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Salvinorin A inhibits colonic transit and neurogenic ion transport in mice by activating κ-opioid and cannabinoid receptors

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Abstract *The major active ingredient of the plant Salvia divinorum, salvinorin A (SA) has been used to treat gastrointestinal (GI) symptoms. As the action of SA on the regulation of colonic function is unknown, our aim was to examine the effects of SA on mouse colonic motility and secretion in vitro and in vivo. The effects of SA on GI motility were studied using isolated preparations of colon, which were compared with preparations from stomach and ileum. Colonic epithelial ion transport was evaluated using Ussing chambers. Additionally, we studied GI motility in vivo by measuring colonic propulsion, gastric emptying, and upper GI transit. Salvinorin A inhibited contractions of the mouse colon, stomach, and ileum in vitro, prolonged colonic propulsion and slowed upper GI transit in vivo. Salvinorin A had no effect on gastric emptying in vivo. Salvinorin A reduced veratridine-, but not forskolin-induced epithelial ion transport. The effects of SA on colonic motility in vitro were mediated by κ-opioid receptors (KORs) and cannabinoid (CB) receptors, as they were inhibited by the antagonists nor-binaltorphimine (KOR), AM 251 (CB_1 receptor) and AM 630 (CB_2 receptor). However, in the colon in vivo, the effects were largely mediated by KORs. The effects of SA on veratridine-mediated epithelial ion transport were inhibited by nor-binaltorphimine and AM 630. Salvinorin A slows colonic motility in vitro and in vivo and influences neurogenic ion transport. Due to its specific regional action, SA or its derivatives may be useful drugs in the treatment of lower GI disorders associated with increased GI transit and diarrhoea.*

Keywords *cannabinoid receptors, gastrointestinal tract, ion transport, kappa opioid receptor, motility, mouse colon, salvinorin A.*

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INTRODUCTION

Salvinorin A (SA), the major active ingredient of the plant *Salvia divinorum* is a potent and highly selective κ-opioid receptor (KOR) agonist.[1] *Salvia divinorum* has been used by the Mazatec Indians of Mexico for ritual and medicinal purposes.[1] The herbal infusion is believed to be helpful in a disease termed 'panzon de barrego' (swollen abdomen) and the plant extracts are also used to relieve diarrhoea.[1]

κ-Opioid receptors mediate important biological functions and are attractive molecular targets in the development of therapeutics. Behavioural processes activated by KOR ligands include pain perception and antinociception, dysphoria, locomotor activity and immunomodulation (for review see Dhawan *et al.*[2]) Over the years, the therapeutic potential of selective KOR ligands to treat pain,[2] drug abuse,[3] HIV infection,[4] and cancer[5] has been examined.

κ-Opioid receptor agonists are potent modulators of gastrointestinal (GI) functions with antidiarrhoeal and constipating effects. The activation of KOR on enteric

neurons inhibits cholinergic excitatory neurotransmission and thus reduces GI motility.[6] κ-Opioid receptor agonists control mucosal ion transport and produce antisecretory effects in the large intestine.[7] Recent studies suggest that KOR ligands may play a role in the treatment of GI disorders, such as postoperative ileus,[8] irritable bowel syndrome (IBS),[9] and intestinal inflammation.[10] The results of clinical trials with asimadoline, a peripherally acting KOR agonist, are promising in patients with IBS.[11] For example, asimadoline was shown to decrease pain and improve abnormal bowel function, thus favourably affecting symptoms of IBS.[12]

There are currently few reports on the activity of SA in the GI tract. Capasso *et al.* evaluated the effect of SA on enteric cholinergic transmission in guinea-pig ileum preparations *in vitro*.[13] In that study SA reduced electrically evoked contractions and this effect was mediated by KOR. Salvinorin A was also shown to inhibit GI motility in croton oil-induced intestinal inflammation in mice.[13,14] Interestingly, the inhibitory effect of SA on motility in inflamed tissue was mediated by KOR and CB_1 receptors.[15] Recent receptor binding studies suggested that SA binds to KOR, CB_1 and CB_2 receptors.[15] For KOR and CB_1 receptors, the functional involvement in the regulation of GI function was previously shown, whether SA acts through CB_2 receptors is uncertain.

As SA-containing products were suggested to reduce diarrhoea, we aimed to characterize the effects of SA on colonic motility and secretion. By using specific opioid and cannabinoid (CB) antagonists, we also wished to examine the potential receptors involved in these effects. This characterization is important to clarify the possible potential of SA or compounds derived from this molecule for future use in the treatment of functional disorders of the human colon.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (CD1, Charles River, Canada), weighing 20–26 g, were used for all experiments. The animals were housed at a constant temperature (22 °C) and maintained under a 12-h light/dark cycle in sawdust-lined plastic cages with access to laboratory chow and tap water *ad libitum*. Animals used for these studies were approved by the University of Calgary Animal Care Committee and the experiments were performed in accordance with institutional animal ethics committee guidelines that follow the guidelines established by the Canadian Council on Animal Care.

Isolated smooth muscle strips

Mice were sacrificed by cervical dislocation. Full-thickness segments of gastric fundus, ileum and distal colon were removed and kept in ice-cold oxygenated Krebs-Ringer solution (NaCl 115 mmol L^{-1}, KCl 8.0 mmol L^{-1}, KH_2PO_4 2.0 mmol L^{-1}, $NaHCO_3$ 25 mmol L^{-1}, $MgCl_2$ 2.4 mmol L^{-1}, $CaCl_2$ 1.3 mmol L^{-1}, and glucose 10 mmol L^{-1}). Luminal contents were gently flushed. All experiments lasted less than 3 h and each preparation was used for a single experiment only.

The preparations were mounted between two platinum electrodes, 1 cm apart and placed in separate organ baths (25 mL; 37 °C; oxygenated with 95% O_2/5% CO_2), as described previously.[16] Using a silk thread, one end of each preparation was attached to the bottom of the organ bath, while the other end was connected to a FT03 force displacement transducer (Grass Technologies, West Warwick, RI, USA). 0.5 g tension was applied and the preparations were allowed to equilibrate for 30 min. Changes in tension were amplified by a P11T amplifier (Grass Technologies, West Warwick, RI, USA) and recorded on a personal computer using the PolyView software (Polybytes Inc., Cedar Rapids, IA, USA).

Electrical field stimulation (EFS; 4 Hz; 24 V; stimulus duration 0.5 or 5 ms; train duration 10 s) was applied by a S88X stimulator (Grass Technologies, West Warwick, RI, USA). EFS of isolated smooth muscle strips caused twitch contractions, which were virtually abolished by the muscarinic receptor antagonist atropine (10^{-6} mol L^{-1}) or the neural blocker TTX (10^{-6} mol L^{-1}) (data not shown).

Salvinorin A, salvinorin B (SB, both 10^{-10}–10^{-6} mol L^{-1}) and JWH 133 (10^{-7}–10^{-5} mol L^{-1}) were added cumulatively into the organ baths and effects on the EFS-induced contractions were recorded. Each concentration was allowed to incubate for 15 min. Before adding drugs, the mean amplitude of four successive twitch contractions was used as an internal control. Changes in contractions were reported as percent of the internal control. In control experiments the effects of the vehicle were tested. To characterize the involvement of opioid and cannabinoid receptors, the following receptor antagonists were added into the organ baths 15 min prior to SA: opioid receptor antagonist naloxone (10^{-6} mol L^{-1}), KOR-selective nor-binaltorphimine (norBNI, 10^{-6} mol L^{-1}), CB_1-selective AM 251 (10^{-7} mol L^{-1}), and CB_2-selective AM 630 (10^{-7} mol L^{-1}).

Epithelial ion transport

The assessment of active ion transport was performed as detailed previously.[17] Briefly, full-wall thickness segments of mouse distal colon were opened along the mesenteric border and mounted in Ussing chambers (0.6 cm^2 opening). Tissues were kept at 37 °C in Krebs buffer (NaCl 115 mmol L^{-1}, KCl 8.0 mmol L^{-1}, KH_2PO_4 2.0 mmol L^{-1}, $NaHCO_3$ 25 mmol L^{-1}, $MgCl_2$ 2.4 mmol L^{-1}, $CaCl_2$ 1.3 mmol L^{-1}). Two tissue segments were used per mouse; one was used as a vehicle control, the other was exposed to drug treatments. Segments receiving vehicle or drug were alternated to eliminate any possible differences in ion transport responses between the mid and the distal regions of the colon.

Tissues were studied under short-circuited conditions in which the voltage was clamped to 0 mV using a WPI EVC-4000 voltage clamp (World Precision Instruments, Sarasota, FL, USA). The tissues were unclamped at the beginning and the end of each experiment to record open potential difference values for the calculation of tissue conductance (in mS cm^{-2}). After baseline I_{sc} was established (15–30 min), either drug or an equal volume of vehicle (100% DMSO) was added. For each challenge, the peak change in I_{sc} (ΔI_{sc}) was measured. In some experiments, tissues were challenged with the cAMP-dependent secretagogue forskolin (10^{-5} mol L^{-1}) or the voltage-dependent Na^+ channel activator veratridine (3×10^{-5} mol L^{-1}).[17]

Colonic expulsion test

Distal colonic expulsion was measured as reported recently.[18] Briefly, after an overnight fasting period, drugs (or vehicle) were injected intraperitoneally (i.p., max volume 100 μL) and 5 min later a prewarmed (37ºC) glass bead (2 mm) was inserted 2 cm into the distal colon using a silicone pusher. After the bead insertion, mice were placed in individual cages and the time to bead expulsion was determined. Mice that did not expel the bead within 30 min were sacrificed to confirm the presence of the bead in the lumen of the intestine.

All colonic expulsion tests were performed 5 min after i.p. administration of SA. The antagonists were administered i.p. 30 min prior to SA injection.

Gastric emptying and geometric centre of upper intestinal transit

Gastric emptying (GE) and geometric centre (GC) experiments were performed according to techniques described earlier.[18–20] Briefly, mice were fasted overnight with free access to tap water. On the day of experiment, the animals received a gavage of 0.2 mL of a marker solution (50 mg phenol red in 100 mL 1.5% methylcellulose, constantly stirred and held at 37 °C). Mice were sacrificed 20 min after administration of the meal. The stomach and the small intestine were carefully removed. The stomach was subsequently opened and its contents transferred to a test tube containing 4 mL of distilled water. After 20 min of sedimentation, 1 mL of supernatant was transferred to another tube containing 1 mL of 1 mol L^{-1} NaOH to develop the maximum intensity of the colour. The solutions were colorimetrically assayed with a Beckman DU 65 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) at 560 nm. Gastric emptying (%) was calculated according to the following formula:

$$GE = 100 \times \left(1 - \frac{amount\ of\ phenol\ red\ after\ 20\ min}{amount\ of\ phenol\ red\ after\ 0\ min}\right)$$

In the GC studies, 20 min after the administration of a meal, the entire small intestine with its content was isolated and divided into 10 segments of equal length. The intestinal contents of each bowel segment were vigorously mixed with 2 mL of distilled water. After 20 min period of sedimentation, 1 mL of supernatant was transferred to another tube containing 1 mL of 1 mol L^{-1} NaOH to develop the maximum intensity of the colour. The solutions were colorimetrically assayed with a Beckman DU 65 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA) at 560 nm. GC of small intestinal transit was calculated according to the following formula:

$$GC = \sum [\%A\ per\ segment \times segment\ number]$$

GC ranged from 1 (minimal motility) to 10 (maximal motility).

In all GE and GC experiments, animals were gavaged 5 min after i.p. administration of SA. The antagonists were administered i.p. 15 min prior to SA injection.

Statistics

In the *in vitro* experiments *n* indicates the number of individual tissues from at least three different animals.

Statistical and curve-fitting analyses were performed using PRISM 4.0 (GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as means ± SEM. Student's *t*-test was used to compare single treatment means with control means. ANOVA followed by Student-Newman-Keuls *post hoc* test was used for analysis of multiple treatment means. *P* values ≤0.05 were considered statistically significant.

Drugs

All drugs and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Oakville, ON, Canada). Salvinorin A (purity: 99% by HPLC) was isolated from *S. divinorum* leaves, purchased from The Sage Wisdom Salvia Shop (Malibu, CA, USA) by one of us (JKZ; 21). Salvinorin B was obtained from SA through hydrolysis and purified.[21] Naloxone hydrochloride, nor-binaltorphimine dihydrochloride, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN 55,212), (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro -6,6,9-trimethyl-6H-dibenzo[b,d]pyran (JWH 133), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251) and 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone (AM 630) were purchased from Tocris Bioscience (Ellisville, MO, USA).

In the *in vitro* experiments (isolated smooth muscle strips, ion transport), all drugs were dissolved in dimethyl sulfoxide. In the *in vivo* assays, drugs were dissolved in vehicle containing 5% dimethyl sulfoxide in saline. The vehicles in the used concentrations had no effects on the observed parameter.

RESULTS

Influence of SA on isolated smooth muscle strips *in vitro*

We first investigated the effects of SA on the isolated mouse colon. Salvinorin A, but not SB, reduced the amplitude of EFS-induced twitch contractions in a concentration-dependent manner (Fig. 1A), with a maximum inhibition of the amplitude of contraction of approximately 50% (Supplementary Table S1). The inhibitory effect of SA was attenuated by about 50% by the opioid receptor antagonist naloxone and to a similar extent by the selective KOR antagonist norBNI (both 10^{-6} mol L^{-1}), indicating the involvement of KORs (Fig. 1A, Supplementary Table S1). Interestingly, the CB_1 receptor antagonist AM 251 (10^{-7} mol L^{-1}) and the CB_2 receptor antagonist AM 630 (10^{-7} mol L^{-1}) also partially reversed the effects of SA (Fig. 1B, Supplementary Table S1). Surprisingly, the effects of the CB_2 receptor antagonist were greater than that of the CB_1 receptor antagonist. Adding AM 251 or AM 630 together with the KOR-selective antagonist norBNI (Fig. 1C, Supplementary Table S1) produced no additive effects, suggesting that the CB_1 and CB_2 receptors are involved in the KOR-sensitive pathways. When given alone, none of the antagonists modified the EFS-induced twitch contractions (data not shown).

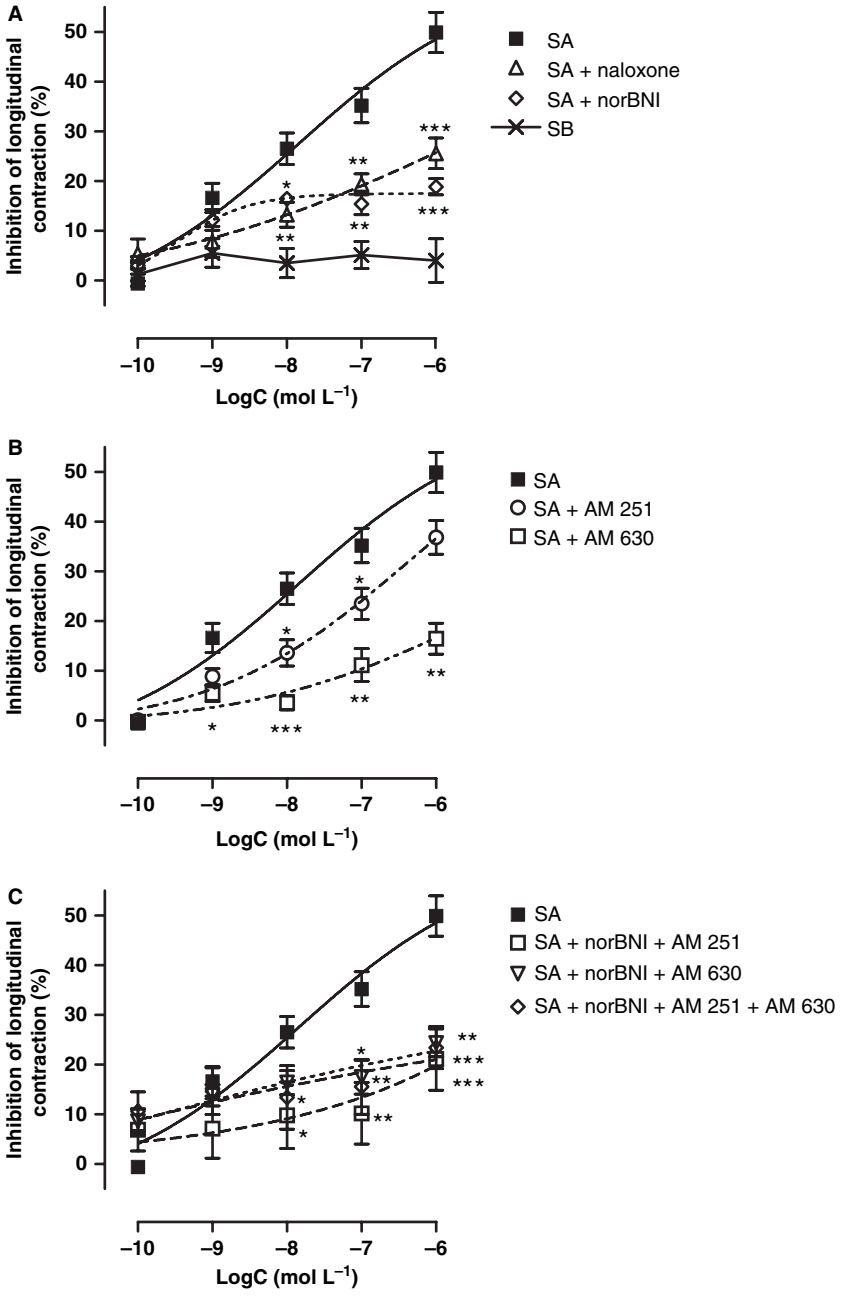

Figure 1 Concentration–response curves showing the inhibitory effect of salvinorin A (SA) and the lack of effect of salvinorin B (SB) on longitudinal smooth muscle contraction in mouse colon. (A) Effect of SA alone or SA in the presence of the opioid antagonist naloxone (10^{-6} mol L^{-1}) and the KOR antagonist nor-binaltorphimine (norBNI, 10^{-6} mol L^{-1}). (B) Effect of SA in the presence of the CB_1 antagonist AM 251 (10^{-7} mol L^{-1}) and the CB_2 antagonist AM 630 (10^{-7} mol L^{-1}). (C) Shows that the combination of norBNI (10^{-6} mol L^{-1}) with either AM 251 (10^{-7} mol L^{-1}) or AM 630 (10^{-7} mol L^{-1}) or both did not increase the blocking effect of norBNI alone. Data represent mean ± SEM for n = 6–10. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as compared with SA alone.

CB_2 receptors are not thought to play a role in the control of contractility under physiological conditions.[22,23] We therefore investigated whether the CB_2 receptor selective agonist JWH 133 altered EFS-evoked contractility. Under our experimental conditions JWH133 did not significantly reduce the amplitude of EFS-induced twitch contractions at high concentrations (13 ± 11% at 10^{-5} mol L^{-1}, n = 4, ns).

Neither SA nor SB (10^{-10}–10^{-6} mol L^{-1}) or the antagonists in the concentrations used had any effect on basal tone, resting phasic activity or smooth muscle precontracted with bethanechol (10^{-7}–2×10^{-5} mol L^{-1}, data not shown; n = 8), making a direct effect on smooth muscle unlikely.

Because we saw the surprising effects of SA acting at CB_2 receptors we further investigated these actions in the stomach and ileum. In mouse stomach, SA caused a concentration-dependent inhibition of the amplitude of EFS-induced twitch contractions, but the maximum inhibitory effect was less than that observed in the colon (Fig. 2A, Supplementary

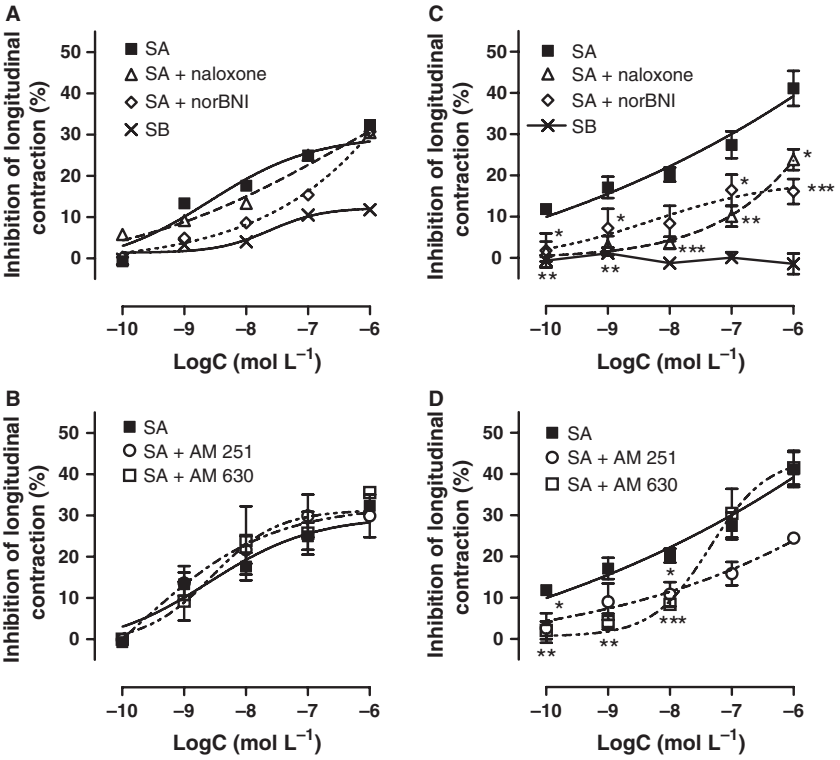

Figure 2 Concentration–response curves showing the inhibitory effect of salvinorin A (SA) and the lack of effect of salvinorin B (SB) on longitudinal smooth muscle contraction in mouse stomach (A and B) and mouse ileum (C and D). A and C: Effect of SA and SA in the presence of the opioid antagonist naloxone (10^{-6} mol L^{-1}) and the KOR antagonist nor-binaltorphimine (norBNI, 10^{-6} mol L^{-1}). B and D: Effect of SA in the presence of the CB_1 antagonist AM 251 (10^{-7} mol L^{-1}) and the CB_2 antagonist AM 630 (10^{-7} mol L^{-1}). Data represent mean ± SEM for n = 6–10. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as compared with SA alone.

Table S1). In the stomach the effects of SA were independent of KOR and cannabinoid receptors, as naloxone (10^{-6} mol L^{-1}), norBNI (10^{-6} mol L^{-1}), and the CB receptor antagonists AM 251 (10^{-7} mol L^{-1}) and AM 630 (10^{-7} mol L^{-1}) had no effect on SA activity (Fig. 2A,B, Supplementary Table S1).

In mouse ileum, SA caused a concentration-dependent inhibition of the amplitude of EFS-induced twitch contractions (Fig. 2C, Supplementary Table S1), as has previously been observed.[13,15] The effect of SA was KOR-mediated, as it was blocked by both, naloxone (10^{-6} mol L^{-1}) and norBNI (10^{-6} mol L^{-1}) (Fig. 2C, Supplementary Table S1) and also seemed to involve cannabinoid receptors, as the CB receptor antagonists AM 251 (10^{-7} mol L^{-1}) and AM 630 (10^{-7} mol L^{-1}) reduced SA-induced inhibition of contractions (Fig. 2D, Supplementary Table S1). Again, we investigated the actions of the CB_2 receptor agonist JWH 133. JWH 133 produced a significant concentration-dependent inhibitory effect on EFS-induced twitch contractions with a maximal inhibition of 37 ± 6% at 10^{-5} mol L^{-1} (n = 4, $P < 0.01$). The effect of JWH 133 was blocked in the presence of the CB_2 receptor antagonist AM 630 (3×10^{-7} mol L^{-1}) (maximum inhibition at 10^{-5}: 90 ± 3%, n = 4, ns).

Salvinorin B did not influence the EFS-induced smooth muscle contractions in mouse colon (Fig. 1A), stomach (Fig. 2A) or ileum (Fig. 2C).

Influence of SA on colonic epithelial ion transport *in vitro*

The reported antidiarrhoeal effects of SA may be due to an action on ion transport. We therefore investigated possible effects on epithelial ion transport. Salvinorin A (10^{-10}–10^{-4} mol L^{-1}) application to the serosal or the mucosal side of non-stimulated colonic epithelia did not influence the transepithelial short-circuit currents (data not shown). Furthermore, no significant differences were observed when tissues were stimulated with the adenylate cyclase activator forskolin (10^{-5} mol L^{-1}) (Fig. 3A), suggesting that the drug does not have a direct action on the colonic epithelium.

We then tested whether SA altered neurogenic secretion. Addition of veratridine to the serosal side of colonic epithelia caused an increase in I_{sc} as previously reported.[17] This response was substantially attenuated by both serosal ($P < 0.05$, Fig. 3B) or mucosal ($P < 0.01$, data not shown) application of SA (10^{-4} mol L^{-1}). The inhibitory effect of SA on veratridine-stimulated increase of colonic ion transport was reversed by the KOR antagonist nor-BNI (10^{-5} mol L^{-1})

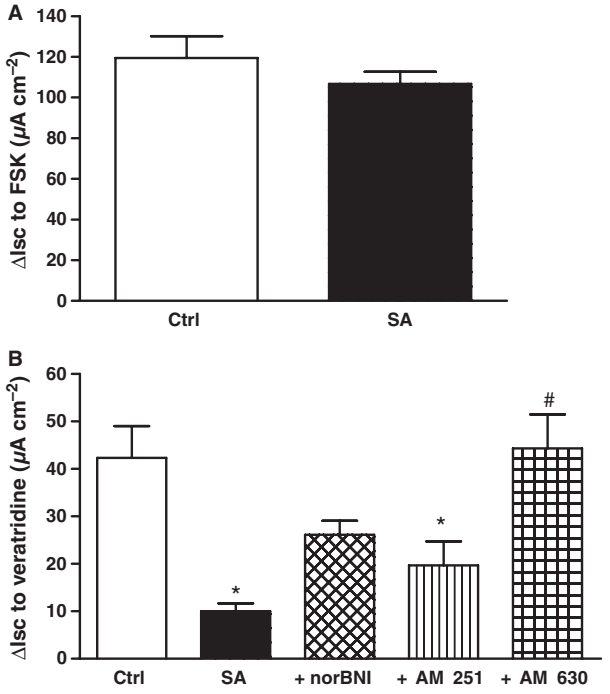

Figure 3 Changes in (A) forskolin (FSK, 10^{-5} mol L^{-1}) and (B) veratridine (3×10^{-5} mol L^{-1})-stimulated short-circuit current (I_{sc}) in mouse distal colon after serosal application of salvinorin A (SA, 10^{-4} mol L^{-1}) alone or in the presence of the KOR antagonist nor-binaltorphimine (norBNI, 10^{-5} mol L^{-1}), the CB_1 antagonist AM 251 (10^{-5} mol L^{-1}) and the CB_2 antagonist AM 630 (10^{-5} mol L^{-1}). Data represent mean ± SEM for n = 4–6 experiments. $^{*}P < 0.05$, as compared with control. $P < 0.05$, for SA *vs* antagonist + SA.

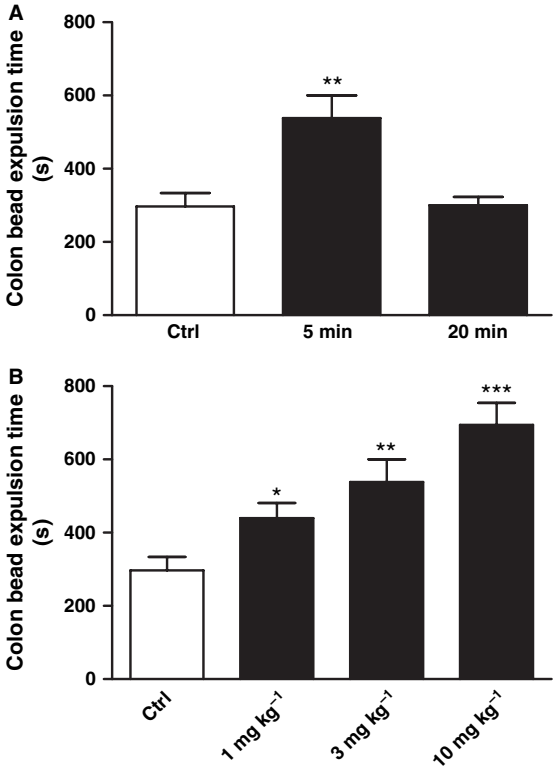

Figure 4 *In vivo* effects of salvinorin A (SA; 3 mg kg^{-1}) on colonic bead expulsion time in mice. (A) The time course of the changes of colonic bead expulsion time. (B) The dose dependence of the effect of SA on colonic bead expulsion. The results are shown as mean ± SEM of n = 8–10 mice for each experimental group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as compared with control.

and the CB_2 antagonist AM 630 (10^{-5} mol L^{-1}, $P < 0.05$) (Fig. 3B).

Influence of SA on colonic expulsion *in vivo*

To extend our findings from the *in vitro* organ bath into the *in vivo* situation, we performed standardized tests of *in vivo* motility. Salvinorin A produced a time- and dose-dependent inhibitory effect on colonic expulsion after i.p. administration. At the dose of 3 mg kg^{-1}, SA significantly reduced the rate of colonic expulsion 5 min after injection and its effect was no longer observed 20 min after administration of the drug (Fig. 4A). In the dose range used 1–10 mg kg^{-1}, the effect of SA on colonic expulsion was dose-dependent (Fig. 4B).

The inhibitory effect of SA on colonic expulsion was completely blocked by naloxone (1 mg kg^{-1}, i.p., n = 8–10, $P < 0.01$) and almost completely by norBNI (10 mg kg^{-1}, i.p., n = 8–10, $P < 0.05$) (Fig. 5). The CB receptor antagonists, AM 251 and AM 630 (both at the dose of 1 mg kg^{-1}, i.p.), did not alter SA-induced inhibition on colonic expulsion (Fig. 5). None of the antagonists used altered colonic expulsion at the doses used in this study (data not shown).

Influence of SA on gastric emptying and upper GI transit *in vivo*

Previously, it has been shown that SA inhibits upper GI transit in a manner that neither involves KORs nor cannabinoid receptors.[15] We have confirmed the main findings of this study. Thus the i.p. administration of SA at the dose of 3 mg kg^{-1} produced a significant slowing of the upper intestinal transit in mice (n = 6–10, $P < 0.01$), as indicated by a lower GC (Fig. 6A). This effect was not reversed by the KOR-selective antagonist norBNI (10 mg kg^{-1}, i.p., n = 6–10) (Fig. 6A). These findings illustrate regional differences of action of SA in the GI tract. To extend these observations, we also studied gastric emptying. Interestingly, SA, at the dose of 3 mg kg^{-1}, had no effect on GE (Fig. 6B). A highly potent CB receptor agonist WIN 55,212 (1 mg kg^{-1}), which was used a positive control, significantly inhibi-

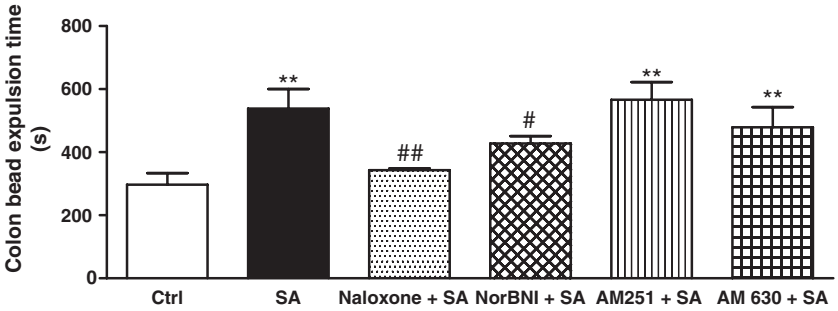

Figure 5 Effects of the opioid antagonist naloxone (1 mg kg^{-1}), the KOR antagonist nor-binaltorphimine (norBNI, 10 mg kg^{-1}), the CB_1 antagonist AM 251 (1 mg kg^{-1}), and the CB_2 antagonist AM 630 (1 mg kg^{-1}) on salvinorin A (SA, 3 mg kg^{-1})-induced increase of colonic bead expulsion time in mice. The results are shown as mean ± SEM of n = 8–10 mice. $^{**}P < 0.01$, as compared with control. $P < 0.05$, $^{\#\#}P < 0.01$ for SA *vs* antagonist + SA.

ted GE (42 ± 4 *vs* 67 ± 3% for control animals, n = 6, $P < 0.05$).

DISCUSSION

Extracts of the plants of the genus *Salvia* exhibit significant effects on muscle relaxation, neuroprotection and analgesia.[24] Among them, *S. divinorum* and its active component SA has recently gained interest because SA was identified as a potent KOR agonist with a selectivity for KOR_1.[1,25] SA has been known for its psychoactivity in the CNS, but new reports point to an additional role of SA, namely as a regulator of intestinal motility and a potential therapeutic in GI disorders.[13,14]

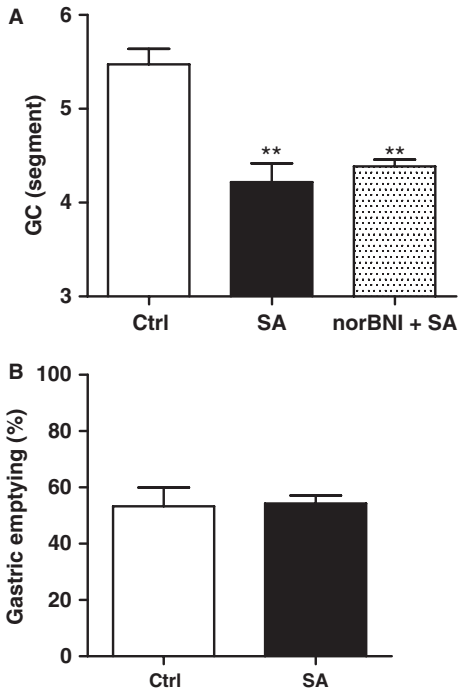

Figure 6 Effect of salvinorin A (SA, 3 mg kg^{-1}) alone or in the presence of the KOR antagonist nor-binaltorphimine (norBNI, 10 mg kg^{-1}) on intestinal transit (A) and gastric emptying (B) in mice. The results are shown as mean ± SEM of n = 6–10 mice for each experimental group. $^{**}P < 0.01$, $^{***}P < 0.001$, as compared with control. $^{\#\#}P < 0.001$, for SA *vs* antagonist + SA.

In this study, we investigated motor and secretory functions of the mouse GI tract in response to SA treatment *in vitro* and *in vivo*, with a focus on the colon. We observed that SA partially inhibited electrically-evoked twitch contractions in mouse stomach, ileum, and colon preparations *in vitro* and significantly prolonged colonic expulsion time. The effects of SA *in vivo* were mediated by KORs, because they were inhibited by a selective KOR antagonist norBNI and by the opioid receptor antagonist naloxone.

The partial inhibitory effect of SA on twitch contractions in mouse ileum and the KOR-mediated delay of colonic expulsion are in good agreement with previous observations.[13,15] Those studies also showed that the inhibitory effects of SA were more prominent in a diarrhoea-induced hypermotility, than in the physiological motility in mice.[14,15] Thus, SA seems to be effective in much lower concentrations in diarrhoea-induced hypermotility than in healthy mice.

In contrast to the absence of effect in forskolin-evoked colonic epithelial ion transport, SA reduced secretory responses to veratridine in the distal colon. Veratridine is a potent activator of voltage-dependent sodium channels and causes depolarisation of enteric neurones, resulting in increased chloride secretion across the colonic mucosa.[17,26] The inhibitory effect of SA on veratridine-mediated epithelial ion transport indicates its interaction with submucosal neurons, but not epithelium in mouse colon.

In our study, SB a de-acetylated derivative of SA and its major metabolite *in vitro* did not show any significant activity in GI tissue. Earlier studies showed that SB has negligible (>10 000 nmol L^{-1}) affinity at KOR[21]

and its administration produced neither antinociceptive, nor hypothermic effects in mice[25]. The lack of the effect of SB indicates that the acetyl group at the C-2 position of SA is crucial for its high affinity and potency at KOR.

Perhaps the most interesting finding of our study is that both the CB_1 antagonist AM 251 and the CB_2 antagonist AM 630 blocked the inhibitory effect of SA on twitch contractions in mouse colon preparations *in vitro* and AM 630 partially reversed the effects of SA on the ion transport. Previous studies suggested that SA may target receptors other than opioid receptors, as high doses caused a non-KOR-mediated decrease in mouse ileal motility. It was found that not only norBNI, but also the CB_1 antagonist rimonabant blocked the inhibitory effects of SA.[14,15] We have demonstrated that both CB_1 and CB_2 receptors are involved in inhibition of smooth muscle activity and the inhibition of secretion, in non-inflamed mouse colon and that these receptors likely interact in some manner with KORs. The CB_2 antagonist AM 630 was more effective in reversing SA effects than the CB_1 antagonist AM 251. This finding is in agreement with data from binding studies in human HEK-293 cells, where SA showed affinity at CB_2 receptors.[15]

The possible crosstalk between KORs and CB receptors was indicated by earlier studies in the CNS. For instance, rewarding effects induced by SA in the zebrafish and the rat were significantly reversed by norBNI and rimonabant.[27,28] Another report focused on a common mechanism of Δ^9-tetrahydrocannabinol and KORs in antinociception.[29] The findings that CB antagonists interfere with KOR-mediated effects suggest an interaction of cannabinoid receptors and KORs in cells expressing both receptors. In the GI tract, such cells could be acetylcholine- and neuropeptide-releasing motor neurons of the enteric nervous system (ENS), which are most likely the site of KOR-CB receptor interaction in our experiments. Both KORs and CB receptors are known to inhibit cholinergic neurotransmission. Thus, KORs have been demonstrated to suppress cholinergic and non-cholinergic excitatory pathways in human colonic muscle strips[6] and to inhibit acetylcholine release prejunctionally in guinea-pig colon and ileum.[13,30] One study showed that in mouse gastric preparations, not only CB_1 but also CB_2 activation inhibits excitatory cholinergic neurotransmission.[22] Our experiments with JWH 133 indicate that CB_2 receptor activation can reduce excitatory contractile response in the ileum *in vitro* which supports our findings with the CB antagonists *in vitro*. In the colon, we did not find evidence that CB_2 receptor activation alone altered colonic expulsion. This observation lends further support to the idea of some sort of interaction between the KOR and the CB_2 receptor whereby KOR activation is necessary to have a CB_2 receptor-mediated effect, whereas activation of CB_2 is not sufficient to alter motility under physiological conditions. Perhaps under pathophysiological conditions this may be different, as we have shown that the CB_2 receptor is now activated.[31] Further experiments to examine this observation are warranted. In line with these data, neurons of myenteric plexus ganglia strongly express both CB receptors and KORs.[31–33] Unfortunately, little information exists on KOR and CB receptor co-localization in ENS neurones that would anatomically validate the interaction of these receptors, except for a single report on cultured myenteric neurons of the pig ileum.[34]

Intracellularly, CB receptors and KORs may share a common signalling pathway. As our experiments showed, the co-application of CB and KOR antagonists did not further increase the inhibtion of SA-inhibited smooth muscle contraction induced by EFS, indicating that KOR and CB signalling could converge on a final pathway (Fig. 1C). κ-Opioid receptors and CB receptors might signal through a common G protein or form functional heterodimers, as recently described for mu-opioid and CB_1 receptors.[35] Most importantly, KORs, CB_1 and to a lesser extent CB_2 receptors, are all able to activate G protein-coupled inwardly-rectifying K^+ channels (GIRKs),[36,37] which fits in the concept that postsynaptic GIRK2 channels represent a common link to the effects of analgesic neurotransmitter receptors, such as opioid and cannabinoid receptors.[38] An interaction of CB receptors and KORs is also conceivable at the level of primary afferent innervation of the GI tract as they largely ramify in intestinal smooth muscle tissue and enteric ganglia. κ-Opioid receptors and CB receptors are expressed in sensory neurons[9,39] and like CB_1 receptor mRNA,[40] KOR mRNA can be transported along their axons.[41]

κ-Opioid receptor agonists could hold a high potential for clinical use in GI disorders because they are located on visceral primary afferents and are able to mediate antinociceptive behaviour.[9] On one hand, KOR agonists may normalize altered visceral hypersensitivity in primary afferents,[42] on the other hand they likely also interfere with cholinergic transmission in the ENS,[13,30] a function they share with CB_1 receptor agonists.[43]They would therefore be useful in GI disorders dominated by diarrhoea and abdominal pain. In this regard, the peripherally acting KOR agonist, asimadoline, has proven quite efficacious in clinical trials by relieving abdominal pain and discomfort associated with mixed IBS.[12] ADL 10-0101,

another peripherally restricted KOR agonist, has been shown to reduce abdominal pain associated with pancreatitis in humans.[44]

The plant *S. divinorum* has been used traditionally as a remedy against 'swollen belly' and diarrhoea. The basis of the anti-diarrhoeal effect of SA most likely lies in its inhibition of muscle contraction, as shown in mouse and guinea pig ileum[13,15] and now in mouse colon (this study). However, unlike asimadoline, SA also affects the CNS, and as with exogenous cannabinoids, the clinical use of SA is hampered by its psychotropic side effects. Although SA has a short half life *in vivo*, it rapidly crosses the blood-brain-barrier[45] and causes short-lived hallucinations. Barring SA from central activity would therefore be a prerequisite for its clinical use in GI disorders.

In summary, we have demonstrated that SA slows colonic expulsion *in vivo* and decreases smooth muscle contractions of the mouse colon and inhibits neurogenic ion transport *in vitro* via three different receptors: KOR, CB_1 and CB_2. Why there are differences *in vivo* and *in vitro* remains to be established, but probably lies with the pharmacokinetics or pharmacodynamics of SA. Our study provides a deeper insight into the mechanisms of SA-mediated inhibitory effect on GI motility and secretion. We have uncovered some interesting interactions between the opioid and cannabinoid receptor systems in our study that warrant further investigation, but may offer promise in the development of drugs to alleviate the symptoms of GI disorders. It seems unlikely that SA would be developed as a drug because of its psychotropic side effects. Derivatives of SA, however, with reduced central activity may prove useful in the treatment of lower GI disorders.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison of the inhibitory effect of SA alone or in the presence of opioid and cannabinoid receptor antagonists on longitudinal smooth muscle contraction in mouse tissues.

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