Kappa-opioid receptor-mediated effects of the plant-derived hallucinogen, salvinorin A, on inverted screen performance in the mouse

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Salvinorin A is a pharmacologically active diterpene that occurs naturally in the Mexican mint Ska Maria Pastora (*Salvia divinorum*) and represents the first naturally occurring κ-opioid receptor agonist. The chemical structure of salvinorin A is novel among the opioids, and thus defines a new structural class of κ-opioid-receptor selective drugs. Few studies have examined the effects of salvinorin A *in vivo*, and fewer still have attempted to assess the agonist actions of this compound at μ-opioid, δ-opioid, and κ-opioid receptors using selective antagonists. In the mouse, salvinorin A disrupted climbing behavior on an inverted screen task, indicating a rapid, but short-lived induction of sedation/motor incoordination. Similar effects were observed with the μ-agonist remifentanil and the synthetic κ-agonist U69,593. When behaviorally equivalent doses of all three opioids were challenged with antagonists at doses selective for μ-opioid, δ-opioid, or κ-opioid receptors, results suggested that the motoric effects of remifentanil were mediated by μ-receptors, whereas those of salvinorin A and U69,593 were mediated via κ-receptors. Despite similar potencies and degrees of effectiveness, salvinorin A and U69,593 differed with regard to their susceptibility to antagonism by the κ-antagonist nor-binaltorphamine. This later finding, coupled with the novel chemical structure of the compound, is consistent with recent findings that the diterpene salvinorin A may bind to the κ-receptor in a manner that is qualitatively different from that of more traditional κ-agonists such as the benzeneacetamide U69,593. Such pharmacological differences among these κ-opioids raise the possibility that the development of other diterpene-based opioids may yield important therapeutic compounds. *Behavioural Pharmacology* 16:627–633 © 2005 Lippincott Williams & Wilkins.

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Introduction

Salvinorin A (SVA) is one of several diterpenes isolated from the Mexican mint Ska Maria Pastora (*Salvia divinorum*). This plant has been used by indigenous peoples in the Oaxaca region of Mexico for hundreds of years (Valdes *et al.*, 1983; Sheffler and Roth, 2003), presumably for its psychoactive effects. Both the plant and SVA extracts are now widely available via the internet (Drug Enforcement Administration, 2002), where they are marketed as legal short-acting hallucinogens. In this regard, the psychoactive potency of SVA rivals that of lysergic acid diethylamide (Valdes *et al.*, 1984) although the hallucinogenic state induced by SVA is reported to be qualitatively different from that produced by the classical serotonergic hallucinogens of ergoline, indolealkylamine, or phenylisopropylamine structure (Siebert, 1994). Despite these qualitative differences, instances of recreational (Giroud *et al.*, 2000; Drug Enforcement Administration, 2002; Sheffler and Roth, 2003) and therapeutic (Hanes, 2001) use of the plant and SVA extracts have been reported, highlighting the easy availability of these preparations and suggesting that there may be some abuse potential for this compound (Bucheler *et al.*, 2005).

The mechanism of action for SVA was unknown until a recent paper by Roth and colleagues (2002) demonstrated that this compound binds as a potent and selective κ-opioid agonist. The agonist effects of SVA at κ-opioid receptors were further elaborated *in vitro* in studies demonstrating that this compound functions as a full agonist at this receptor (Chavkin *et al.*, 2004). SVA is thus the first naturally occurring exogenous κ-opioid receptor agonist to be discovered. Similarly, SVA is the only non-nitrogenous compound known to bind to opioid receptors. The structure of SVA is lipid-like, completely distinct from those of all previously identified opioid ligands, and thus defines a new structural class of κ-opioid-receptor selective drugs (see Fig. 1).

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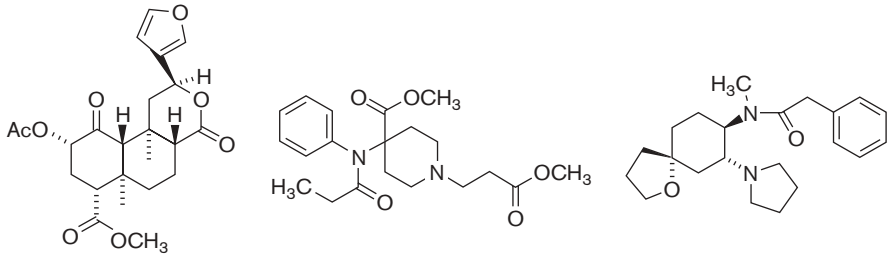
Fig. 1



Chemical structures of salvinorin A (left), remifentanil (middle), and U69,593 (right).

Few studies with SVA have been performed *in vivo*, but Butelman and coworkers (2004) have shown that the interoceptive cue induced by SVA is similar to that of the highly selective synthetic κ-opioid agonist U69,593 in rhesus monkeys. Additionally, the toxic effects of chronic SVA administration on the brain, heart, kidney, bone marrow, blood, and spleen were studied in mice, and found to be quite low (Mowry *et al.*, 2003). Recently, SVA has been shown to reduce caudate levels of dopamine, induce conditioned place aversion, and attenuate locomotor activity in the mouse (Zhang *et al.*, 2005). The results of these few reports are consistent with the idea that the effects of SVA *in vivo* are mediated by κ-opioid mechanisms.

In the present studies, we attempted to further characterize the effects of systemically administered SVA in mice using an inverted screen procedure to measure sedation/motor incoordination in a climbing task (e.g. Coughenour *et al.*, 1977). In this test, compounds with sedative and/or ataxic properties produce a dose-dependent impairment of climbing behavior, whereas drugs without sedative/ataxic effects (such as the psychomotor stimulants) do not (Ginski and Witkin, 1994). The ultra-short acting μ-opioid agonist remifentanil (Glass *et al.*, 1993; Rosow, 1999) and the high-efficacy brain-penetrating κ-opioid agonist U69,593 (Remmers *et al.*, 1999; Butelman *et al.*, 2002) were used as positive controls. Studies with receptor subtype-selective doses of opioid antagonists were also conducted to gauge the contribution of μ-opioid, δ-opioid, and κ-opioid receptors to the effects of all three compounds on climbing behavior (see Drugs section below for information on antagonist dose selection).

Methods

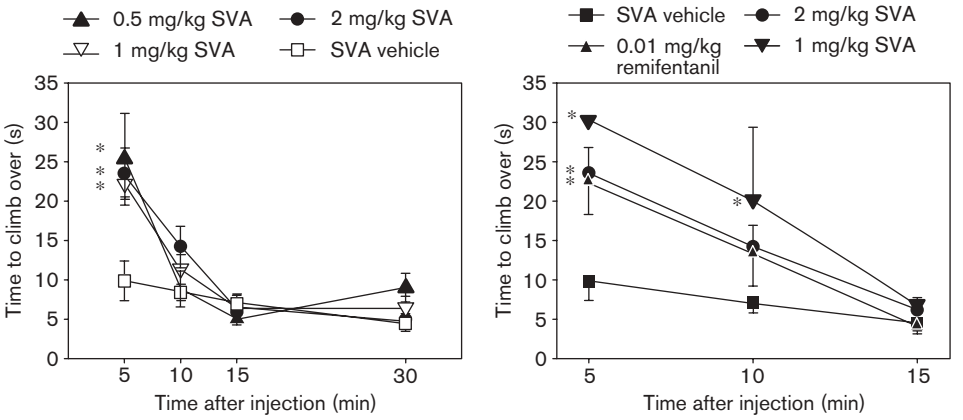
Subjects and apparatus

Male NIH Swiss mice (Harlan Sprague–Dawley Inc., Indianapolis, Indiana, USA) weighing approximately 20–30 g were housed (12 animals per $44.5 \times 22.3 \times 12.7$ cm Plexiglas cage) in a temperature-controlled room maintained at an ambient temperature of $22 \pm 2^{\circ}$C at 45–50% humidity. Lights were set to a 12-h light/dark cycle. Animals had free access to Lab Diet rodent chow (Laboratory Rodent Diet #5001, PMI Feeds Inc., St Louis, Missouri, USA) and water until immediately before testing. Animals were not used in experiments until at least 2 days after arrival in the laboratory. All studies were carried out in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, Maryland, USA). Experimental protocols were approved by the Animal Care and Use Committee at the University of Michigan.

Procedure

On experimental days, mice were weighed, marked, and returned to the home cage. Doses were then calculated and prepared for injection. Individual animals were subsequently removed from the home cage, injected intraperitoneally with saline or an opioid antagonist, and returned to the home cage again for the duration of the pretreatment interval. After the appropriate pretreatment interval had elapsed, mice were injected with various doses of SVA, U69,593, remifentanil, the SVA vehicle, or saline, and then returned once more to the home cage until testing. The SVA dose range used here has been shown to be neurochemically and behaviorally active in the mouse following intraperitoneal injection (Zhang *et al.*, 2005). During testing, mice were placed on a 15×15 cm wire mesh screen elevated 40 cm above the apparatus floor. The screen was slowly inverted by 180°, and time to climb over onto the top of the screen (all four paws on the upper surface) was recorded for each animal. Animals falling off the inverted screen were not assigned a time or included in any statistical analyses, but are noted in the figures by a fraction over the applicable point or bar corresponding to number of mice successfully climbing/total mice studied; these animals appeared to be profoundly impaired, typically 'sliding off' the screen immediately on inversion. For time course studies, the same animal was tested on the apparatus at multiple post-injection time points. For such studies, data from repeated saline or SVA vehicle control injections are

Fig. 2



Left panel: effects of three salvinorin A (SVA) doses, or the SVA vehicle, on inverted screen performance in mice. Each data point represents the mean time to climb over the inverted screen for six mice, while vertical error bars represent the SEM values. Right panel: comparison of behaviorally equivalent doses of SVA, U69,593, and remifentanil on inverted screen performance. For both panels, horizontal axes define time after injection, vertical axes represent time to climb over the inverted screen, and asterisks indicate significant differences from vehicle-injected mice at $P<0.05$.

presented so that the effect of previous experience with the task can be gauged. Similarly, the effects of repeated exposure to U69,593 on inverted screen performance were determined in order to assess the development of tolerance to the motoric effects of this compound.

Drugs

Remifentanil (GlaxoWellcome, Research Triangle Park, North Carolina, USA), U69,593 (Pharmacia & Upjohn, Kalamazoo, Michigan, USA), naloxone (National Institute on Drug Abuse, Bethesda, Maryland, USA), nor-binaltorphimine (nor-BNI, provided by Dr H.I. Mosberg, Division of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan, USA), and naltrindole (National Institute on Drug Abuse, Research Triangle Park, North Carolina, USA) were dissolved in sterile water. Salvinorin A was isolated from the leaves of *Salvia divinorum*, as described previously (Valdes *et al.*, 1984), and dissolved in a solution composed of ethanol (10% of volume), Alkamuls (10% of volume), and sterile water (80% of volume) to a final concentration of 0.2 mg/ml. As SVA tended to precipitate out of this preparation, fresh SVA solutions were prepared before each experiment and used immediately. All injections were administered intraperitoneally in a volume equal to body weight (g)/100.

Doses of the μ-opioid and κ-opioid antagonists, naloxone and nor-BNI, respectively, were chosen on the basis of published literature and pilot studies with the inverted screen procedure (see Fig. 3). Antagonist doses of the δ-opioid receptor blocker naltrindole could not be empirically determined because δ-opioid agonists stimulate locomotor activity in rodents, and therefore would not be expected to alter climbing behavior in the inverted screen task (Ginski and Witkin, 1994). The dose of naltrindole employed in the present studies (3.2 mg/kg) has, however, previously been used to selectively block antinociception produced by various peptidic δ-agonists in mice, whereas doses as high as 20 mg/kg have proven ineffective against other types of opioid-induced antinociception (Suzuki *et al.*, 1995; Wells *et al.*, 2001; Lattanzi *et al.*, 2002).

Data analysis and statistics

Data are presented as mean time to climb over ± SEM for groups in which $n = 6$ mice (unless otherwise indicated, as described above). For SVA time course studies, data were compared using a two-way (dose × time) ANOVA and analyzed *post hoc* with Tukey's honestly significant difference test. To determine the behavioral equivalency of selected doses of remifentanil and U69,593 with a reference dose of SVA, data were analyzed by a two-way repeated-measures ANOVA, then compared *post hoc* with SVA using the method of Dunnett. For antagonist experiments, SVA data satisfied conditions of normality and were therefore analyzed by a one-way ANOVA; remifentanil and U69,593 data for these experiments did not satisfy conditions of normality and were therefore analyzed with the Kruskal–Wallis one-way ANOVA on ranks. Antagonist data for all three opioids were compared *post hoc* with the appropriate saline-pretreated group using the method of Dunnett. All statistical tests were performed with commercially available software and significance was judged at $P < 0.05$.

Results

Salvinorin A time course study

Following the injection of SVA vehicle, mice rapidly climbed over the inverted screen at all time points. With successive testing (i.e. later time points), time to climb

over decreased for all animals, indicating a mild 'practice effect' (Fig. 2, left panel, inverted triangles). Statistical testing revealed a significant effect of time ($P < 0.05$) and dose ($P < 0.05$), as well as significant interaction ($P < 0.05$). All doses of SVA significantly increased climbing time to a similar degree at 5 min after injection ($P < 0.05$), but there was no significant effect of SVA dose at this time point. In contrast, at 10 min after injection, a non-significant trend towards dose dependence emerged ($P = 0.09$). Animals receiving the lowest dose were least impaired and performed at saline-like levels, animals receiving the intermediate dose were not fully recovered, and animals receiving the highest dose remained most impaired. By 15 min following SVA, and at later time points, climb times had returned to control levels in all animals (Fig. 2, left panel). Higher doses of SVA could not be tested because of the poor solubility of this compound.

Comparison with remifentanil and U69,593

Remifentanil and U69,593 had similar effects to SVA in the inverted screen test (Fig. 2, right panel). Statistical testing revealed a significant effect of time ($P < 0.05$), but not of drug, and there was no significant interaction effect. A dose of 0.01 mg/kg remifentanil (open triangles) and a dose of 1.0 mg/kg U69,593 (filled inverted triangles) thus induced motoric effects in the inverted screen procedure that were behaviorally equivalent to those induced by 2.0 mg/kg SVA (filled circles) in terms of time course and effectiveness, whereas higher doses of both remifentanil and U69,593 elicited more profound effects (data not shown). These equivalent doses were used for further comparisons among compounds following antagonist pretreatments.

Antagonist pretreatments and repeated agonist injections

The motor incoordinating effects of 2.0 mg/kg SVA (Fig. 3, top panel, filled bar) were not altered by 3.2 mg/kg naltrindole (30 min pretreatment) or 0.1 mg/kg naloxone (15 min pretreatment), but were significantly blunted following 24 h pretreatment with 30.0 mg/kg nor-BNI ($P < 0.05$). In contrast, the effects of 0.01 mg/kg remifentanil were not altered by pretreatment with naltrindole or nor-BNI, but were significantly reduced ($P < 0.05$) by naloxone (Fig. 3, middle panel). Finally, the effects of U69,593 on motor performance were not attenuated by naltrindole, naloxone, or nor-BNI when tested 24 h after pretreatment (see Fig. 4, left panel). Interestingly, the effects of U69,593 on inverted screen performance were significantly attenuated ($P < 0.05$) when the nor-BNI pretreatment period was extended to 5 days (Fig. 3, bottom panel).

This differential rate of development of the antagonist effects of nor-BNI against SVA and U69,593 was an unexpected result, and thus prompted further study to more fully characterize the time course of antagonist effects to all three opioids. Following 30 mg/kg nor-BNI, the effects of remifentanil on climbing behavior were unaltered over a period of at least 7 days, while the effects of SVA were completely antagonized at all time points (Fig. 4, left panel). In contrast, significant antagonism ($P < 0.05$) of the motoric effects of U69,593 again did not develop until 5 days after nor-BNI administration (Fig. 4, left panel). Repeated injections of U69,593 administered to a separate group of mice in the absence of antagonist pretreatment (Fig. 4, right panel) revealed that significant ($P < 0.05$) tolerance developed to the motoric effects of U69,593 at day 7, on the fourth exposure to the drug. The effects of repeated SVA injections in the absence of nor-BNI were not assessed in further studies, as antagonist effects were evident on the first injection of SVA, thus ruling out a tolerance-based interpretation of the data.

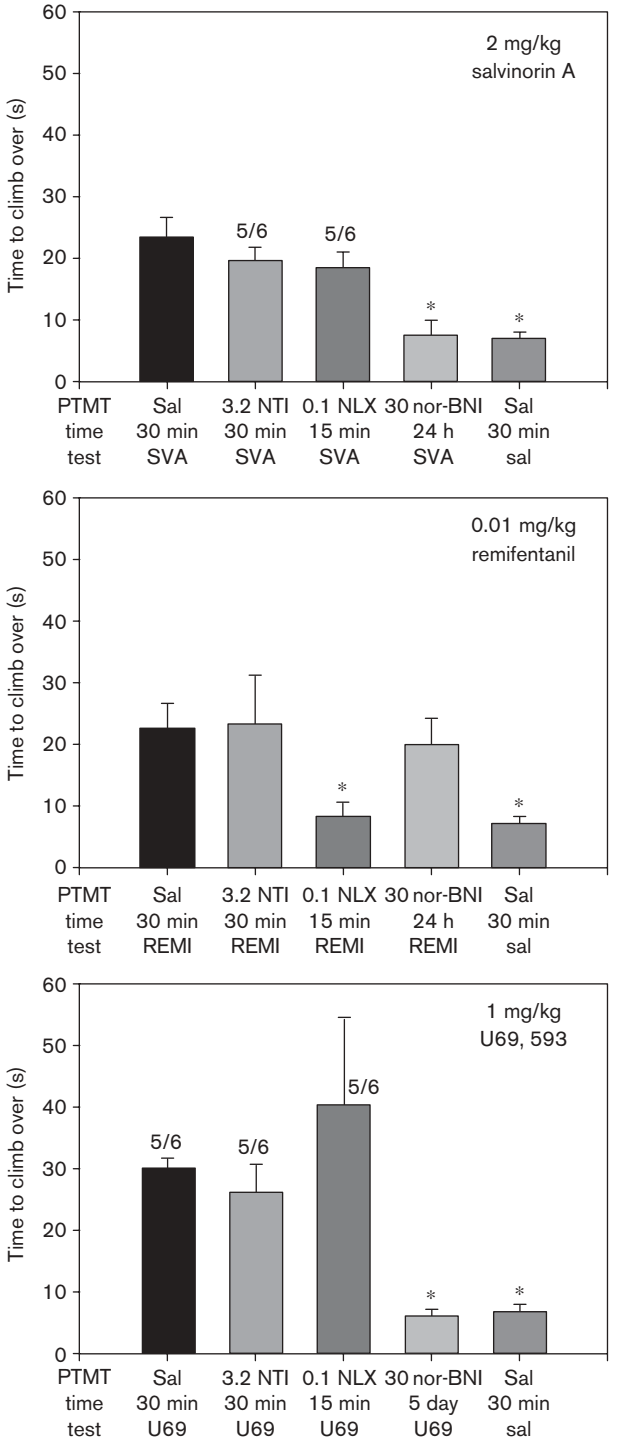
Discussion

In the present studies, systemically injected SVA induced κ-opioid receptor-mediated motoric effects similar to those of the brain-penetrating κ-agonist U69,593. SVA did not produce dose-dependent effects on climbing behavior, probably because of the limited range of doses that were studied. Although differences between this compound and other opioids might have been observed had the lower doses of SVA (0.5 or 1.0) been compared with remifentanil or U69,593, the present results nevertheless identified behaviorally equivalent doses of all three agonists.

Although SVA was somewhat less potent than U69,593 in these studies, previous in-vitro (Roth *et al.*, 2002) and in-vivo (Butelman *et al.*, 2004) assays of κ-receptor interaction have shown that SVA and U69,593 are equally potent. One reason for differences in potency in the present and previous studies might be that the present formulation of SVA was not optimal for the present experiments. A comparable range of doses of SVA, prepared similarly, was recently shown to be neurochemically and behaviorally active in the mouse, suggesting that the formulation of SVA for the present experiments was not problematic (Zhang *et al.*, 2005). Large differences in potency (on a mg/kg basis) also are observed between active SVA doses in the monkey (as reported by Butelman *et al.*, 2004) and those in the mouse (present results). Such differences are probably owing to a higher ratio of κ-receptors to total opioid receptors in the primate brain (e.g. Ko *et al.*, 2003) than in the mouse brain (e.g. Yoburn *et al.*, 1995). Alternatively, pharmacokinetic factors might also underlie this species difference. As yet, there are no relevant data to bolster the latter conjecture.

Time course studies with all three opioid agonists revealed fast onsets (peak effects within 5 min after injection) and short durations of action (15 min or less) in this assay. A similarly rapid onset for the interoceptive

Fig. 3

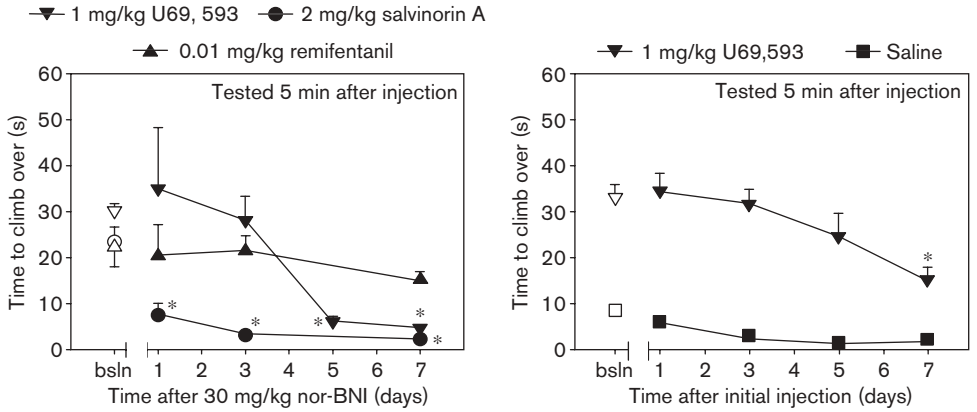


Antagonist effects of various opioid antagonists on inverted screen performance 5 min after injection of behaviorally equivalent doses of salvinorin A (SVA) (top panel), remifentanil (middle panel), or U69,593 (bottom panel). Each bar represents the mean time to climb over the inverted screen. Each group was composed of six mice, and fractions above bars indicate number of mice completing task/number of mice tested. Vertical error bars delineate the SEM values. For all panels, horizontal axes define pretreatment condition (top line), pretreatment time (middle line), and test compound (bottom line); asterisks indicate significant differences from saline pretreated mice at $P<0.05$.

effects of SVA has been previously described in the rhesus monkey (Butelman *et al.*, 2004) and in the human (Siebert, 1994). At behaviorally equivalent doses, the effects of remifentanil on inverted screen performance were mediated by μ-receptors, while those of SVA and U69,593 were mediated by κ-receptors. Yet, despite this common mechanism of action for SVA and U69,593, antagonism of the effects of these two compounds by nor-BNI differed with respect to the time course. One interpretation of such data could be that the effects of U69,593 on climbing behavior are *not* κ-receptor mediated, and that the observed changes in inverted screen performance over successive days are due to the development of tolerance to the motoric effects of U69,593 with repeated injection rather than by antagonism by nor-BNI. This interpretation seems unlikely, as complete antagonism of the motoric effects of U69,593 by nor-BNI was observed at time points days earlier than the partial tolerance demonstrated by repeated injections (compare left and right panels of Fig. 4). Similarly, in separate experiments with different mice, the antagonist effects of nor-BNI were evident upon the *first* exposure to U69,593 5 days after antagonist injection (see Fig. 3, bottom panel), precluding any involvement of tolerance. These findings seem to imply that the differential susceptibility of SVA and U69,593 to nor-BNI antagonism is due to dissimilar interactions of these structurally-distinct agonists with the κ-receptor.

Further differences between the molecular effects of SVA and U69,593 have previously been outlined. In this regard, it has been reported that SVA has a decreased capacity to internalize and downregulate κ-receptors *in vitro* as compared with synthetic κ-receptor agonists such as U69,593 (Wang *et al.*, 2005). Such pharmacodynamic differences might contribute to the differential susceptibility to nor-BNI antagonism reported here. Alternatively, the agonist binding at κ-receptors exhibited by SVA and U69,593 might differ in some other qualitative dimension that renders U69,593 less prone to displacement by antagonists. This later point is particularly intriguing in its implication that more than one receptor conformation (induced by differential binding properties of agonists with diverse chemical structures) may yield full agonist effects within the κ-opioid system. Consistent with this idea, molecular mechanisms by which SVA binds to κ-opioid receptors have recently been shown to differ from those for binding by U69,593 and the endogenous peptide dynorphin; more specifically, SVA binds to the same recognition site as these other κ-opioid agonists, but appears to interact with unique residues within this shared binding pocket (Yan *et al.*, 2005). Conceivably, the further development of such novel diterpene-based compounds, particularly those with high affinity and selectivity for μ-receptors, may yield a novel set of clinically useful opioids.

Fig. 4



Left panel: development of nor-binaltorphamine (nor-BNI) antagonism against the effects of behaviorally equivalent doses of remifentanil, salvinorin A (SVA), and U69,593 on inverted screen performance. Each data point represents mean time to climb over the inverted screen for six control mice (open symbols) or six mice previously treated with 30 mg/kg nor-BNI (closed symbols), then challenged with saline (squares), 0.01 mg/kg remifentanil (triangles), 2 mg/kg SVA (circles), or 1 mg/kg U69,593 (inverted triangles). Vertical error bars represent the SEM values. Right panel: development of tolerance to the effects of U69,593 on inverted screen performance. Each data point represents mean time to climb over the inverted screen for six single-injection control mice (open symbols) or six mice repeatedly injected with saline (squares) or 1 mg/kg U69,593 (inverted triangles). Vertical error bars represent the SEM values. For both panels, horizontal axes define time after nor-BNI (left) or first injection of saline or U69,593 in days ('bsln' represents baseline data obtained from separate groups of mice, in the absence of antagonist pretreatment). Vertical axes represent time to climb over the inverted screen, and asterisks indicate significant differences from baseline performance at $P<0.05$.

To our knowledge, the present studies provide the first functional in-vivo assessment of the agonist actions of SVA at μ-opioid, δ-opioid, and κ-opioid receptors. These findings recapitulate the results of previous in-vitro (Roth *et al.*, 2002; Sheffler and Roth, 2003; Chavkin *et al.*, 2004) and in-vivo (Butelman *et al.*, 2004; Wang *et al.*, 2005; Zhang *et al.*, 2005) research that has described SVA as a selective and efficacious κ-agonist. Furthermore, these studies suggest that the agonist binding properties of SVA at κ-opioid receptors differ from those of U69,593 *in vivo* in ways that may be particularly relevant to medications development efforts.

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