLEPAHISPAIPV
Rat OPRK1        1 MESPIQIFRGEPGPTCAPSACLLPNSSSWFPNWAESDSNGSVGSEDQQLEPAHISPAIPV

Rhesus OPRK1    61 IITAVYSVVFVVGLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSTVYL
Human OPRK1     61 IITAVYSVVFVVGLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSTVYL
Rat OPRK1       61 IITAVYSVVFVVGLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSAVYL

Rhesus OPRK1   121 MNSWPFGDVLCKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINI
Human OPRK1    121 MNSWPFGDVLCKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINI
Rat OPRK1      121 MNSWPFGDVLCKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINI

Rhesus OPRK1   181 CIWLLSSSVGISAIVLGGTKVREDVDVIECSLQFPDDDYSWWDLFMKICVFVFAFVIPVL
Human OPRK1    181 CIWLLSSSVGISAIVLGGTKVREDVDVIECSLQFPDDDYSWWDLFMKICVFIFAFVIPVL
Rat OPRK1      181 CIWLLASSVGISAIVLGGTKVREDVDVIECSLQFPDDEYSWWDLFMKICVFVFAFVIPVL

Rhesus OPRK1   241 IIIVCYTLMILRLKSVRLLSGSREKDRNLRRITRLVLVVVAVFIVCWTPIHIFILVEALG
Human OPRK1    241 IIIVCYTLMILRLKSVRLLSGSREKDRNLRRITRLVLVVVAVFVVCWTPIHIFILVEALG
Rat OPRK1      241 IIIVCYTLMILRLKSVRLLSGSREKDRNLRRITKLVLVVVAVFIICWTPIHIFILVEALG

Rhesus OPRK1   301 STSHSTAALSSYYFCIALGYTNSSLNPILYAFLDENFKRCFRDFCFPLKMRMERQSTSRV
Human OPRK1    301 STSHSTAALSSYYFCIALGYTNSSLNPILYAFLDENFKRCFRDFCFPLKMRMERQSTSRV
Rat OPRK1      301 STSHSTAVLSSYYFCIALGYTNSSLNPVLYAFLDENFKRCFRDFYFPIKMRMERQSTNRV

Rhesus OPRK1   361 RNTVQDPAYLRDVDGINKPV   374/380 Homology vs. human(98.4%)
Human OPRK1    361 RNTVQDPAYLRDIDGMNKPV
Rat OPRK1      361 RNTVQDPASMRDVGGMNKPV   360/380 Homology vs. human (94.7%)
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Fig. 7. Amino acid coding sequence for *OPRK1* cloned from cDNA. Rhesus monkey sequence is compared with published sequence for human *OPRK1* (GenBank accession no. NM_000912), and rat *OPRK1* (GenBank accession no. NM_017167) (Yakovlev et al., 1995).

present conditions. This dose of ketanserin was sufficient to block the reinforcing effects of the stimulant/hallucinogen methylenedioxymethamphetamine (Ecstasy) in this species (Fantegrossi et al., 2002); methylenedioxymethamphetamine is also known to cause prolactin release in humans (Grob et al., 1996). Overall, this experiment supports the conclusion that salvinorin A produces this neuroendocrine effect through κ- and not 5-HT2 receptors in primates.

Cloning of the rhesus monkey *OPRK1* gene revealed greater predicted homology to human κ-receptor (374 of 380 residues; 98.4% homology) compared with that of other experimental species previously reported (for review, see Liu-Chen, 2004). Rhesus monkey *OPRK1*, as determined from cDNA and confirmed by genotyping the present subjects, exhibit the S358 residue in the C terminus, which is present in human *OPRK1*. Studies indicate that this residue is of critical importance for the maintenance of adaptations including receptor desensitization and internalization (Liu-Chen, 2004). Interestingly, this residue is not conserved in rat *OPRK1*, and this may underlie the lesser propensity for such adaptations in rat *OPRK1* in vitro. Overall, these initial studies suggest that rhesus monkey *OPRK1* may have greater functional similarity to human *OPRK1* than those of other experimental species. This is the first report, to our knowledge, of full-length cloning of a nonhuman primate κ-receptor. As expected based on studies of μ-receptors, nonhuman primates may provide valuable insights into species differences that may occur with other experimental subjects such as rodents (Miller et al., 2004).

In summary, the widely available hallucinogen salvinorin A produced effects consistent with high-efficacy agonist actions at κ-receptors, in a neuroendocrine biomarker of translational value. This confirms the κ-receptor as the primary site of action in vivo of this unique hallucinogen. These are, to our knowledge, the first data on the neuroendocrine effects of salvinorin A in any species. Salvinorin A's effects in this assay are consistent with reports of fast onset and relatively short duration of salvinorin A-containing preparations in humans (http://biopsych.com/cpdd/CPDD04_PDFs/CPDD04_981339497336.pdf; Gonzales et al., 2006).

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