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Short communication

Blockade of μ-opioid receptor-mediated G-protein activation and antinociception by TRK-820 in mice

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Abstract

The effects of κ-opioid receptor agonists *trans*-3,4-dichloro-*N*-(2-(1-pyrollidinyl)-cyclohexyl) benzeneacetamide ((−)-U50,488H) and 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl)acrylamido]morphinan hydrochloride (TRK-820) on the G-protein activation and antinociception induced by the selective μ-opioid receptor agonist, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), were determined in mice. G-protein activation was measured by monitoring the guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding in the mouse pons/medulla. DAMGO (10 μM) produced a marked increase of [^{35}S]GTPγS binding to the mouse pons/medulla membrane. On the other hand, both TRK-820 and (−)-U50,488H produced small but significant increases of [^{35}S]GTPγS binding to the mouse pons/medulla membrane. These increases by both TRK-820 and (−)-U50,488H were completely reversed by the selective κ-opioid receptor antagonist, norbinaltorphimine. Under these same conditions, the DAMGO-induced increase of [^{35}S]GTPγS binding was significantly attenuated by TRK-820 in a concentration-dependent manner, but not by (−)-U50,488H. In the tail-flick test, DAMGO (16 ng) given intracerebroventricularly (i.c.v.), produced a marked antinociception. The antinociception induced by DAMGO was dose-dependently blocked by co-treatment with TRK-820, but not (−)-U50,488H, in mice pretreated with norbinaltorphimine (5 μg, i.c.v.). The present results provide direct evidence for the antagonistic property of TRK-820 for μ-opioid receptors, in addition to the full agonistic property for κ-opioid receptors.

Keywords: μ-opioid receptor; κ-opioid receptor; G-protein activation; Antinociception; (Mouse)

1. Introduction

Opioid receptors have been classified into at least three types; the μ-, δ- and κ-opioid receptor. These opioid receptors belong to the superfamily of seven-transmembrane domain receptors that are coupled to G-proteins (Chen et al., 1993; Evans et al., 1992; Kieffer et al., 1992; Yasuda et al., 1993). The occupation of these opioid receptors by agonists leads to the activation of the Gi/Go class of G proteins, which can be measured by assessing agonist stimulation of membrane binding of the non-hydrolyzable analog of GTP, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS). The increases of [^{35}S]GTPγS binding induced by selective opioid receptor agonists have been reported in membranes from cells expressed with respective endogenous δ- and μ-opioid receptors (Szekeres and Traynor, 1997; Traynor and Nahorski, 1995), cloned κ-opioid receptors (Zhu et al., 1997), and from rodent brain (Mizoguchi et al., 2000; Narita et al., 2000; Sim et al., 1996) or spinal cord (Narita et al., 1999) tissues. The measurement of selective opioid receptor agonist-induced increase of [^{35}S]GTPγS binding in membrane preparations has provided quantitative analysis of respective opioid receptor occupation by agonist and agonist efficacy for the activation of G-proteins.

The novel κ-opioid receptor agonist, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl)acrylamido]morphinan hydrochloride (TRK-820), has a unique chemical structure different from the traditional κ-opioid receptor agonists, *trans*-3,4-dichloro-*N*-

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(2-(1-pyrollidinyl)-cyclohexyl) benzeneacetamide ((−)-U50,488H) or (+)-(5α,7α,8β)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69, 593) (Nagase et al., 1998). TRK-820 contains a 4,5-epoxymorphinan structure with a tyrosine–glycine moiety, commonly found in κ-opioid receptor antagonists (Nagase et al., 1998). Neither (−)-U50,488H nor U69,593 contain this epoxymorphinan structure, nor do they contain any tyrosine–glycine sequence, believed necessary for endogenous opioid activity. TRK-820 also has similar pharmacological properties with other traditional κ-opioid receptor agonists. In the guinea pig ileum and mouse vas deferens, TRK-820 shows a high selectivity and potency to the κ-opioid receptor, as does (−)-U50,488H or U69,593 (Nagase et al., 1998). Moreover, in antinociceptive studies including acetic acid writhing, tail-flick and paw pressure tests, TRK-820 produced a potent antinociception that was mediated by the κ-opioid receptor (Endoh et al., 1999, 2000). However, unlike (−)-U50,488H, TRK-820 failed to prevent the development of antinociceptive tolerance to morphine (Tsuji et al., 2000b). On the contrary, TRK-820, but not (−)-U50,488H, suppressed the development of physical dependence on morphine (Tsuji et al., 2000a). Moreover, TRK-820 neither induces aversive nor reinforcing effects in a conditioned place preference experiment paradigm (Tsuji et al., 2001), while traditional κ-opioid receptor agonists, at doses that produce an antinociception, induce an aversive effect (Funada et al., 1993). Seki et al. (1999) have reported that TRK-820 shows a moderate affinity to μ-opioid receptors in addition to the high affinity to κ-opioid receptors. The present study was designed to further investigate the antagonistic property of TRK-820 for μ-opioid receptors using the [^{35}S]GTPγS binding in a brain membrane preparation and the antinociceptive tail-flick response induced by the selective μ-opioid receptor agonist, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), in mice.

2. Material and methods

2.1. Animals

All experiments were approved by and conformed to the guidelines of the Medical College of Wisconsin Animal Care Committee. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Male CD-1 mice weighing 25–30 g (Charles River Breeding Laboratories, Wilmington, MA) were used. Animals were housed five per cage in a room maintained at 22 ± 0.5 °C with an alternating 12-h light–dark cycle. Food and water were available ad libitum.

2.2. Membrane preparation

Mice were sacrificed by decapitation, the pons/medulla was rapidly excised at 4 °C, and the tissue was homogenized with 15 volumes (w/v) of ice-cold 0.32 M sucrose using a Potter–Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at 4 °C for 10 min at 1000 × *g*. The pellet was discarded and supernatant was centrifuged at 4 °C for 20 min at 20,000 × *g*. The pellet was resuspended in 15 volumes of an ice-cold 50 mM Tris–HCl buffer (pH 7.4) and centrifuged at 4 °C for 20 min at 20,000 × *g*. The resultant pellet was resuspended in an ice-cold assay buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl, and then stored at −70 °C until used.

2.3. [^{35}S]GTPγS binding assay

The homogenized membrane fractions (3–8 μg of protein/assay) were incubated at 25 °C for 2 h in assay buffer (above) with various concentrations of the agonist, 30 μM guanosine-5′-diphosphate (GDP: Sigma, St. Louis, MO) and 50 pM [^{35}S]GTPγS (1000 Ci/mmol; Amersham, Arlington Heights, IL) in a total volume of 1 ml. The reaction was terminated by filtering through Whatman GF/B glass filters (previously soaked in buffer containing 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ at 4 °C for 2 h) using a Brandel cell harvester (Model M-24; Brandel, MD). The filters were then washed three times with 5 ml of ice-cold Tris–HCl buffer (pH 7.4) and transferred to scintillation counting vials. Then 0.5 ml of the tissue solubilizer, Soluene-350 (Packard Instrument, Meriden, CT), and 4 ml of the scintillation cocktail, Hionic Fluor (Packard Instrument), were added to the vials. After a 12-h equilibration period, the radioactivity in the samples was determined with a liquid scintillation analyzer (Model 1600CA, Packard Instrument). Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

2.4. Assessment of antinociception

Antinociception was determined by the tail-flick test (D'Amour and Smith, 1941). For measurement of the latency of the tail-flick response, mice were gently held by hand with their tail positioned in an apparatus (Model TF6, EMDIE Instrument, Maidens, VA, USA) for radiant heat stimulation on the dorsal surface of the tail. The intensity of heat stimulus was adjusted so that the animal flicked its tail after 3 to 5 s for a baseline reading. The inhibition of the tail-flick response was expressed as percent maximum possible effect, %MPE, which was calculated as: $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where T_0 and T_1 were the tail-flick latencies before and after the treatments, respectively, and T_2 was the cutoff time, set at 10 s to avoid injury of the tail.

2.5. Intracerebroventricular injection

Intracerebroventricular (i.c.v.) injection was performed following the method described by Haley and McCormick

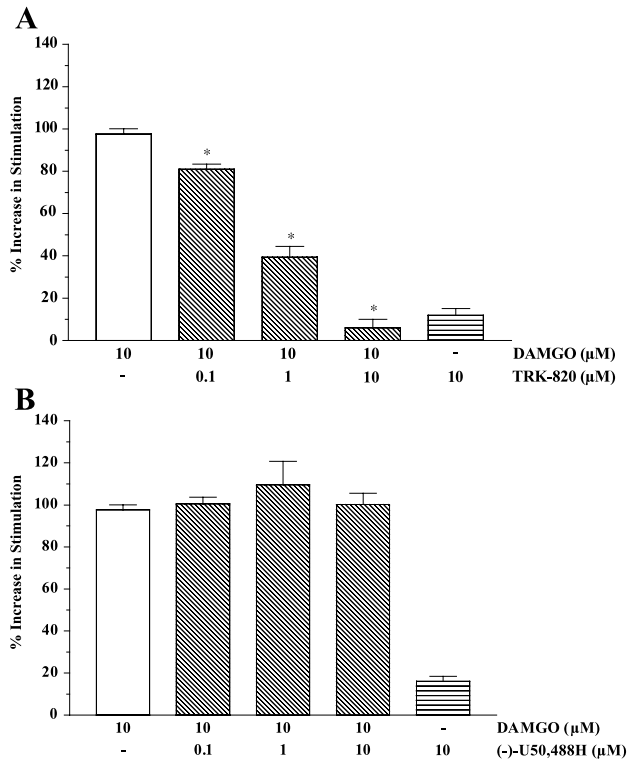

Fig. 1. Effects of TRK-820 (A) and (−)-U50,488H (B) on the DAMGO-stimulated increase of [^{35}S]GTPγS binding to the mouse pons/medulla membranes. Membranes were incubated with 50 pM [^{35}S]GTPγS and 30 μM GDP with or without DAMGO (10 μM), TRK-820 (0.1 – 10 μM), and/or (−)-U50,488H (0.1 – 10 μM) for 2 h at 25 °C. The data are expressed as the percentage increase of [^{35}S]GTPγS binding when compared to baseline binding (that measured in the presence of GDP and absence of agonist), and was calculated as: [binding of treatment group]/[baseline binding] × 100. Nonspecific binding was subtracted from specific binding when determining this. The data represent the mean ± S.E.M. from at least three independent experiments. The statistical significance of differences between the groups was assessed with one-way analysis of variance (ANOVA) followed by Dunnett's test. *$P < 0.05$ vs. DAMGO alone. (A) The F values of one-way ANOVA is $F(3,8) = 132.2$.

(1957) using a 10-μl Hamilton syringe. Each mouse received two i.c.v. injections, 24 h apart, in which the pretreatment and treatment drugs were delivered. The volume for i.c.v. injection was 4 μl.

2.6. Drugs

The drugs used were DAMGO (Bachem California, Torrance, CA), (−)-U50,488H (RBI, Natick, MA), TRK-820 (Toray Industries, Kamakura, Japan), norbinaltorphimine (RBI), GTPγS (RBI), and GDP (Sigma).

2.7. Statistical analysis

Comparable results were expressed as the mean ± S.E.M. The statistical significance of differences between the groups was assessed with one-way analysis of variance (ANOVA) followed by Dunnett's test.

3. Results

DAMGO produced a concentration-dependent increase of [^{35}S]GTPγS binding to the mouse pons/medulla membrane with 97.67 ± 2.41% of maximal increase at 10 μM. The DAMGO-induced increase of [^{35}S]GTPγS binding had an ED_{50} value(95% confidence limit) of 0.158(0.1047 – 0.2371) μM, and was selectively mediated by the stimulation of μ-opioid receptors, because this increase was completely blocked by the selective μ-opioid receptor antagonists, β-funaltrexamine (β-FNA) and D-Phe-Cys-Tyr-D-Tyr-Orn-Thr-Phe-Thr-NH_2 (CTOP) (data not shown). On the other hand, both TRK-820 and (−)-U50,488H produced a small but significant increase of [^{35}S]GTPγS binding (11.84 ± 3.18% or 16.10 ± 2.35%, respectively) to the mouse pons/medulla membrane (Fig. 1A and B). The increase of [^{35}S]GTPγS binding induced by both TRK-820 and (−)-U50,488H was completely eliminated by the selective κ-opioid receptor antagonist, norbinaltorphimine (data not shown). Interestingly, the increase of [^{35}S]GTPγS binding induced by DAMGO (10 μM) was significantly attenuated by TRK-820 (0.1 – 10 μM) in a concentration-dependent manner (Fig.

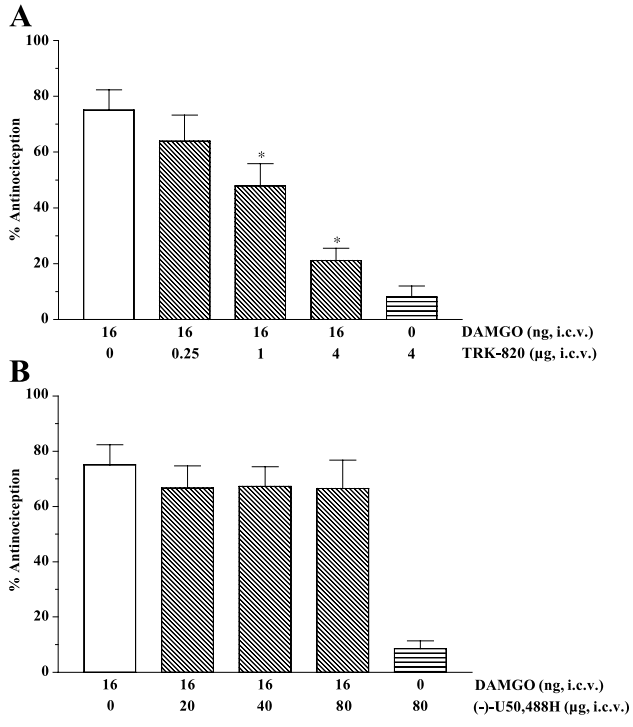

Fig. 2. Effects of TRK-820 (A) and (−)-U50,488H (B) on the DAMGO-induced antinociception in mice. Groups of mice were pretreated i.c.v. with norbinaltorphimine (5 μg), 24 h before i.c.v. injection with DAMGO (16 ng), DAMGO + TRK-820 (0.25 – 4 μg), DAMGO+(−)-U50,488H (20 – 80 μg), TRK-820 (4 μg), or (−)-U50,488H (80 μg). The tail-flick inhibition was measured 20 min after the treatment. The data represent the mean ± S.E.M. The statistical significance of differences between the groups was assessed with one-way ANOVA followed by Dunnett's test. *$P < 0.05$ vs. DAMGO alone. (A) The F values of one-way ANOVA is $F(3,35) = 9.682$.

1A). On the contrary, (−)-U50,488H (0.1–10 μM) failed to attenuate the DAMGO-induced increase of [^{35}S]GTPγS binding (Fig. 1B).

DAMGO (16 ng), given i.c.v., produced a marked inhibition of the tail-flick response in mice. The inhibition of the tail-flick response developed slowly, reached a peak at 20 min after injection, and returned to the preinjection level at 60 min post injection (data not shown). All mice were pretreated i.c.v. with norbinaltorphimine (5 μg), 24 h prior the i.c.v. injection of DAMGO, to eliminate the κ-opioid receptor-mediated component of the antinociception by TRK-820 and (−)-U50,488H. As shown in Fig. 2A and B, DAMGO (16 ng), given i.c.v., produced the marked tail-flick inhibition (75.09 ± 7.29% MPE) in mice pretreated with norbinaltorphimine. Neither TRK-820 (4 μg, i.c.v.) nor (−)-U50,488H (80 μg, i.c.v.) produced any significant tail-flick inhibition in mice pretreated with norbinaltorphimine (Fig. 2A and B, respectively). The tail-flick inhibition induced by DAMGO was dose-dependently attenuated by the co-treatment with TRK-820 (0.25–4 μg, i.c.v.), but not (−)-U50,488H (20–80 μg, i.c.v.) (Fig. 2A and B, respectively).

4. Discussion

TRK-820 as well as (−)-U50,488H produced a small but significant increase of [^{35}S]GTPγS binding to the mouse pons/medulla membrane. This increase was completely blocked by the selective κ-opioid receptor antagonist, norbinaltorphimine. Furthermore, the increase of [^{35}S]GTPγS binding induced by TRK-820 in the mouse pons/medulla membrane was not eliminated in μ-opioid receptor knockout mice (unpublished observation). These findings indicate that G-protein activation induced by TRK-820 in the mouse pons/medulla is mediated by the stimulation of the κ-opioid receptor, but not the μ-opioid receptor.

It is of interest to note that TRK-820, but not (−)-U50,488H, concentration-dependently attenuated the increase of [^{35}S]GTPγS binding induced by DAMGO in the mouse pons/medulla membrane. TRK-820 has been previously reported to have a moderate affinity to the μ-opioid receptor in addition to the high affinity to κ-opioid receptor (Seki et al., 1999). Considering the evidence that the G-protein activation induced by TRK-820 in the mouse pons/medulla is mediated by the stimulation of the κ-opioid receptor, but not μ-opioid receptor, the attenuation of the DAMGO-stimulated increase of [^{35}S]GTPγS binding by TRK-820 observed in the present study should be interpreted as a result of the antagonistic property of TRK-820 for the μ-opioid receptor.

TRK-820 as well as (−)-U50,488H produce potent antinociception that is mediated by the stimulation of the κ-opioid receptor (Endoh et al., 1999, 2000). Therefore, all mice used in the behavioral experiment were pretreated with the selective κ-opioid receptor antagonist, norbinaltorphimine, to eliminate the κ-opioid receptor-mediated antinociception induced by TRK-820 and (−)-U50,488H. In the mice pretreated with norbinaltorphimine, TRK-820, but not (−)-U50,488H, dose-dependently attenuated the DAMGO-induced antinociception. The findings obtained from the in vivo behavioral study is consistent with the result of the in vitro G-protein assay that indicates the antagonistic property of TRK-820 for the μ-opioid receptor.

Seki et al. (1999) previously reported that TRK-820 shows a partial agonistic property for μ-opioid receptor in cell membranes expressed with cloned μ-opioid receptors. However, no intrinsic activity of TRK-820 mediated by the μ-opioid receptor was detected in the present study. The discrepancy between the present study and their report is likely due to the different membrane preparations and efficacy requirements used in both studies.

In conclusion, the G-protein activation and antinociception induced by DAMGO were attenuated by TRK-820. The present results provide direct evidence for antagonistic property of TRK-820 on μ-opioid receptors in addition to full agonistic property on κ-opioid receptors.

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