Salvinorin A Produces Cerebrovasodilation through Activation of Nitric Oxide Synthase, $\kappa$ Receptor, and Adenosine Triphosphate–sensitive Potassium Channel

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ABSTRACT

Background: Salvinorin A is a nonopioid, selective $\kappa$ opioid–receptor agonist. Despite its high potential for clinical application, its pharmacologic profile is not well known. In the current study, we hypothesized that salvinorin A dilates pial arteries via activation of nitric oxide synthase, adenosine triphosphate–sensitive potassium channels, and opioid receptors.

Methods: Cerebral artery diameters and cyclic guanosine monophosphate in cortical periarachnoid cerebrospinal fluid were monitored in piglets equipped with closed cranial windows. Observation took place before and after salvinorin A administration in the presence or absence of an opioid antagonist (naloxone), a $\kappa$ opioid receptor–selective antagonist (norphinaltorphimine), nitric oxide synthase inhibitors (N(G)-nitro-L-arginine and 7-nitroindazole), a dopamine receptor $D_2$ antagonist (sulpiride), and adenosine triphosphate–sensitive potassium and Ca$^{2+}$–activated K channel antagonists (glibenclamide and iberiotoxin). The effects of salvinorin A on the constricted cerebral artery induced by hypocarbia and endothelin were investigated. Data were analyzed by repeated measures ANOVA (n = 5) with statistical significance set at a $P$ value of less than 0.05.

Results: Salvinorin A induced immediate but brief vasodilatation that was sustained for 30 min via continual administration every 2 min. Vasodilatation and the associated cyclic guanosine monophosphate elevation in cerebrospinal fluid were abolished by preadministration N(G)-nitro-L-arginine, but not 7-nitroindazole. Although naloxone, norbinaltorphimine, and glibenclamide abolished salvinorin A–induced cerebrovasodilation, this response was unchanged by iberiotoxin and sulpiride. Hypocarbia and endothelin-constricted pial arteries responded similarly to salvinorin A, to the extent observed under resting tone.

Conclusions: Salvinorin A dilates cerebral arteries via activation of nitric oxide synthase, adenosine triphosphate–sensitive potassium channel, and the $\kappa$ opioid receptor.

Salvinorin A is an active component of Salvia divinorum, a perennial herb of the Lamiaceae (mint) family, indigenous to Mexico. Salvia divinorum has long been traditionally used by local people to produce hallucinogenic experiences essential for spiritual divination. Salvinorin A is a highly efficacious, naturally occurring nonpeptide, and it is the only known nonnitrogenous $\kappa$ opioid receptor (KOR) agonist. Similar to the history of opium, Salvia divinorum is a naturally abundant plant that has been used by human beings for recreational purposes for several centuries. Salvinorin A has been proposed as a new opioid receptor agonist for clinical use. However, none of KOR agonists have been used clinically because of their known adverse effects. Although Salvinorin A does not belong to the opioid drug class, despite its KOR-agonist properties, it is banned in many counties and much of the United States. Unlike the KOR agonists, salvinorin A does not produce frank hallucinatory effects, and has no dysphoric actions. Many intrinsic characters of the compound (e.g., quick on-
set, short-acting sedative effect, no respiratory depression)\textsuperscript{7–9} make it attractive for use in well-controlled perioperative settings. Therefore, its physiologic profile and related mechanism should be well investigated—especially its effects on the central nervous system, including cerebral vasculature in normal and pathologic conditions. Although some neurologic effects of salvinorin have been well documented,\textsuperscript{1,10,11} no data exist for the cerebral vasculature effect of salvinorin A.

We have demonstrated that other KOR agonists (dynorphin and U50488) can dilate the pial artery through the nitric oxide pathway.\textsuperscript{10,12} In the current study, we hypothesized that salvinorin A might dilate pial arteries under resting and increased tone conditions induced by hypocarbia or endothelin \textit{via} activation of nitric oxide synthase, adenosine triphosphate–sensitive potassium (K\textsubscript{ATP}) channels, and \kappa receptors.

Materials and Methods
Salvinorin A (purity \textgeq 98%), sodium nitroprusside (SNP), \textit{N}(G)-nitro-L-arginine (L-NNA), glibenclamide, iberiotoxin, cromakalim, calcitonin gene–related polypeptide, NS1619, naloxone, methionine enkephalin, nornaltorphimine, 7-nitroindazole, sulpiride, and isoproterenol are obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were also obtained from Sigma-Aldrich and were of reagent grade.

Animals and Surgery
Newborn pigs (aged up to 6 days, 1.3–1.8 kg) of both genders were used for this study. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Philadelphia, Pennsylvania). Animals were induced with isoflurane (1–2 minimum alveolar concentration) and maintained with room air after tracheal cannulation. Rectal temperature was maintained at 37–39°C by a heating pad. A closed cranial window consisted of three parts: a stainless steel ring, a circular glass cover slip, and three ports consisting of 17-gauge hypodermic needles attached to three precut holes in the stainless steel ring. Cortical periarachnoid cerebrospinal fluid (CSF) was collected through the cranial window port for cyclic guanosine monophosphate (cGMP) determination. Before placing the window, the scalp was reflected and an opening was made in the skull over the parietal cortex. Then the dura mater was cut and retracted over the bone edge. The cranial window was placed on the cranial opening and cemented in place with dental acrylic. The space under the window was filled with artificial CSF with the following composition (in mM): 3.0 KCl; 1.5 MgCl\textsubscript{2}; 1.5 calcium chloride; 132 NaCl; 6.6 urea; 3.7 dextrose; 24.6 NaHCO\textsubscript{3} per liter; pH 7.33; POC\textsubscript{2}, 46 mmHg; and PO\textsubscript{2} 43 mmHg. Artificial CSF was warmed to 37–38°C before application to the cerebral cortical surface. Pial arteries were observed with a television camera mounted on a dissecting microscope. Vascular diameter was measured from a video monitor connected to the camera with a video microscler (FOR-A, IV550, Tokyo, Japan).

Experimental Protocols
Pial artery diameter (small artery, 120–160 \textmu m; arteriole, 50–70 \textmu m) was monitored and recorded every half minute for 10 min after injection of artificial CSF in the presence or absence of the investigated drug. In general, the window was flushed in 30 s with 1–2 ml CSF through the port connected into the side of the window. CSF samples were collected for cGMP analysis before medication administration and 10 min afterward. We collected the cerebral cortical periarachnoid CSF by slowly infusing CSF into one port of the window and allowing the CSF to drip freely into a collection tube on the opposite port.

Responses to salvinorin A (10 nM and 1 \mu M, dissolved with alcohol) and SNP (10 nM and 1 \mu M), were obtained in the absence and presence of L-NNA (1 \mu M), a nitric oxide synthase (NOS) inhibitor, and 7-nitroindazole (100 nM), an antagonist of neuronal NOS (nNOS). To distinguish the direct versus permissive role of nitric oxide in the salvinorin A–induced dilation response, pial artery diameter changes were recorded after SNP (100 \mu M), a subthreshold vascular concentration of a nitric oxide donor, as well as after L-NNA and salvinorin A coadministration. Also determined were the influences of the following on pial artery response: sulpiride (100 \mu M), a dopamine receptor \textsubscript{D} \textsubscript{2} antagonist; glibenclamide (100 nM), a K\textsubscript{ATP} channel antagonist; iberiotoxin (100 nM), a Ca\textsuperscript{2+}–activated K\textsuperscript{+} channel antagonist; cromakalim (1 \mu M); calcitonin gene–related polypeptide (10 nM and 1 \mu M), a K\textsubscript{ATP} agonist; and NS1619 (10 nM and 1 \mu M), a Ca\textsuperscript{2+}–activated K\textsuperscript{+} channel agonist. Finally, the effects of the following were investigated: naloxone (1 mg/kg IV) and topical nornaltorphimine (1 \mu M), KOR antagonists; and methionine enkephalin and isoproterenol (10 nM and 1 \mu M each), the latter being a \beta–adrenergic receptor agonist. All tested drug solutions were made fresh on the day of use. Pial artery response to continuous administration of salvinorin A every 2 min, was recorded for 30 min to determine whether sustained vascular dilatation could be achieved.

cGMP Determination
To determine the role of the nitric oxide pathway in the observed effects of salvinorin on cerebral vasculature, CSF samples were collected for cGMP determination before and after salvinorin A administration with or without L-NNA and nornaltorphimine pretreatment. Commercially available ELISA kits (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) were used to quantify cGMP concentrations.

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Salvinorin A on Constricted Vessels

To test the cerebrovascular effect of salvinorin during increased cerebrovascular tone, we induced vasoconstriction via hypocarbia (PaCO₂ reduced 20–30% for 10 min) and endothelin (0.1 pM). Pial artery diameter was monitored at baseline, after hypocarbia (n=3) or endothelin (n=5), and after administration of salvinorin A (10 nM and 1 µM).

Statistical Analysis

All data (diameters and cGMP) were analyzed using ANOVA with repeated measures (two-tailed) followed by Bonferroni post hoc test (SPSS 10.0 for Windows; International Business Machines Corporation, Armonk, NY). A P of less than 0.05 was considered statistically significant. Values are represented as mean ± SEM of the absolute value or as percentage change from baseline values. Distributions of all values were evaluated by histogram.

Results

Dilation Effect and the Role of Nitric Oxide Pathway

In a dose-dependent manner, salvinorin A dilated the pial artery (fig. 1A). This effect was observed immediately after salvinorin administration and lasted less than 5 min for both test doses. When salvinorin A was administered every 2 min, sustained dilation effects were observed for 30 min (fig. 1B). This response was abolished by L-NNA, the NOS inhibitor, but not by 7-nitroindazole, the nNOS antagonist (figs. 1A and C). The dilation response to SNP was not affected by L-NNA. Likewise, 100-pM SNP had no effects on the pial artery, but it restored the constriction induced by L-NNA although it did not restore the dilation response of salvinorin A as blocked by L-NNA (fig. 1D). Dilation in response to salvinorin A was associated with increased cGMP in CSF; L-NNA blocked cGMP elevation (fig. 2). No significant blood pressure changes occurred during salvinorin administration.

K<sub>ATP</sub> Channel Involves in the Dilation Effect

Glibenclamide, the K<sub>ATP</sub> channel inhibitor— but not iberiotoxin, the Ca<sup>2+</sup>-activated K<sup>+</sup> channel inhibitor—blocked the dilation effects of salvinorin A. Combined glibenclamide and iberiotoxin in any sequence also blocked salvinorin A–induced dilation (fig. 3). Glibenclamide, but not iberiotoxin, blocked dilation in response to both test doses of cromakalim and calcitonin gene–related polypeptide. Iberiotoxin, but not glibenclamide, blocked the dilation effects of NS1619 at both dosing levels (fig. 4).

Opioid Receptor Blocked Dilation Effect of Salvinorin

Norbinaltorphimine, the KOR-selective antagonist, and naloxone blocked the dilation effects of salvinorin A and methionine enkephalin. This response was unaffected by isoproterenol (figs.

Fig. 1. In the newborn piglet, salvinorin A dilated the brain pial artery in a dose-dependent manner (n = 5). (A) N(G)-nitro-L-arginine (L-NNA), a nitric oxide synthase inhibitor, blocks the dilation effects of salvinorin A, but not sodium nitroprusside (SNP). (B) Continual administration every 2 min of salvinorin A can sustain dilation of the pial artery for 30 min. (C) The neuronal NOS inhibitor 7-nitroindazole (7-NINA) did not block the dilation effects of salvinorin A. (D) Although 100-pM SNP restored the construction effects of L-NNA, it did not restore the dilation effects induced by salvinorin A, which was blocked by L-NNA.
5A and B). Likewise, sulpiride had no effect on the dilation response of salvinorin A (fig. 5C). Norbinaltorphimine blocked salvinorin A–induced cGMP elevation in the CSF. At both dosing levels, there were no differences among cGMP concentrations before versus after salvinorin A administration with norbinaltorphimine pretreatment ($P = 1.0$).

**Salvinorin Dilates Pial Arteries in Increased Cerebrovascular Tone Conditions**

Hypocarbia and endothelin significantly decreased the diameter of pial arteries (fig. 6). Salvinorin diluted pial arteries under increased tone conditions similar to that observed under normocapnic (resting tone) conditions.

No significant differences between responses of the small artery and arteriole were observed in any of above experiments. Thus, only changes in the small artery are presented.

**Discussion**

In the current study, we demonstrated that salvinorin A is a potent pial artery dilator in piglets under normal and vessel-constricted conditions as induced by endothelin and hypocarbia. Dilatation effects were observed immediately after salvinorin administration, lasted less than 5 min for each test dose (10 nM and 1 μM), and were dose dependent. Sustained dilatation effects were observed for 30 min with continual administration every 2 min. The activation of the opioid receptor, NOS, and $K_{ATP}$ channel were involved in the signal pathway of dilatation effects.

We previously demonstrated that U50488, an exogenous KOR agonist, and dynorphin, an endogenous KOR agonist, dilate the pial artery. $^1^2$ Pei et al. demonstrated that U50488 relaxes the isolated aortic artery in rat in a dose-dependent manner. Different from other KOR agonists, the dilatation effect of salvinorin is very short-lived, lasting less than 5 min. Therefore, salvinorin is the shortest acting agent known among the $\kappa$ agonists—presenting researchers with an opportunity to explore this novel agent that would allow for easy management and titration in perioperative and critical care settings. $^1^4$ To achieve longer lasting effects, infusion is the most common technique used in clinical anesthesia. In the current study, we found that persistent vascular dilatation can be achieved for 30 min via continual administration every 2 min.

Because continual administration every 2 min results in a sustained dilatatory effects, the unique structure of salvinorin A, not tachyphylaxis, contributes to its short-acting character. Ester linkage in its structure can be easily metabolized by esterase in the blood and tissues. Tsujikawa et al. demonstrated that carboxylesterase mainly involved in the salvinorin A hydrolysis in rat plasma and the degradation products including salvinorin B which are not pharmacologically active.

Similar to other KOR agonists, the activation of NOS, $K_{ATP}$ channels, and opioid receptors mediated salvinorin A’s dilatation effects. Goyagi et al. demonstrated that selective KOR agonist BRL 52537 protected the ischemic brain by attenuating nitric oxide production in the ischemic striatum. Zeynalov et al. proved that the neuroprotection of BRL 52537 was lost in nNOS-null mice. Therefore, the KOR agonist BRL 52537 attenuated nNOS activity and ischemia-evoked nitric oxide production. However, in the current study, pial artery dilatation to salvinorin A was abolished by the L-NNA, a nonspecific NOS inhibitor, but not 7-nitroindazole, a selective nNOS antagonist. This result indicates that nNOS is not involved in the dilative effective of salvinorin A. Because a subthreshold amount of the nitric oxide donor SNP failed to restore the dilation response, the elevation of cGMP in CSF is likely the result of stimulation, rather than inhibition, of nitric oxide production and direct activation of salvinorin on endothelial NOS rather than permissive enabling. The experimental paradigm used in this study does not allow us to determine the cellular site of origin for the cGMP detected in CSF, but the origin may include endothelial, vascular smooth muscle, and/or neuronal cells.

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**Fig. 2.** Salvinorin A increases cyclic guanosine monophosphate (cGMP) in the cerebrospinal fluid (CSF). N(G)-nitro-L-arginine (L-NNA) blocked salvinorin A–induced cGMP elevation and vascular dilation ($n = 5$).

**Fig. 3.** Glibenclamide (Glib), but not iberiotoxin (Iberi), blocked the dilation effects of salvinorin A (S). In any dosing sequence, combined glibenclamide and iberiotoxin blocked the dilation effects of salvinorin A ($n = 5$). * First-administered agent.
K\textsubscript{ATP} channel activation may result in hyperpolarization of the membrane of vascular smooth muscle cells. Membrane potential changes would then regulate muscle relaxation through alterations in Ca\textsuperscript{2+} influx through voltage-dependent Ca\textsuperscript{2+} channels.\textsuperscript{17,18} To our knowledge, this is the first study to demonstrate that salvinorin A activates the K\textsubscript{ATP} channel directly or indirectly. Different from many other agents that can activate the K\textsubscript{ATP} channel, salvinorin A can easily penetrate the blood-brain barrier.\textsuperscript{7,8} Because the K\textsubscript{ATP} channel plays a crucial protective role against brain injury from hypoxia, ischemia, and metabolic inhibition,\textsuperscript{19–22} salvinorin A might be a potential neuroprotective agent for future clinical use.

Although salvinorin A shows a high affinity for dopamine D\textsubscript{2} receptors,\textsuperscript{23} sulpiride, the dopamine D\textsubscript{2} receptor selective antagonist, has no effects on the dilation effects of salvinorin A. On the contrary, naloxone and norbinaltorphimine, the KOR-selective antagonist, abolished the vascular dilative effects of salvinorin A, suggesting the important role of κ receptor rather than the dopamine D\textsubscript{2} receptor.

The vascular dilative effect of salvinorin A observed in normal and constricted cerebral vessels by hypocarbia and endothelin opens new possibilities for clinical applications. Likely targets include treatment of cerebral vessel spasm in clinical situations such as migraine and after subarachnoid hemorrhage, where increase of endothelin plays an important role.\textsuperscript{24} More studies are necessary to demonstrate the dilation effects of salvinorin A on other pathologic conditions. Similar to other short-acting agents, continuous infusion could be used to titrate and achieve sustained effects.

In the current study, newborn piglets were used as study subjects. The piglet brain is gyrencephalic, similar in maturity to human infant, and contains more white than grey matters, the former being selectively vulnerable to injury.\textsuperscript{25,26} The newborn piglet was also used because it is large enough for easy placement of a cranial window and vascular visualization. Cerebral vascular
responses in the newborn piglet are similar to those of human subjects in many clinical situations; no reports indicate a difference in responses. However, it is advisable to retain caution in interpreting these data for use in adult subjects.

In conclusion, salvinorin A is a fast, short-acting, potent pial artery dilator in piglets in normal and vessel-constricted conditions induced by endothelium or hypocarbia. The mechanism involves the activation of NOS, K$_{\text{ATP}}$ channels, and the $\kappa$ opioid receptor. These findings suggest that salvinorin A might have clinical value in clinical settings that require cerebral vascular dilation.

References