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Comparison of Pharmacological Activities of Three Distinct κ Ligands (Salvinorin A, TRK-820 and 3FLB) on κ Opioid Receptors in Vitro and Their Antipruritic and Antinociceptive Activities in Vivo

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ABSTRACT

Salvinorin A, TRK-820 (17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl) acrylamido]morphinan hydrochloride), and 3FLB (diethyl 2,4-di-[3-fluorophenyl]-3,7-dimethyl-3,7-diazabicyclo[3.3.1]nonane-9-one-1,5-dicarboxylate) are structurally distinctly different from U50,488H [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate], the prototypic selective κ agonist. Here, we investigated their in vitro pharmacological activities on receptors expressed in Chinese hamster ovary cells and in vivo antiscratch and antinociceptive activities in mice. All three compounds showed high selectivity for the κ opioid receptor (KOR) over the μ opioid receptor (MOR) and δ opioid receptor (DOR) and nociceptin or orphanin FQ receptors. In the guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding assay, all three were full agonists on the KOR. The rank order of affinity and potency for the KOR was TRK-820 ≫ U50,488H ~ salvinorin A ≫ 3FLB. TRK-820 acted as a partial agonist on MOR and DOR, whereas salvinorin A and 3FLB showed no activities on these receptors. Salvinorin A, TRK-820, and 3FLB caused internalization of the human KOR in a dose-dependent manner. Interestingly, although salvinorin A and U50,488H had similar potencies in stimulating [^{35}S]GTPγS binding, salvinorin A was about 40-fold less potent than U50,488H in promoting internalization. Following 4-h incubation, all three compounds induced down-regulation of the human KOR, with salvinorin A causing a lower extent of down-regulation. Although TRK-820 was potent and efficacious against compound 48/80-induced scratching, salvinorin A showed low and inconsistent effects, and 3FLB was inactive. In addition, salvinorin A and 3FLB were not active in the acetic acid abdominal constriction test. The discrepancy between in vitro and in vivo results may be due to in vivo metabolism of salvinorin A and 3FLB and possibly to their effects on other pharmacological targets.

At least three types of opioid receptors, μ, δ, and κ, mediate pharmacological effects of opioid drugs and physiological actions of endogenous peptides (for review, see Chang, 1984; Mansour et al., 1988). Opioid receptors are coupled to G_i/G_o proteins to affect several different effectors, including inhibition of adenylyl cyclase, enhancement of K^+ conductance, decrease in Ca^{2+} conductance, and activation of p42/p44 mitogen-activated protein kinases (for review, see Law et al., 2000). In addition, opioid receptors are shown to act through Gz to inhibit adenylyl cyclase and G_{16} to activate phospholipase C (Lai et al., 1995; Lee et al., 1998), and κ opioid receptors stimulate Na^+, H^+-exchanger 3 activity via Na^+, H^+-exchanger regulatory factor-1/Ezrin-radixin-moesin-binding phosphoprotein-50 independent of pertussis toxin-sensitive G proteins (Huang et al., 2004).

μ, δ, and κ opioid receptors of several species have been cloned (for review, see Kieffer, 1995; Knapp et al., 1995). In addition, a receptor with high sequence similarity to the opioid receptors, termed the ORL1 receptor, was cloned and found to be coupled to G_i/G_o proteins (for review, see Kieffer, 1995; Knapp et al., 1995). Subsequently, the endogenous ligand for the ORL1 receptor was identified and named no-

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ABBREVIATIONS: N/OFQ, nociceptin/orphanin FQ; (−)U50,488H, (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate; U69,593, (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; TRK-820, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl) acrylamido]morphinan hydrochloride; HZ2, 2,4-di-2-pyridyl-3,7-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-one 1,5-diester; 3FLB, diethyl 2,4-di-[3-fluorophenyl]-3,7-dimethyl-3,7-diazabicyclo[3.3.1]nonane-9-one-1,5-dicarboxylate; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; CHO, Chinese hamster ovary; DAMGO, Try-D-Ala-Gly-(Me)Phe-Gly-ol; DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen-OH (disulfide bridge between D-Pen^2 and D-Pen^5); hKOR, human κ opioid receptor; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; ICI 204,448, (*R,S*)-*N*-[2-(*N*-methyl-3,4-dichlorophenylacetamido)-2-(3-carboxyphenyl)ethyl]pyrrolidine.

ciceptin or orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995). In this paper, the term N/OFQ receptor is used. All four receptors have seven transmembrane domains and belong in the rhodopsin subfamily of G protein-coupled receptors (Schwartz and Holst, 2002).

Activation of κ opioid receptors produces many effects in vivo including antinociception (VonVoigtlander et al., 1983; Dykstra et al., 1987), dysphoria (Pfeiffer et al., 1986; Dykstra et al., 1987), and water diuresis (VonVoigtlander et al., 1983; Dykstra et al., 1987). Studies on mice deficient in the κ opioid receptor have demonstrated that κ opioid receptors are involved in the perception of visceral chemical pain and mediates the hypolocomotor, antinociceptive and aversive actions of U50,488H [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate], a selective κ opioid agonist (Simonin et al., 1998). In addition, the κ opioid receptor participates in the expression of chronic morphine-induced withdrawal syndromes (Simonin et al., 1998) and mediates the aversive effects of Δ9-tetrahydrocannabinol (Ghozland et al., 2002). κ agonists may be useful as analgesics (VonVoigtlander et al., 1983; Wheeler-Aceto and Cowan, 1991), water diuretics (Bhargava and Gulati, 1988; Reece et al., 1994), and antipruritic drugs (Gmerek and Cowan, 1988).

U50,488H (Fig. 1), an arylacetamide compound, was the prototypic selective κ opioid receptor agonist (VonVoigtlander et al., 1983). Subsequently, many compounds were synthesized based on the arylacetamide structure, and some were found to be selective κ agonists, including U69,593 ((+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide), spiradoline, enadoline, ICI 204,448, and asimadoline (Szmuszkovicz, 1999). Recently, several compounds that do not have the arylacetamide structure were reported to be κ opioid receptor agonists. Salvinorin A, TRK-820 (17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl) acrylamido]morphinan hydrochloride), and HZ2 (2,4-di-2-pyridyl-3,7-dimethyl-3,7-diazabicyclo-[3.3.1]nonan-9-one 1,5-diester) are among these compounds.

Salvinorin A (Fig. 1), a neoclerdane diterpene, is extracted from the leaves of the mint family plant *Salvia divinorum*. The leaves have been used in religious rituals and cause hallucinations. Roth et al. (2002) conducted a large-scale screening of target molecules by binding, including cloned human G protein-coupled receptors, transporters, and ligand-gated ion channels, and found that salvinorin A was selective for the κ opioid receptor. Salvinorin A is the only non-nitrogenous selective κ opioid receptor agonist known to date (Sheffler and Roth, 2003). It has been reported that salvinorin A, currently unregulated in the United States, has similar potency to lysergic acid diethylamide in producing hallucinations (Siebert, 1994).

TRK-820 is a 4,5-epoxymorphinan compound with substitutions at the 6 position (Fig. 1) (Nagase et al., 1998). TRK-820 showed potent κ agonist activity in vitro and exhibited high antinociceptive and antipruritic activities, which are mediated by the κ opioid receptor (Endoh et al., 1999, 2000; Togashi et al., 2002). TRK-820 has been registered for use in Sweden as an antipruritic drug for the treatment of uremic pruritus in kidney dialysis patients (Scrip, 2003).

3FLB (diethyl 2,4-di-[3-fluorophenyl]-3,7-dimethyl-3,7-diazabicyclo[3.3.1]nonane-9-one-1,5-dicarboxylate) (Fig. 1) is a fluorphenyl derivative of HZ2, which was synthesized based on the 3,7-diazabicyclo[3.3.1]nonan-9-one skeleton (Siener et al., 2000). HZ2 exhibited high affinity for the κ opioid receptor in rat brain membranes (Siener et al., 2000). HZ2 also showed a strong and long-lasting antinociceptive effect in the mouse tail-flick test (Kogel et al., 1998). To the best of our

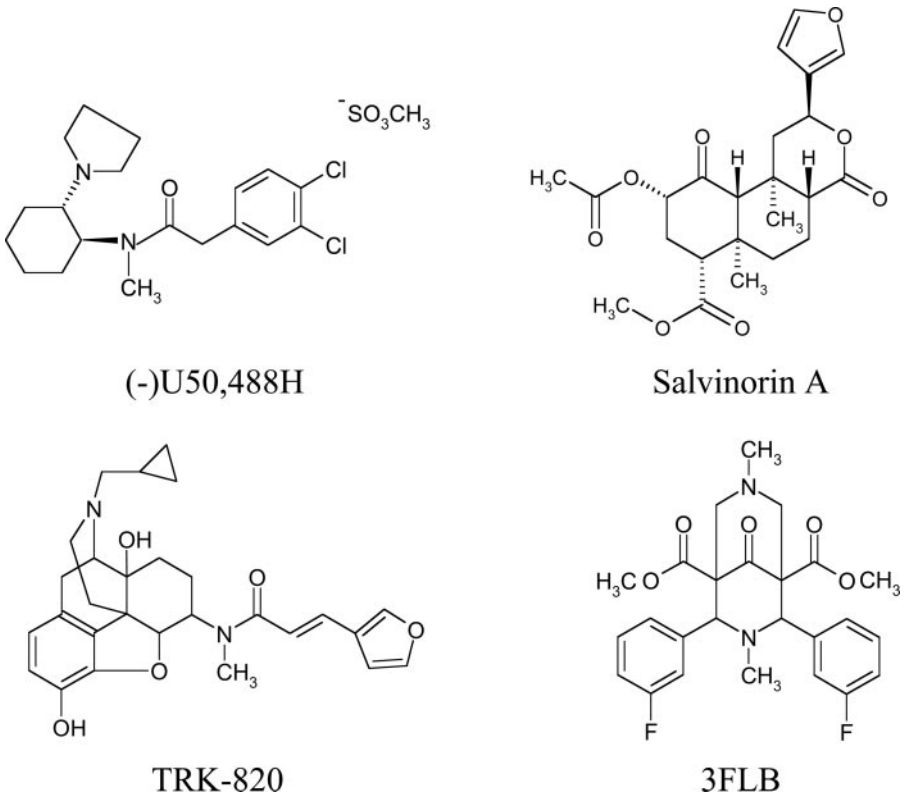

Fig. 1. Chemical structures of (−)U50,488H, salvinorin A, TRK-820, and 3FLB.

knowledge, pharmacological properties of 3FLB have not been characterized in detail.

In this study, we compared the biochemical pharmacological activities of salvinorin A, TRK-820, and 3FLB on opioid and N/OFQ receptors. We determined their binding affinities to the receptors and their potencies and efficacies in activating the receptors to enhance guanosine 5′-*O*-(3-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) binding in CHO cells stably transfected with the μ, δ, or κ opioid or N/OFQ receptor. In addition, because κ agonists differ in their abilities to regulate the human κ opioid receptor (Li et al., 1999, 2003), we examined whether these three compounds caused internalization and down-regulation of the human κ opioid receptor in CHO cells. Finally, we examined the three compounds for their effects against compound 48/80-induced scratching in mice and whether or not salvinorin A and 3FLB were antinociceptive in the mouse abdominal constriction test.

Materials and Methods

Materials. TRK-820 HCl was a generous gift from Adolor Corporation (Exton, PA). U50,488H was obtained from the National Institute on Drug Abuse (Bethesda, MD). 3FLB was synthesized in Ulrike Holzgrabe's laboratory, and salvinorin A was supplied by Daniel Siebert. [15,16-^{3}H]Diprenorphine (56 Ci/mmol), [leucyl-3,4,5-^{3}H]nociceptin/orphanin FQ (N/OFQ) (94 Ci/mmol), and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). DAMGO and nociceptin/OFQ were purchased from Phoenix Pharmaceuticals (Belmont, CA). Naloxone HCl, GDP, M1 mouse anti-FLAG monoclonal antibody, rabbit anti-FLAG polyclonal antibody, normal goat serum, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). The following reagents were obtained from indicated companies: GTPγS, Roche Diagnostics (Indianapolis, IN); geneticin (G418), Cellgro Mediatech, Inc. (Herndon, VA); DPDPE, ICI (Downingtown, PA); goat anti-mouse IgG (H+L) conjugated with Alexa Fluo 488, Molecular Probes (Eugene, OR); SuperSignal wet pico chemiluminescent substrate kit, Restroe Western blot stripping buffer, Pierce Chemical (Rockford, IL); and Opti-MEMI reduced serum, Invitrogen (Carlsbad, CA). Lab-Tek II Slide Chambers were purchased from Lab-Tek (Naperville, IL).

Cell Lines. CHO cells stably transfected with the rat μ opioid receptor were established previously (Chen et al., 1995). CHO cells stably expressing the mouse δ opioid receptor were kindly supplied by Dr. Ping-Yee Law (Department of Pharmacology, University of Minnesota, School of Medicine, Minneapolis, MN). CHO cells with stable expression of the human κ opioid receptor were established previously (Zhu et al., 1997). CHO cells stably transfected with the human N/OFQ receptor were a gift from Dr. Lawrence Toll (SRI International, Menlo Park, CA; Adapa and Toll, 1997). Cells were cultured in 100-mm culture dishes in Dulbecco's modified Eagle's medium/F-12 HAM supplemented with 10% fetal calf serum, 0.4 mg/ml geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

HEK 293 cells stably expressing the FLAG-hKOR (HEK-FLAG-hKOR) were established previously (Li et al., 2003). Cells were cultured in 100-mm culture dishes in minimum essential medium supplemented with 10% fetal calf serum, 0.4 mg/ml geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

Cell Membrane Preparation. Membranes were prepared according to a modified procedure of Zhu et al. (1997). Cells were washed twice and harvested in Versene solution (0.54 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 1 mM glucose) and centrifuged at 500*g* for 3 min. The cell pellet was suspended in lysis buffer (5 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride), passed through a 26 × three-eighths-gauge needle 10 times and then centrifuged at 46,000*g* for 30 min. The pellet was resuspended in lysis buffer and centrifuged again. The membrane pellet was resuspended in 50 mM Tris-HCl buffer (50 mM Tris, pH 7.4, and 1 mM EGTA), aliquoted and frozen in dry ice/ethanol, and stored at −80°C. All procedures were performed at 4°C.

Receptor Binding. Ligand binding experiments were carried out with [^{3}H]diprenorphine for opioid receptors and [^{3}H]nociceptin/OFQ for the N/OFQ receptor. Saturation binding of [^{3}H]diprenorphine to μ, δ, and κ opioid receptors was performed with at least six concentrations of [^{3}H]diprenorphine (ranging from 25 pM to 2 nM), and K_d and B_{max} values were determined (Huang et al., 2001). Competition inhibition by salvinorin A, TRK-820, and 3FLB of [^{3}H]diprenorphine (0.4 nM) binding to opioid receptors was performed in the absence or presence of various concentrations of each drug. Binding was carried out in [^{35}S]GTPγS binding buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA with 10 μM GDP freshly added) at room temperature for 1 h in duplicate in a final volume of 0.5 ml with ~10 μg of membrane protein. Naloxone was used to define nonspecific binding: 10 μM for κ and δ opioid receptors and 1 μM for μ opioid receptor. Bound and free [^{3}H]diprenorphine were separated by filtration under reduced pressure with GF/B filters presoaked with 50 mM Tris, pH 7.4, 0.1 mg/ml bovine serum albumin, and 0.2% polyethyleneimine. Radioactivity on filters was determined by liquid scintillation counting. Each experiment was performed in duplicate and repeated at least three times. Binding data and K_i values of each drug were analyzed and determined with the Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

Saturation binding of [^{3}H]nociceptin/OFQ to the N/OFQ receptor was performed with at least six concentrations of [^{3}H]nociceptin/OFQ (ranging from 25 pM to 2 nM), and K_d and B_{max} values were determined (Huang et al., 2001). Competition inhibition by 3FLB, TRK-820, and salvinorin A of [^{3}H]nociceptin/OFQ (0.2 nM) binding to the N/OFQ receptor was performed, and K_i values of each drug were determined. Nociceptin/OFQ (1 μM) was used to define nonspecific binding.

[^{35}S]GTPγS Binding. Determination of [^{35}S]GTPγS binding to G proteins was carried out using a modified procedure of Zhu et al. (1997). Immediately before the [^{35}S]GTPγS binding assay, membranes were thawed at 37°C, chilled on ice, and diluted with binding buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA). Membranes (~10 μg) were incubated in binding buffer containing [^{35}S]GTPγS (~80 pM) and 10 μM GDP with or without a ligand in a total volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was defined by incubation in the presence of 10 μM GTPγS. Bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure. Radioactivity on filters was determined by liquid scintillation counting. EC_{50} values, and maximal responses (E_{max}) of drugs were determined by curve fitting to the equation for a sigmoidal curve: Y = bottom + (top − bottom)/(1 + 10^(LogEC$_{50}$ − X)). X is the logarithm of concentration, and Y is the response.

Fluorescence Flow Cytometry. The procedure was modified from that of Li et al. (2003). CHO cells stably transfected with the FLAG-hKOR cultured in 12-well plates were left untreated or were treated for 30 min at 37°C with U50,488H, TRK-820, salvinorin A or 3FLB at indicated concentration. Cells were lifted with PBS containing 0.5 mM EDTA, 58 mM Na_2HPO4, 17 mM NaH_2PO4, 68 mM NaCl, and 0.5 mM EDTA, pH 7.4, and washed twice with TBS (50 mM Tris, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.4). Cells were incubated with M1 anti-FLAG antibody (1.0 μg/ml) in 500 μl of Opti-MEM I at 4°C for 1 h. After three washes with TBS, cells were incubated with goat anti-mouse IgG (H + L)-conjugated Alexa Fluo 488 (1 μg/ml) in 500 μl of Opti-MEM I at 4°C for 45 min. Cells were washed three times with ice-cold TBS and then resuspended with 300 μl of TBS. Receptor immunofluorescence was quantitated by FACScan (BD Biosciences, San Jose, CA). Fluorescence intensity of

10,000 cells was collected for each sample. Cellquest software (BD Biosciences) was used to calculate the mean fluorescence intensity of single cells in each population. Internalized receptors (percentage of surface receptors) = 100% − (the mean fluorescence of live cells with drug treatment/the mean fluorescence of live cells without drug) × 100%. The dose-response relationship was fitted to the equation: $y = [E_{max}/[1 + (x/EC_{50})s]] +$ background, in which y is the response at the dose x, E_{max} is the maximal response, and s is a slope factor.

Fluorescence Microscopy. Endocytic trafficking of receptors was visualized using an "antibody feeding" method described by Chu et al. (1997). Briefly, HEK 293 cells stably transfected with the FLAG-hKOR were grown on Lab-Tek II Slide Chambers, and the surface receptors were specifically labeled by incubating cells with M1 anti-FLAG monoclonal antibody (2.0 μg/ml) at 4°C for 30 min. Labeled cells were subsequently treated with or without (control) a test compound at indicated concentration at 37°C for 30 min. Cells were washed with TBS (50 mM Tris, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.4), fixed in 4% paraformaldehyde for 15 min at room temperature, then quenched with three washes of TBS. Cells were permeabilized with 0.1% Triton X-100 in a blocking solution (4% normal goat serum in TBS) and incubated with goat anti-mouse IgG (H + L)-conjugated Alexa Fluo 488 (1:500 dilution) for 1 h. Cells were mounted with Slow Fade mounting medium, and coverslips were sealed with nail polish. Confocal fluorescence microscopy was performed using an Olympus FluoView 300 microscope fitted with a 60× objective.

Receptor Down-Regulation Measured by Immunoblotting Assay. CHO cells stably expressing the Flag-hKOR were treated with agonists at 37°C for 4 h, washed with ice-cold PBS, and lifted with PBS containing 0.5 mM EDTA. After pelleting by centrifugation at 500*g* for 5 min on a bench-top microcentrifuge, the cells were solubilized with 2× Laemmli sample buffer and subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corporation, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in TBS (25 mM Tris-HCl and 150 mM NaCl, pH 7.5, with 0.1% Tween 20), incubated with rabbit polyclonal antibodies against Flag (1:5000) in the blocking solution for overnight at 4°C on a shaker. Then the membranes were washed with TBS, incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:10,000) for 1 h at room temperature. Blots were stripped in Restore Western stripping buffer (Pierce Chemical) for 15 min and probed for β-actin (1:50,000) as loading controls. The target protein bands were detected using SuperSignal west chemiluminescent substrate (Pierce Chemical) with Image Reader LAS-1000 plus image system (Fuji Film, Tokyo, Japan) and analyzed using ImageGauge 4.1 software (Fuji Film).

Animals. Male Swiss albino mice (Ace Laboratories, Boyertown, PA) weighing 25 to 30 g were used. The animals were housed five per cage with free access to food and water. A standard light/dark cycle was maintained with a timer-regulated light period from 7:00 AM to 7:00 PM. Experimental procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Determination of Antiscratching Activities in Mice. Each mouse was weighed and allowed to acclimate for at least 1 h in individual, rectangular observation boxes. TRK-820 and U50,488H were dissolved in saline; 3FLB was suspended in 1% Tween 80 and water; and salvinorin A was dissolved in ethanol/Tween 80/water (1:1:8 proportion by volume). Vehicle (0.25 ml/25 g) or test agent was injected s.c. to groups of eight mice 20 min before challenging the animals s.c. with 0.10 ml of the pruritogen, compound 48/80 (0.50 mg/ml; 0.05 mg), into the back of the neck (Kuraishi et al., 1995). The number of hind leg scratching movements directed to the neck was counted for 30 min. Experiments took place between 2:00 PM and 5:00 PM. For each group of mice, the mean values for scratching were normalized (relative to the appropriate vehicle group) to percent antagonism of scratching and A50 values with 95% confidence limits were calculated using linear regression analysis (PharmTools Pro software; The McCary Group, Elkins Park, PA). In our laboratory, typical saline-treated control mice scratched 230 ± 15 (mean ± S.E.M.) times in the 30 min after compound 48/80.

Mouse Abdominal Constriction Test. Groups of eight mice were injected s.c. with vehicle, salvinorin A (15, 30, and 50 mg/kg), or 3FLB (40 mg/kg) 20 min before i.p. challenge with 0.6% acetic acid (0.25 ml/25 g mouse). After a further 5 min, groups of two individually housed mice were observed for 10 min, and the number of abdominal constrictions/hind leg extensions (termed responses) was counted. Mean percent antinociception was expressed as follows: [(mean responses in vehicle group) − (responses by individual mouse)/(mean responses in vehicle group)] × 100 (Tomizawa et al., 2001).

Results

Binding Affinities of Salvinorin A, TRK-820, and 3FLB for μ, δ, and κ Opioid and N/OFQ Receptors Expressed in CHO Cells. Saturation binding of [^{3}H]diprenorphine to μ, δ, or κ opioid receptors was performed previously, and K_d and B_{max} values were determined: for the μ opioid receptor, K_d, 0.14 ± 0.03 nM, B_{max}, 2.1 ± 0.5 pmol/mg protein; for the δ opioid receptor, K_d, 0.33 ± 0.04, B_{max}, 1.1 ± 0.3 pmol/mg protein; and for the κ opioid receptor, K_d, 0.15 ± 0.03, B_{max}, 1.3 ± 0.2 pmol/mg protein (Huang et al., 2001). K_d and B_{max} values of [^{3}H]N/OFQ binding to the N/OFQ receptor were determined to be 0.14 ± 0.02 and 5.0 ± 0.7 pmol/mg protein, respectively (Huang et al., 2001).

Affinities of the three compounds for each receptor were determined by competitive inhibition of [^{3}H]diprenorphine binding to opioid receptors and [^{3}H]N/OFQ binding to the N/OFQ receptor. Receptor binding and [^{35}S]GTPγS binding were performed in the same buffer to allow comparison of binding affinity and potency. TRK-820 exhibited the highest affinity for the κ opioid receptor with K_i of 75 pM, which was about 70- or 2000-fold selective for the κ over the μ (K_i = 5.2 nM) and δ (K_i = 161 nM) opioid receptors, respectively. However, TRK-820 displayed no significant affinity for the N/OFQ receptor (Fig. 2; Table 1). Salvinorin A bound to the κ opioid receptor with K_i of 7.9 nM but did not show significant affinities for the μ, δ, opioid or N/OFQ receptors (Fig. 2; Table 1). 3FLB showed significant affinity only for the κ opioid receptor (K_i, 248 nM). 3FLB, at 1 μM, did not bind to the μ or δ opioid receptors or the N/OFQ receptor (Table 1; Fig. 2).

The affinity of TRK-820 for the κ opioid receptor was about 150-fold higher than that of U50,488H (K_i 11 nM). Salvinorin A exhibited a similar affinity for the κ opioid receptor as U50,488H, whereas the affinity of 3FLB was ~20-fold lower than that of U50,488H (Fig. 2; Table 1).

Efficacies and Potencies of Salvinorin A, TRK-820, and 3FLB in Stimulating [^{35}S]GTPγS Binding via μ, δ, and κ Opioid and N/OFQ Receptors. Activation of μ, δ, and κ opioid and N/OFQ receptors has been shown to enhance [^{35}S]GTPγS binding to pertussis toxin-sensitive G proteins in membranes (Traynor and Nahorski, 1995; Befort et al., 1996; Zhu et al., 1997; Quock et al., 1997). [^{35}S]GTPγS binding has been used a functional measure for determination of potencies and efficacies of agonists when a reference compound is defined as a full agonist.

Salvinorin A, TRK-820, and 3FLB stimulated the κ opioid receptor to enhance [^{35}S]GTPγS binding to membranes, with the maximal effects being 93, 110, and 90%, respectively, of that of the full agonist U50,488H (Fig. 3; Table 2), indicating that the three compounds are full agonists. The potency in

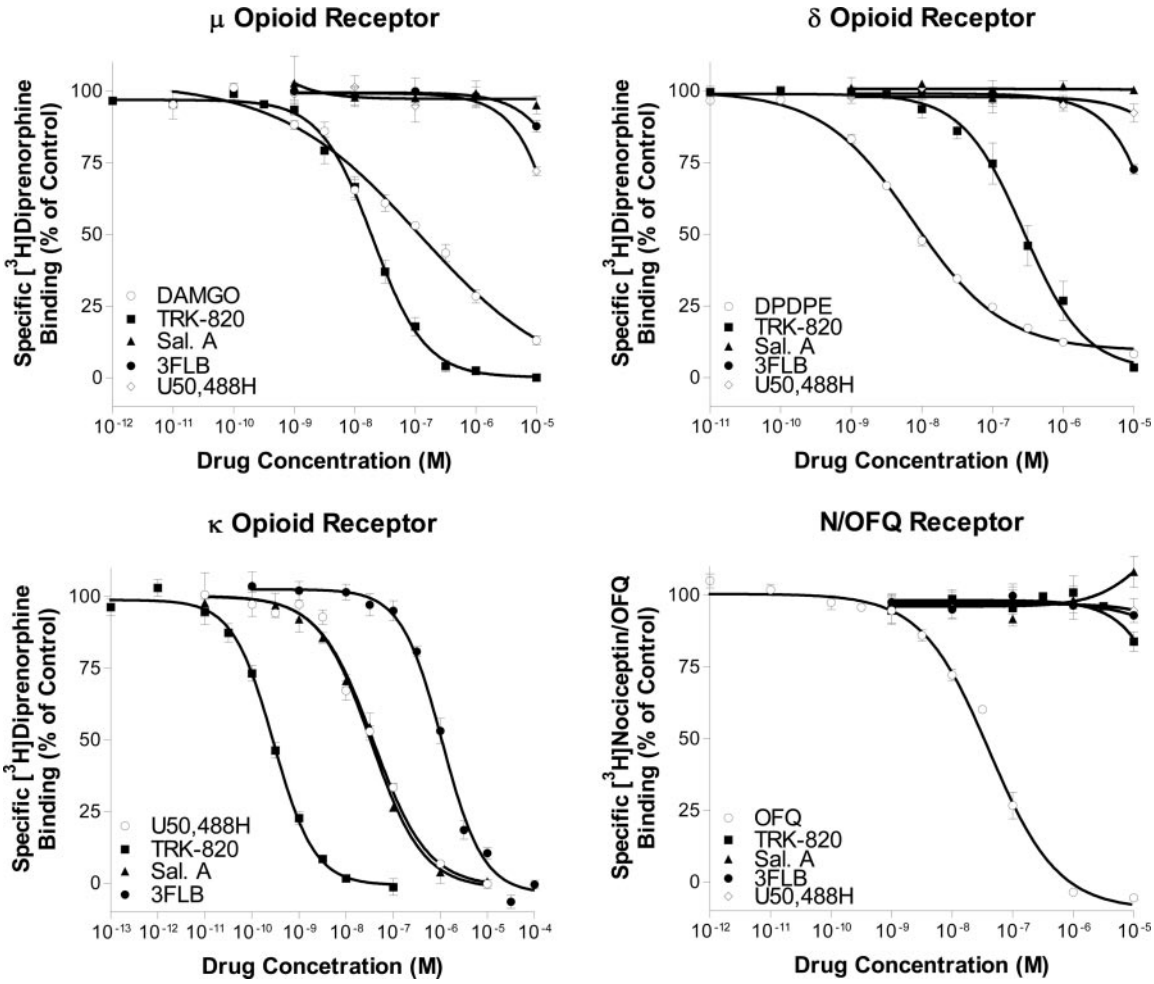

Fig. 2. Competitive inhibition by salvinorin A, TRK-820, 3FLB, or U50,488H of [^{3}H]diprenorphine binding to μ, δ, and κ opioid receptors and [^{3}H]N/OFQ binding to the N/OFQ receptor. Membranes were prepared from CHO cells expressing each receptor. Binding of opioid receptors and N/OFQ receptor was carried out with ~0.4 nM [^{3}H]diprenorphine and ~0.2 nM [^{3}H]nociceptin/OFQ, respectively, in the presence and absence of various concentrations of salvinorin A, TRK-820, 3FLB, or U50,488H as described under *Materials and Methods*. Specific [^{3}H]diprenorphine and [^{3}H]nociceptin/OFQ binding was 2000 to approximately 3000 dpm/tube, and the corresponding nonspecific binding was ~ 200 dpm/tube. Data were normalized to the percentage of specific binding. Each value represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. Apparent K_i values are shown in Table 1.

TABLE 1

Apparent K_i values (nanomolar) of salvinorin A, TRK-820, 3FLB, and U50,488H for the opioid receptors and the N/OFQ receptor in [^{35}S]GTPγS binding buffer

K_i values were calculated from the equation $K_i = IC_{50}/(1 + [L]/K_d)$ (Cheng and Prusoff, 1973). IC_{50} values were derived from the competition curves shown in Fig. 2.

	μ	δ	κ	N/OFQ
TRK-820	5.2 ± 0.8	161 ± 42	0.075 ± 0.007	N.A.
Salvinorin A	N.A.	N.A.	7.9 ± 0.8	N.A.
3FLB	N.A.	N.A.	248 ± 23	N.A.
U50,488H	N.A.	N.A.	11.0 ± 0.2	N.A.

N.A., did not bind at 1 μM.

[^{35}S]GTPγS binding was in the order of TRK-820 (EC_{50} = 25 pM) ≫ U50,488H (EC_{50} = 2.2 nM) and salvinorin A (EC_{50} = 4.6 nM) > 3FLB (EC_{50} = 73.6 nM).

For the μ opioid receptor, the maximal effect of TRK-820 on [^{35}S]GTPγS binding was 54% of that of the full agonist DAMGO. For the δ opioid receptor, TRK-820 was 51% as efficacious as DPDPE, a full agonist (Fig. 3). TRK-820 was more potent at the μ than at the δ opioid receptor in stimulating [^{35}S]GTPγS binding with EC_{50} values of 3.2 and 289 nM, respectively (Table 3). Neither 3FLB nor salvinorin A activated μ or δ opioid receptors at concentrations up to 10 μM.

For the N/OFQ receptor, TRK-820 slightly increased [^{35}S]GTPγS binding, with the maximal effect about 27% of that of N/OFQ, a full agonist. Salvinorin A and 3FLB exhibited no activities at the N/OFQ receptor up to 10 μM (Fig. 3).

Salvinorin A, TRK-820, and 3FLB Promoted Internalization of the hKOR. Activation of the hKOR by U50,488H has been shown to promote phosphorylation, desensitization, internalization, and down-regulation of the receptor (Blake et al., 1997; Li et al., 1999, 2000, 2003). These regulatory processes are thought to be related to receptor resensitization and tolerance to drugs. Full agonists acting on the κ opioid receptor showed differential abilities to regulate the hKOR (Blake et al., 1997; Li et al., 1999, 2000, 2003). Although U50,488H and U69,593 induced internalization and down-regulation of the hKOR, etorphine and levorphanol did not. We thus examined whether the three agonists caused internalization and down-regulation of the hKOR.

When nonpermeabilized CHO-FLAG-hKOR cells were incubated with M1 anti-FLAG monoclonal antibody followed by

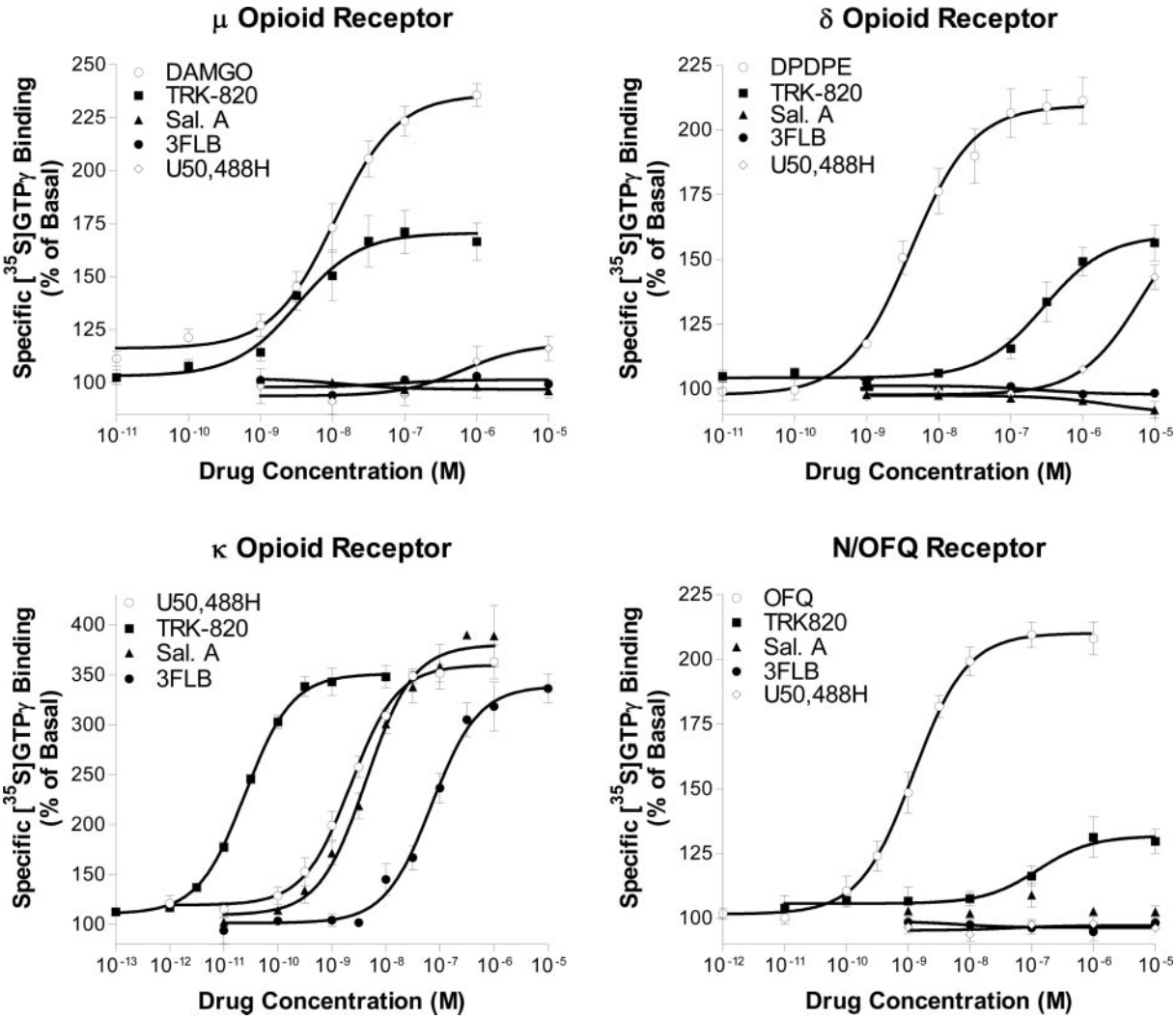

Fig. 3. Stimulation of [^{35}S]GTPγS binding to membranes of CHO cells stably transfected with μ, δ, or κ opioid or N/OFQ receptor by salvinorin A, TRK-820, 3FLB, U50,488H, and a full agonist for each receptor. [^{35}S]GTPγS binding to membranes was performed with various concentrations of each compound as described under *Materials and Methods*. Nonspecific binding, determined in the presence of 10 μM cold GTPγS, was ~500 dpm. Basal [^{35}S]GTPγS binding in the absence of added compounds was ~3000 dpm. Data were normalized to percentage of the basal [^{35}S]GTPγS binding. Each value represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. EC_{50} values and maximal responses are shown in Table 2.

TABLE 2

EC_{50} values and maximal effects of salvinorin A, TRK-820, and 3FLB in stimulating [^{35}S]GTPγS binding to membranes of CHO cells stably expressing the μ, δ, and κ opioid or N/OFQ receptors

DAMGO, DPDPE, U50,488H ,and N/OFQ were used as the full agonists for the μ, δ, and κ opioid and N/OFQ receptors, respectively. Data were derived from the curves in Fig. 3.

	μ		δ		κ		N/OFQ	
	EC_{50}	Maximal Effect	EC_{50}	Maximal Effect	EC_{50}	Maximal Effect	EC_{50}	Maximal Effect
	nM	%	*nM*	%	*nM*	%	*nM*	%
DAMGO	8.9 ± 2.1	100						
DPDPE			3.4 ± 0.7	100				
U50,488H					2.2 ± 0.3	100		
Nociceptin/OFQ							1.2 ± 0.2	100
TRK-820	3.2 ± 1.3	54 ± 7	289 ± 60	51 ± 6	0.025 ± 0.003	93 ± 5	147 ± 30	27 ± 4
Salvinorin A	N.S.		N.S.		4.6 ± 1.2	110 ± 12	N.S.	
3FLB	N.S.		N.S.		73.6 ± 12	90 ± 5	N.S.	

N.S., no stimulation up to 1 μM.

Alexa Fluo 488-conjugated goat anti-mouse IgG, cells exhibited surface fluorescence as detected by fluorescence flow cytometry, whereas cells incubated with nonimmune serum displayed little fluorescence. Incubation of CHO-FLAG-hKOR cells with salvinorin A, TRK-820, and 3FLB reduced cell surface fluorescence in a dose-dependent manner, similar to incubation with U50,488H, demonstrating that these compounds promoted internalization of FLAG-hKOR. The EC_{50}

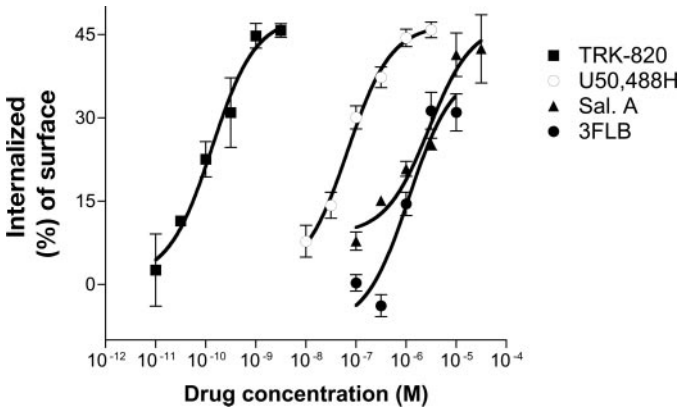

Fig. 4. Dose-response relationship of U50,488H-, TRK-820-, salvinorin A-, and 3FLB-induced internalization of FLAG-hKOR expressed in CHO cells. CHO-FLAG-hKOR cells grown on 12-well plates were left untreated or treated with different concentrations of U50,488H, TRK-820, salvinorin A, and 3FLB at 37°C for 30 min. Cells were washed, cooled to 4°C, and processed for surface immunofluorescence staining using M1 anti-FLAG mouse monoclonal antibody as the primary antibody and goat anti-mouse IgG (H+L) conjugated with Alexa Fluo 488 as the secondary antibody. Cells were analyzed by fluorescence flow cytometry on a FACScan, and internalized receptor was determined as described under *Materials and Methods*. Each value represents the mean ± S.E.M. of three independent experiments.

values were as follows: salvinorin A, 2.86 ± 1.23 μM; TRK-820, 0.134 ± 0.025 nM; 3FLB, 1.14 ± 0.57 μM; and U50,488H, 68.8 ± 18.5 nM (n = 3–4 each, mean ± S.E.M.). The maximum percent receptor internalized induced by 3FLB was less than U50,488H and TRK-820 ($P < 0.01$, two-tailed Student's t test) (Fig. 4).

Immunofluorescence staining was carried out with M1 anti-FLAG antibody in the presence of Triton X-100 to permeabilize cells for detection of surface and intracellular FLAG-hKOR in HEK 293 cells according to the method of Zhang et al. (2002) with modifications. HEK 293 cells were used instead of CHO cells because HEK293 cells have much smaller nuclei than CHO cells, making it easier to visualize internalized receptors. Without drug treatment, immunofluorescence staining of FLAG-hKOR was mostly on the cell surface (Fig. 5, control). At a concentration that caused maximal internalization of FLAG-hKOR, salvinorin A, TRK-820, 3FLB, and U50,488H decreased cell surface staining and caused punctate staining intracellularly following incubation at 37°C for 30 min (Fig. 5), indicating internalization of FLAG-hKOR.

Salvinorin A, TRK-820, and 3FLB Caused Down-Regulation of the FLAG-hKOR. We showed by receptor binding that a 4-h incubation with a saturation concentration of U50,488H (1 μM) caused ~30% down-regulation of the hKOR, whereas etorphine did not (Zhu et al., 1998; Li et al., 2000; Zhang et al., 2002). Here, we used western blotting to investigate whether salvinorin A (10 μM), TRK-820 (1 nM), and 3FLB (10 μM) also caused down-regulation of FLAG-hKOR after a 4-h incubation. The concentration used for each compound induced the maximal level of internalization of the hKOR. SDS-PAGE followed by immunoblotting revealed FLAG-hKOR as a relatively broad band of molecular mass 55 kDa (Fig. 6A), most likely because of different extents of

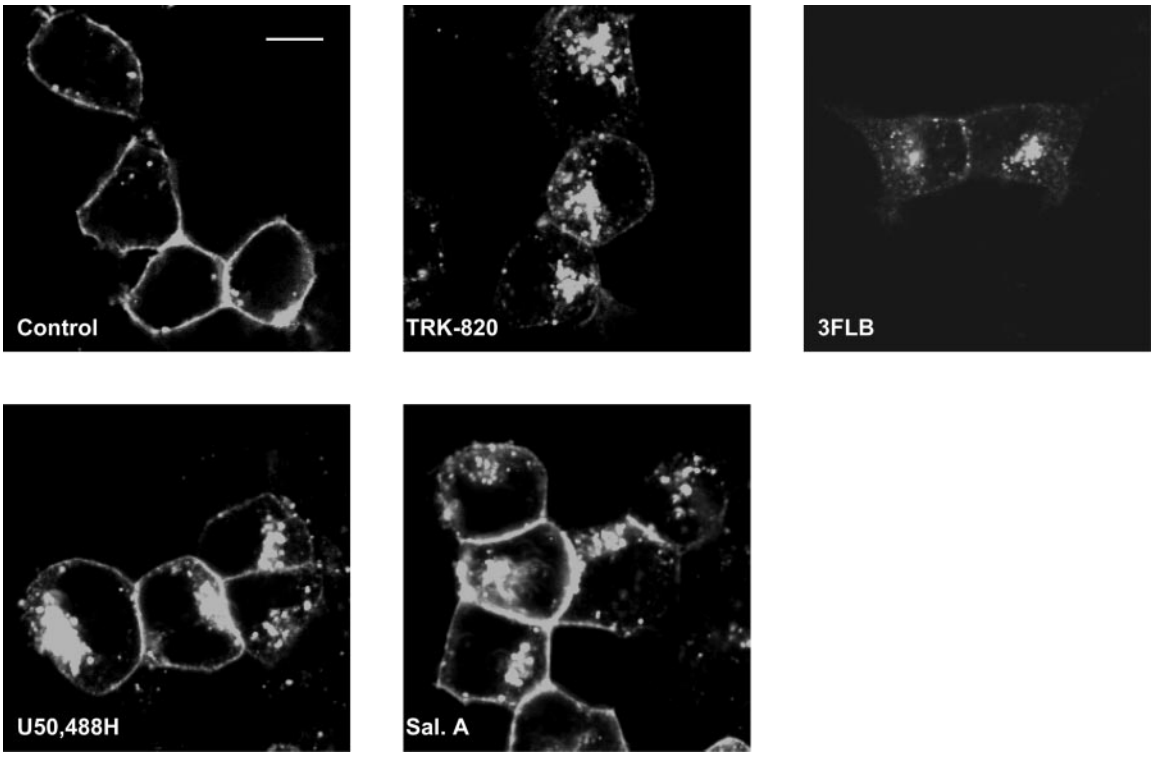

Fig. 5. TRK-820, salvinorin A, and 3FLB caused internalization of FLAG-hKOR stably transfected into HEK 293 cells: immunofluorescence microscopy. Cells grown on chamber slides were left untreated or incubated with U50,488H (1 μM), TRK-820 (1 nM), salvinorin A (10 μM), or 3FLB (10 μM) at 37°C for 30 min. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and processed for immunofluorescence microscopy using M1 anti-FLAG mouse monoclonal antibody and goat anti-mouse IgG (H+L) conjugated with Alexa Fluo 488 as described under *Materials and Methods*. Confocal fluorescence microscopy was carried out using an Olympus FluoView 300 and a 60× objective. Scale bar = 10 μm. The figures represent one of the three experiments performed with similar results. After incubation with agonists for 30 min, receptors redistributed from plasma membranes into cytoplasmic vesicles.

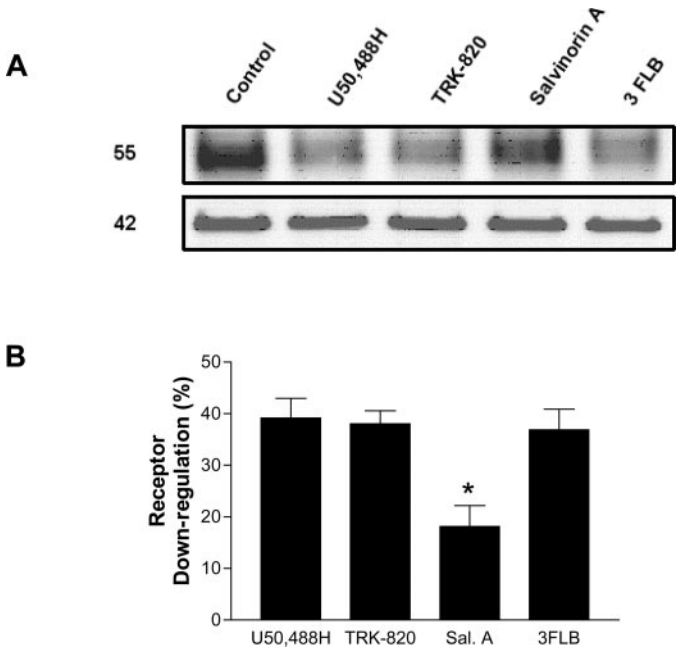

Fig. 6. Effects of test compounds on down-regulation of FLAG-hKOR expressed in CHO cells determined by Western blotting (A and B). CHO-FLAG-hKOR cells were left untreated or treated with U50,488H (1 μM), TRK-820 (1 nM), salvinorin A (10 μM), and 3FLB (10 μM) at 37°C for 4 h. A, cells were washed, harvested, solubilized with 2× Laemmli sample buffer, and subjected to SDS-PAGE. The receptors were detected by sequential incubation with rabbit anti-FLAG antibodies and HRP-conjugated goat anti-rabbit antibody followed by enhanced chemiluminescence (top panel). Following stripping, membranes were probed for actin as an internal standard (bottom panel). The figure represents one of the four experiments with similar results. B, Western blot results shown in A were quantitated by ImageGauge software and plotted relative to the amount of receptors detected in control cells. Receptor down-regulation was determined as described under *Materials and Methods*. Each value represents mean ± S.E.M. of four independent experiments. *, $P < 0.01$ compared with those treated with U50,488H, TRK-820, or 3FLB by one-way analysis of variance followed by Dunnett's post hoc test.

glycosylation. Quantitation by densitometry demonstrated that treatment with U50,488H, TRK-820, or 3FLB caused similar levels of down-regulation, 35 to 40%, whereas salvinorin A induced a much lower level of down-regulation, ~15% (Fig. 6B).

TRK-820 Showed Antiscratching Activity, but Salvinorin A and 3FLB Did Not. κ Opioid receptors have been implicated in the itch/scratch cycle in both animals (Gmerek and Cowan, 1984; Ko et al., 2003; Umeuchi et al., 2003) and humans (Sorbera et al., 2003). Therefore, we examined the antiscratching effects of these test compounds with a view to extending structure-activity relationships for this endpoint. An excellent dose-response curve was obtained with TRK-820 (5–20 μg/kg) in the compound 48/80-induced scratch assay (Fig. 7) with the antiscratching A50 value of 6.64 (3.70–8.93) μg/kg. Doses of 50 and 100 μg/kg TRK- 820 caused obvious hypoactivity and behavioral depression. U50,488H was equiefficacious with TRK-820 but approximately 190 times less potent with an A50 value of 1.34 (1.23–4.54) mg/kg. The dose-response relation for salvinorin A was unimpressive at doses that did not induce any overt behavioral changes (locomotion, grooming), associated with large S.E.s, and an A50 value was unobtainable (>20 mg/kg). 3FLB was inactive against compound 48/80 at the doses tested (10–40 mg/kg).

Salvinorin A and 3FLB Lacked Antinociceptive Activity. Activation of κ opioid receptors by agonists, including U50,488H and TRK-820, has been shown to elicit antinociceptive effects in the mouse acetic acid abdominal constriction test (Endoh et al., 1999). Thus, we examined whether salvinorin A and 3FLB have activities in this test. Salvinorin A (15–50 mg/kg) elicited no dose-related effects in the mouse abdominal constriction procedure. The maximum mean percent antinociception was only 16.5% (with the 50 mg/kg dose). The corresponding figure for the one dose of 3FLB tested (40 mg/kg) was 21.5%.

Discussion

To the best of our knowledge, our study represents the first comparison of pharmacological properties of the three nonarylacetamide κ agonists. Of particular interest are the findings that although salvinorin A was similarly potent as U50,488H in stimulating [^{35}S]GTPγS binding, salvinorin A was 40-fold less potent in promoting internalization of the hKOR, caused less down-regulation of surface receptors than U50,488H, and showed little antiscratching and no antinociceptive activities in mice.

Salvinorin A. The high selectivity of salvinorin A for the κ opioid receptor (K_i = 7.9 nM) among the four homologous receptors is consistent with the observation of Roth et al. (2002), who found that salvinorin A at 10 μM bound only to the κ opioid receptor among 50 potential targets, and its K_i value for the κ opioid receptor was 16 nM. The difference in *K*i values may be due to the different radiolabeled ligand used in the binding assay: [^{3}H]diprenorphine in our study and [^{3}H]bremazocine in that of Roth et al. (2002). Diprenorphine and bremazocine, having different structures, most likely have distinct contact points within the binding pocket of the κ receptor; thus, salvinorin A may have differential abilities to displace the two ligands.

Our finding that salvinorin A has similar potency and efficacy as U50,488H in activating the κ opioid receptor is in accord with those of Roth et al. (2002) and Chavkin et al. (2004). Roth et al. (2002) reported salvinorin A was slightly more potent than U69,593 in inhibiting forskolin-stimulated cAMP accumulation in HEK-293 cells and stimulating [^{35}S]GTPγS binding to guinea pig brain membranes. Chavkin et al. (2004) showed that salvinorin A was equally or more efficacious than U69,593 and U50,488 but 2- to 3-fold more potent than the two compounds in activating G_i proteins and in enhancing Kir3 current in *Xenopus* oocytes.

In contrast to U50,488H, salvinorin A showed very low and inconsistent activity against compound 48/80-induced scratching in mice and was essentially inactive in the abdominal constriction test with this species. In preliminary experiments, we have demonstrated that pretreating mice with norbinaltorphimine (20 mg/kg i.p. at −20 h) reverses the (albeit modest) antiscratching activity of salvinorin A (30 mg/kg s.c.) against compound 48/80 (S. Inan and A. Cowan, unpublished data). Salvinorin A has been reported to cause hallucinations in humans (Siebert, 1994; Sheffler and Roth, 2003). However, there is no definitive evidence that hallucinations are mediate by κ opioid receptors. Recently, Butelman et al. (2004) showed that salvinorin A produced discriminative stimulus effects similar to U69,593 in rhesus monkeys.

TRK-820 exhibited high selectivity for the κ opioid receptor, having ~70- and ~2000-fold higher affinity for the μ and

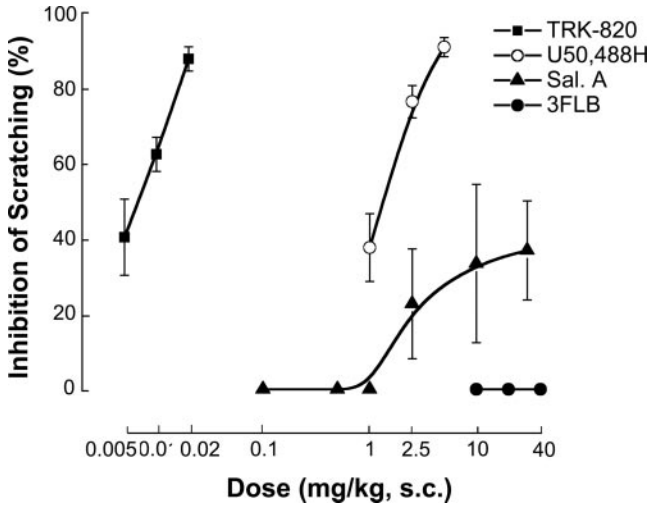

Fig. 7. Dose-response relationships for TRK-820, U50,488H, salvinorin A, and 3FLB against compound 48/80-induced scratching in mice. The animals were pretreated s.c. with either vehicle or test compound 20 min before challenge with compound 48/80 (0.05 mg s.c.). Hind leg scratching movements were then counted for 30 min. Percent inhibition of scratching was calculated as described under *Materials and Methods*. Each value represents mean ± S.E.M. of data obtained from eight mice.

δ opioid receptors, respectively, and no significant affinity for the N/OFQ receptor. These results showed higher selectivity of TRK-820 for the κ over the μ and δ opioid receptors than those of Seki et al. (1999), who demonstrated 15- and 340-fold selectivity, respectively. However, the K_i value of TRK-820 for the κ opioid receptor was 3.5 nM in the study by Seki et al. (1999), which is about 50-fold higher than the K_i value in our study. The discrepancy may be due to the different radiolabeled ligands used in the competitive inhibition assay, [^{3}H]diprenorphine in the present study, and [^{3}H]bremazocine in that of Seki et al. (1999) (see above).

Our observation that TRK-820 is a full agonist at the κ opioid receptor and is ~90-fold more potent than U50,488H is consistent with that of Seki et al. (1999), who used inhibition of forskolin-stimulated cAMP accumulation as the endpoint. TRK-820 has also been shown to increase [^{35}S]GTPγS binding to mouse pons/medulla membranes via the κ opioid receptor (Mizoguchi et al., 2003).

TRK-820 is a partial agonist at μ and δ opioid receptors in the [^{35}S]GTP binding assay, which is consistent with the study of Seki et al. (1999). In addition, Mizoguchi et al. (2003) reported that TRK-820 decreased DAMGO-induced [^{35}S]GTP binding in the mouse pons/medulla membranes and antinociception, demonstrating its partial agonist activity at the μ opioid receptor. Our findings that TRK-820 exhibited no significant affinity or efficacy for the N/OFQ receptor are consistent with those of Seki et al. (1999).

We found that TRK-820 potently inhibited scratching caused by compound 48/80 in mice. TRK-820 was previously demonstrated to reduce scratching induced by either substance P or histamine in mice (Endoh et al., 1999; Togashi et al., 2002). TRK-820 has been shown to have potent antinociceptive effects in several tests, including acetic acid abdominal constriction, low temperature hot plate (51°C), thermal tail-flick, mechanical tail pressure, tail pinch, and formalin tests (Endoh et al., 1999, 2000). The antinociceptive activities of TRK-820 and U50,488H were antagonized when the mice were pretreated s.c. with the κ antagonist norbinaltorphimine (Endoh et al., 1999). Similarly, the antipruritic activity of TRK-820 (against substance P- or histamine-induced scratching) in mice was antagonized by pretreatment with this antagonist (Togashi et al., 2002). These results indicate that these effects of TRK-820 are mediated by κ opioid receptors.

3FLB is a fluorphenyl derivative of HZ2. To the best of our knowledge, this is the first examination of pharmacological properties of 3FLB. The affinity and potency of 3FLB for the κ opioid receptor were ~20- and ~30-fold, respectively, lower than that of U50,488H in this study. HZ2, the parent compound of 3FLB, was reported to be selective for the κ opioid receptor in rat brain membranes with K_i 15 nM, and its affinity was 2- to approximately 4-fold lower than those of U50,488H and U69,593 (Kogel et al., 1998). Thus, 3FLB exhibited lower affinity than HZ2 for the κ opioid receptor.

In vivo, 3FLB did not show any activity in countering scratching induced by compound 48/80 or in reducing responses to acetic acid in the abdominal constriction test. It was demonstrated that HZ2 produced a strong antinociceptive effect in acetic acid abdominal constriction, hot plate, tail-flick, and tooth pulp stimulation tests (Kogel et al., 1998). HZ2 showed an unusually long duration of action in the mouse tail-flick test. HZ2 caused other κ opioid receptor-related effects, such as sedation and diuresis (Kogel et al., 1998). HZ2 also elicited emesis, which is not typical for κ opioid receptor agonists (Kogel et al., 1998).

Internalization and Down-Regulation of the hKOR. Full agonists exhibited differential abilities to regulate the hKOR. Although U50,488H, U69,593, and dynorphin A(1–17) cause phosphorylation, internalization, and down-regulation of the hKOR, levorphanol and etorphine did not (Blake et al., 1997; Li et al., 1999, 2000, 2003). The mechanism underlying the differential effects of agonists is suggested to be related to the different receptor conformations required for activation of G proteins versus receptor phosphorylation and internalization (Li et al., 2003). Although U50,488H and dynorphin A(1–17) induce both conformations, etorphine and levorphanol elicit conformations able to activate G proteins but not those to promote phosphorylation and internalization (Li et al., 2003). Here, we found that like U50,488H, salvinorin A, TRK-820, and 3FLB caused internalization and down-regulation of the hKOR.

The EC_{50} values of TRK-820, U50,488H, salvinorin A, and 3FLB in promoting internalization of the hKOR are 5-, 31-, 622-, and 15-fold, respectively, of those in enhancing [^{35}S]GTPγS binding. The results with U50,488H and 3FLB and, to a lesser extent, with TRK-820, are similar to our previous observations on U50,488H and dynorphin A(1–17) (Li et al., 2003). These findings support the notion that different activated receptor conformations are required for the two processes. However, although salvinorin A is similar in potency to U50,488H in [^{35}S]GTPγS binding, salvinorin A is much less potent than U50,488H in inducing internalization of the hKOR. Thus, salvinorin A fits in between full agonists that cause internalization [such as U50,488H and dynorphin A (1–17)] and those that do not (such as etorphine and levorphanol).

Salvinorin A, TRK-820, and 3FLB induced down-regulation of the hKOR as determined by immunoblotting. The 55-kDa band in Western blots represents fully glycosylated FLAG-hKOR, mostly in plasma membranes (Li et al., 2002;

Jian-Guo Li, unpublished data). The extent of U50,488H-induced down-regulation (35–40%) determined by the intensity of the 55-kDa band is somewhat higher than that measured by [^{3}H]diprenorphine binding (25–30%) (Zhu et al., 1998; Li et al., 2000; Zhang et al., 2002). [^{3}H]Diprenorphine binding measures receptors in plasma membranes and in membranes of intracellular organelles. The intracellular receptors include those in the endocytic pathway and those in the biosynthetic pathway. This may explain the somewhat higher levels of down-regulation detected by Western blot. After a 4-h incubation, salvinorin A, TRK-820, and 3FLB, like U50,488H, caused down-regulation, but salvinorin A reduced receptors to a less extent than others. Since the salvinorin A concentration used induced a similar degree of internalization as the others (Fig. 4), one possibility is that receptors internalized by salvinorin A may be preferentially recycled. This remains to be investigated. It has been shown that δ opioid receptors internalized by etorphine are recycled to a greater extent, whereas those internalized by DPDPE or deltorphin II are mostly sorted to lysosomes for degradation (Marie et al., 2003).

Antiscratching and Antinociceptive Effects of κ Agonists. Our observation that the chemically diverse TRK-820 and U50,488H demonstrate dose-related activity against compound 48/80-induced scratching (Fig. 7) is in accord with previous findings that activation of κ opioid receptors produces antiscratching effects in animals (Gmerek and Cowan, 1984; Togashi et al., 2002; Ko et al., 2003; Umeuchi et al., 2003) and in humans (Sorbera et al., 2003). TRK-820 and U50,488H have been shown to have antinociceptive effects (VonVoigtlander et al., 1983; Dykstra et al., 1987; Endoh et al., 1999, 2000, 2001), which are mediated by the κ opioid receptor. Both central and peripheral sites of action may contribute to these endpoints. This conclusion is based on studies with centrally penetrating compounds such as TRK-820 and U50,488H and with agonists such as asimadoline that target peripheral κ receptors exclusively (Barber et al., 1994; Kehner et al., 1999).

Previous experiments have revealed a strong correlation between the relative potencies of arylacetamide κ agonists in mouse antiscratching (compound 48/80) and antinociceptive (abdominal constriction) tests (Kehner et al., 1999). This correlation has been maintained in the present study. Thus, our antipruritic potencies for TRK-820, U50,488H, salvinorin A, and 3FLB (Fig. 7) are ranked as follows: 0.007, 1.34, >20, and >40 mg/kg s.c., respectively. The corresponding rank order of potency in the mouse abdominal constriction test for TRK-820 and U50,488H (Endoh et al., 1999) and for salvinorin A and 3FLB is: 0.003, 1.16, >50, and >40 mg/kg s.c.

Discrepancy between in Vitro and in Vivo Results on Salvinorin A and 3FLB. In our study, there is a lack of correlation between the relative potencies of κ agonists in vivo and in vitro. Although salvinorin A displayed similar potency and efficacy to U50,488H in the [^{35}S]GTPγS binding assay, it showed unimpressive activity in countering compound 48/80-induced scratch and, surprisingly, essentially no activity in the abdominal constriction test in mice. In addition, 3FLB was inactive in the two in vivo tests. The divergence between in vivo and in vitro results may be due to in vivo metabolism of salvinorin A and 3FLB to metabolites that are inactive at the κ opioid receptor. The metabolism of the two compounds has not been investigated in laboratory animals or humans. Another possibility that cannot be excluded is that salvinorin A and 3FLB may act on other pharmacological targets, in addition to the κ opioid receptor. After screening 50 receptors, ion channels, and transporters, Roth et al. (2002) found that salvinorin A showed high affinity only to the κ receptor. Since there are more than 1000 receptors, ion channels, and transporters, there are many more pharmacological targets to be examined. Even less is known about other pharmacological targets of 3FLB. The possible effects of salvinorin A and 3FLB on other targets in vivo may reduce or counter their activities on the κ opioid receptor. Also, 3FLB may have too low a potency on the κ opioid receptor to elicit in vivo effects.

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