Study the Mechanisms of U-50,488 to Prevent the Development of Morphine Tolerance in Guinea Pigs

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Abstract

Previously we have shown that low dose of [trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide hydrochloride] (U-50,488) could prevent the development of morphine tolerance in guinea pigs. In the present study we tried to investigate the role of glutamate and nitric oxide in this process. Male Hartley guinea pigs (200-300 g) were chronically treated s.c. with either saline or morphine (15 mg/kg) or morphine + U-50,488 (0.003 mg/kg) twice a day for 7 days. Antinociceptive activity was assessed by hot-plate test on the first, fourth and seventh day. Spinal cord slices (450 μm) were prepared 30 min after drug treatment on eighth day and [^{3}H] glutamate and nitric oxide (NO) released were determined.

We found that coadministration of U-50,488 (0.003 mg/kg) suppressed the development of morphine tolerance to antinociceptive effect as we reported before. The percentage of *in vitro* spinal release of [^{3}H] glutamate by 100 μM morphine was significantly higher in the chronic morphine group than the control group. On the other hand, coadministration of U-50,488 with morphine for 7 days blocked this effect significantly. The basal NO level released from the spinal cord slices was significantly higher in chronic morphine group but not in chronic (morphine + U-50,488) group. *In vitro* morphine (100 μM) increased the NO level in control group and chronic (morphine + U-50,488) group and also further increased NO in chronic morphine group. From the NMDA-displaced [^{3}H] glutamate binding in guinea pig spinal cord, we found that the B_{max} decreased in chronic morphine group but not in the chronic (morphine + U-50,488) group.

In conclusion, chronic morphine treatment may activate the NMDA receptors by increasing the release of glutamate which causes the increase of synthesis and release of NO and following uncertain mechanisms to induce the development of morphine tolerance. And the mechanisms of U-50,488 to prevent the development of morphine tolerance may involve the inhibition of glutamate released by chronic morphine and also the decrease of NO induced by chronic morphine.

Key Words: morphine, tolerance, U-50,488, N-methyl-D-aspartate, glutamate, nitric oxide

Introduction

U-50,488, i.e. [trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide hydrochloride], a selective κ-opioid receptor agonist, has been reported to suppress the development of antinociceptive tolerance to morphine in rats (22), mice (17) and guinea pigs (18). However the mechanism involved is not clear yet. Both competitive and non-competitive NMDA receptor antagonists (20, 12, 19) have been also reported to prevent the development of morphine tolerance, suggesting that the development of morphine tolerance may involve the activation of NMDA receptor. However how chronic morphine activates the NMDA receptors is not clear either. Perhaps chronic morphine treatment increases glutamate release or increases the affinity or density of NMDA receptors. The activation of NMDA receptors can induce the synthesis of NO through the activation of nitric oxide (NO) synthase (4, 2). It has

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also been reported that co-administration of NO sythase inhibitors, such as N^G-nitro-arginine, along with morphine prevents morphine tolerance (8, 11).

The purpose of the present study is to investigate how the NMDA receptors are activated after chronic morphine treatment and what is the role of U-50,488 in this process.

Materials and Methods

Animals

Male Hartley guinea pigs weighing 200-300 g were used in this study. The animals were housed two or three to a cage, in a room maintained at a temperature of 23 ± 2°C with a 12 h light-dark cycle. Food and water were available ad libitum throughout the experiment.

Materials

U-50,488 was prepared according to the literature (15, 16) and isolated as the hydrochloride salt, mp 221.5-222.5°C, 99.5% by HPLC (RP-select B; H_2O/CH_3OH = 1:4 buffered at pH 7.52; 254 nm). Morphine hydrochloride was purchased from the Narcotics Bureau of the National Health Administration, Taipei, R.O.C.; [^{3}H] glutamate (49.4 Ci/mmol,) was purchased from New England Nuclear; all other chemicals used were reagent grade and were supplied by Sigma Chemical Co. (St. Louis, MO).

Quantification of the Antinociceptive Response

Antinociceptive activity was assessed by the hot-plate test as we described before (18). The temperature of the plate was maintained at 55 ± 0.5 °C. The latency until the animal either lifted its paw or moved around the plate was determined. Animals not responding within 50 sec (cut-off time) were removed from the hot-plate and were assigned a response latency of 50 sec. Antinociceptive activity was determined just prior to and 30, 60, 90, 120, 180 and 240 min after drug injection. The basal latencies were found to be 6-10 sec. The basal latency was subtracted from the responded latency induced by the drug and then represented as the response time (sec). The area under the time (min) - response (sec) curve (AUC) was calculated for each animal by using Trapezoidal rule.

Induction and Assessment of Morphine Tolerance

Guinea pigs were separated randomly into 3 groups. The animals of the chronic morphine group were rendered tolerant to morphine by s.c. administration of 15 mg/kg of morphine HCl twice a day for 7 days. The animals of the chronic (morphine and U-50,488) group were coadministered morphine HCl (15 mg/kg) and U-50,488 (0.003 mg/kg) s.c. and the animals of control group were given saline instead of drugs twice a day for 7 days. The antinociceptive effect was measured on the first, fourth and seventh day to see whether tolerance developed.

In Vitro Study of Glutamate and NO Release in Spinal Cord Slices after Chronic Treatment of Drugs in Vivo.

After chronic treatment of saline or drugs for 7 days, the animals were sacrificed by decapitation 30 min after drug treatment on eighth day and the spinal cords were removed and quickly dissected at 4°C in artificial cerebrospinal fluid (aCSF; NaCl, 124 mM; KCl, 3.3 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 2.4 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25.7 mM; D-glucose, 10 mM) which was pregassed with 95% O_2 and 5% CO_2. The lumbar part of spinal cord was cut into 450 μm sections and every 10 pieces were put into a culture dish (35 mm) for totally two dishes per animal. The sections were incubated in aCSF for 10 min and then change medium which contained [^{3}H] glutamate (35, 000 dpm) and incubated for another 20 min allowing the uptake of [^{3}H] glutamate into nerve endings. After three washes to remove free [^{3}H] glutamate, the slices were incubated with either aCSF (dish 1) or morphine (100 μM; dish 2) for 10 min. Samples were taken to determine the level of [^{3}H] glutamate by liquid scintillation counter (Beckman, LS 6000TA) and the level of NO as described in the following section.

Measurement of NO Level

The samples (200 μl) taken from culture dishes were centrifuged at 7,200 g for 3 min to remove particles or debris. Six to ten μl supernatants were taken to measure the amounts of nitrite and nitrate level by adding a reducing agent (0.8% VCl_3 in 1 N HCl) to the purge vessel to convert them to NO by using a helium purge gas. The NO is then drawn into the Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers Inc., Boulder, Co., U.S.A.). NO concentrations were calculated by comparison with standard solutions of sodium nitrate.

NMDA Displaced [^{3}H] Glutamate Binding

In order to study the chronic effects of morphine on the affinity and density of spinal NMDA receptors, another experiment was carried out and the lumbar part of spinal cords of each group were removed rapidly after 7 days treatment and kept in a -70°C

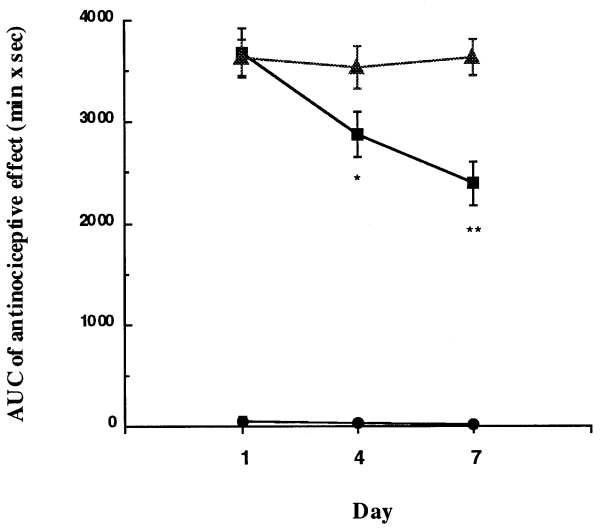

Fig. 1. The effect of U-50,488 on the development of tolerance to morphine antinociception in guinea pigs (n>6). (●) control group; (■) chronic morphine group; (▲) chronic (morphine + U-50,488) group. Significant difference ($^{*}p<0.05$; $^{**}p<0.01$) between chronic morphine group and chronic (morphine + U-50, 488) group was analyzed by ANOVA and Newman-Keuls test.

freezer before use. Spinal tissue was homogenized in 10 volumes of 50 mM Tris acetate buffer containing 1 mM EDTA (pH=7.4) with a PT20 polytron (setting at 6 for 20 sec) and centrifuged at 35,000 × g for 20 min at 4°C twice. The pellets were resuspended with 50 mM Tris acetate buffer and froze in a methanol bath chilled with dry ice, and then thawed at room temperature and centrifugated under 35,000 × g for 20 min to remove excess endogenous excitatory amino acids. These pellets were stored at -70°C before used for binding assay. On the day of receptor binding assay, membranes were thawed and centrifuged at 35, 000 × g for 20 min. The pellets were resuspended with 50 mM Tris acetate buffer (containing 1 mM EDTA) and incubated at 37°C for 20 min and centrifuged again at 35,000 × g for 20 min. Final pellets were resuspended with 50 mM Tris acetate buffer (pH 7.4) for receptor binding assay.

NMDA receptor binding assays were carried out in triplicate with [^{3}H] glutamate (30-600 nM) in a final volume of 1.0 ml of 50 mM Tris acetate buffer at pH 7.4 for 10 min at 4°C. Non-specific binding was determined by inclusion of 100 μM NMDA in the binding mixtures. After 10 min, incubations were terminated by rapid centrifugation (10,600 g, 10 min). Supernatant was removed and the pellet was washed carefully by 2 ml cold 5 mM Tris acetate buffer thrice. Protosol (NEN) was used to dissolve the pellet and liquid counting solution (Ready Safe, Beckman) was added and counted at 40-50% efficiency in a LS 6000TA counter (Beckman). The amount of membrane protein used in each assay was approximately 100 μg and was determined by the method of Lowry (9).

Statistical Analysis

The results are expressed as the means ± S.E.M. Analysis of variance was used to assess the statistical significance for repeated measures of the overall data, and differences between the individual mean values in different groups were analyzed by Newman-Keuls test. The difference was considered to be significant at $p<0.05$ or $p<0.01$.

Results

The Effect of U-50,488 on the Development of Tolerance to Morphine Antinociception in Guinea Pigs

Daily treatment with 15 mg/kg (s.c) morphine HCl twice resulted in the development of tolerance to the antinociceptive effect of morphine as shown in Fig. 1. Coadministration of U-50,488 (0.003 mg/kg) suppressed the development of tolerance to morphine without affecting morphine antinociception as we reported previously (18).

The Effect of Morphine on [^{3}H] Glutamate Release in Guinea Pig Spinal Cord Slices after Chronic Drug Treatment in Vivo

The uptake efficiency of [^{3}H] glutamate in this study is about 20%. Since different dishes of spinal cord slices showed large variation on released amount of [^{3}H] glutamate, the percentage of [^{3}H] glutamate released by 100 μM morphine (dish 2) compared to the basal release of [^{3}H] glutamate (dish 1) was calculated to represent the effect of morphine. As shown in Table 1, the percentage of in vitro spinal release of [^{3}H] glutamate by 100 μM morphine was significantly higher in the chronic morphine group than in the control group. On the other hand, coadministration of U-50,488 with morphine for 7 days reversed this effect significantly.

The Effect of Morphine on NO Release in Guinea Pig Spinal Cord Slices after Chronic Drug Treatment in Vivo

As shown in Fig. 2, the basal NO level was significantly higher in chronic morphine group but not in the chronic (morphine + U-50,488) group compared to the control group. When 100 μM morphine was added to the medium of spinal cord slices, it increased the level of NO in each group. However the increase of NO level in the chronic

Table 1. The Effect of Morphine on [^{3}H] Glutamate Release in Guinea Pig Sipinal Cord Slices after Chronic Drug(s) Treatment *in Vivo*

Treatment	n	% of basal release
Control	13	90.4 ± 4.4
Chronic M	14	154.8 ± 8.1**
Chronic M + U	15	117.9 ± 10.6$^{*\#\#}$

Chronic saline (control) or morphine (M;15 mg/kg, s.c.) or morphine + U-50,488 (M + U; 0.003 mg/kg, s.c.) was treated as described in Methods. Percentage of [^{3}H]-glutamate release in spinal cord slices stimulated by 100 μM morphine compared to basal release was calculated. Values are means ± S.E.M. ANOVA and Newman-Keuls test were used to analyze the data. *P<0.05, **p<0.01 represent significant differences from control group, and $^{\#\#}$p<0.01 represents significant difference when chronic (morphine + U-50, 488) group was compared to chronic morphine group.

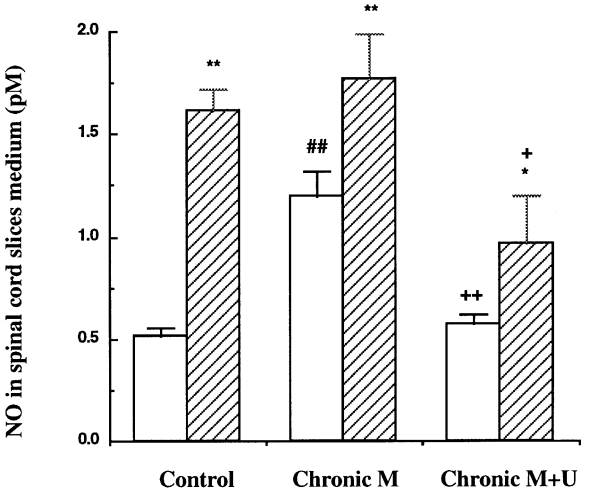

Fig. 2. The effect of in vitro morphine on NO release in guinea pig spinal cord slices from saline or chronic morphine or chronic (morphine + U-50,488) treated animal. Empty bars represent the basal levels of NO; hatched bars represent the NO levels in the presence of 100 μM morphine. Values are means ± S.E.M. ANOVA and Newman-Keuls test were used to analyze the data. $^{\#\#}$P<0.01 represent significant differences when basal level of NO was compared to the control group. *P<0.05, **p<0.01 represent significant differences when compared to the basal level in the same group. $^{+}$P<0.05, $^{++}$p<0.01 represent significant differences when (chronic M+U) group compared to (chronic M) group.

(morphine + U-50,488) group was significantly less than the chronic morphine group.

The Effect of Chronic Morphine or Morphine + U-50,488 on NMDA Displaced [^{3}H] Glutamate Binding in Guinea Pig Spinal Cord

Table 2. The NMDA-Displaced [^{3}H] Glutamate Binding in Guinea Pig Spinal Cord Membrane

	n	K_d (nM)	B_{max} (pmol/mg)
Control	6	180.5 ± 34.9	1.01 ± 0.15
Chronic M	6	201.2 ± 42.1	0.59 ± 0.05*
Chronic M + U	6	183.5 ± 45.0	0.72 ± 0.12

Values are means ± S.E.M. ANOVA and Newman-Keuls test were used to analyze the data. * P<0.05 represents significant difference from control group.

As shown in Table 2, the K_d of NMDA-displaced [^{3}H] glutamate binding in guinea pig spinal cord membrane did not change after chronic morphine or (morphine + U-50,488) treatment. The B_{max} of NMDA-displaced [^{3}H] glutamate binding decreased in chronic morphine group but not in the chronic (morphine + U-50,488) group.

Discussion

Previously we have reported that low dose of U-50,488 blocks the development of morphine tolerance in guinea pigs (18). In rat hippocampal slice model, we also found U-50,488 could effectively block the development of morphine tolerance and this effect could be antagonized by a κ-opioid receptor antagonist, nor-binaltorphimine (nor-BNI), indicating this is an opioid effect (14).

The site of action of U-50,488 in suppressing the development of tolerance to morphine antinociceptive effect has been shown at the spinal level by interacting with κ-opioid receptors in this area (17). Therefore we chose guinea pig spinal cord slices after chronic treatment *in vivo* to investigate the possible mechanisms of U-50.488 to block the development of morphine tolerance in the present study.

First we found 100 μM morphine increased [^{3}H] glutamate release in guinea pig spinal cord slices taken from chronic morphine treated animals but not from control animals. When U-50,488 was coadministered with morphine chronically, the effect of 100 μM morphine on spinal [^{3}H] glutamate release was significantly reduced (Table 1).

Ueda et al. (21) reported that μ- and δ-opioid agonists modulate pain transmission in rat spinal dorsal horn, at least in part, by inhibiting the release of glutamate from capsaicin-sensitive primary afferents. Gannon and Terrian (3) reported that U-50, 488H inhibits glutamate release from guinea pig hippocampal mossy fiber terminals. Hill and Brotchie

(7) reported that κ-opioid receptor agonist (enadoline) decreased 4-aminopyridine-stimulated glutamate release in rat and marmoset striatal synaptosomes and this effect was reversed by nor-BNI (a κ-opioid receptor antagonist). Nicol, et al. (13) reported that μ- and κ-opioids inhibit K^+ evoked glutamte release but not the basal glutamate release from rat cerebrocortical slices. All these reports suggested that either morphine (a μ-opioid agonist) or U-50,488 (a κ-opioid agonist) may inhibit the release of glutamate acutely. However our present data indicated that the effect of morphine on glutamate release had changed from acute inhibition to chronic stimulation. U-50,488 prevented this change of chronic morphine treatment. Perhaps chronic morphine treatment only affected μ-opioid receptors but not κ-opioid receptors, so that U-50,488 could still effectively inhibit glutamate release and then counteracted the chronic effect of morphine on glutamate release.

Gudehithlu et al. (6) reported that chronic administration of morphine to rats down-regulated the NMDA receptors in the cerebral cortex. However they did not find significant difference in the spinal cord. In their study, [^{3}H] MK-801 binding in the absence of glycine and glutamate was used. Most NMDA receptor channels under this condition may be not opened and therefore not available for [^{3}H] MK-801 binding. Later on they reported that the binding of [^{3}H] MK-801 was also decreased in the midbrain and spinal cord by 40 and 33% in morphine tolerant rats when the binding was carried out in the presence of glutamate and glycine (1). In our present studies, NMDA displaced [^{3}H] glutamate binding was carried out and we also found a significant decrease of NMDA receptor density in the spinal cord by 42% after chronic morphine treatment without any change in the affinity. It means that chronic morphine treatment may overactivate the spinal NMDA receptors by increasing the glutamate release and results in the down-regulation of spinal NMDA receptors. Coadministration of U-50,488 with morphine blocked the effect of chronic morphine on overacting the NMDA receptors by decreasing the glutamate release and therefore NMDA receptors were not down-regulated (Table 2).

Activation of NMDA receptors can induce the synthesis of nitric oxide (NO) through the activation of NO synthase (NOS; 2, 5). Our data (Fig. 2) have shown that chronic morphine treatment caused the basal spinal release of NO to be higher than the control group. This is consistent with our previous data and suggestion on the overactivation of the NMDA receptors after chronic morphine treatment (Table 1). Chronic coadministration of U-50,488 with morphine prevented this effect on basal NO level (Fig. 2).

When we added 100 μM morphine in the medium of spinal cord slices, the NO level increased significantly in all the three groups comparing to their basal level (Fig. 2). This implies that morphine can stimulate the synthesis of NO either by acute or chronic administration. Since acute morphine usually inhibits glutamate release, the increase of NO by acute morphine in the control spinal cord slices may be through mechanisms other than activation of NMDA receptors. We also found that the increased NO level by 100 μM morphine in chronic (morphine + U-50,488) group is significantly less than the chronic morphine group. It means that chronic coadministration of U-50,488 with morphine partially antagonized the stimulation effect of morphine on NO. This is consistent with our previous report in rat hippocampal slices (10).

In conclusion, we think the inhibition of both glutamate release and NO induced by chronic morphine may be involved in the mechanisms of U-50,488 to prevent the development of morphine tolerance.

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