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Chronic intracerebroventricular administration of morphine down-regulates spinal adenosine A_1 receptors in rats

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Abstract

Previous studies from our laboratory have shown that systemic chronic morphine treatment causes down-regulation of spinal adenosine A_1 receptors in rats. In this study, we further investigated whether supraspinal morphine treatment causes this effect. Adult male Sprague-Dawley rats were rendered tolerant to morphine by multiple intracerebroventricular (i.c.v.) injections for 2 or 4 days. Adenosine A_1 receptor binding activities were measured with [^{3}H]cyclohexyladenosine in the spinal cord and midbrain. A significant decrease in [^{3}H]cyclohexyladenosine binding was found in the spinal cord but not in the midbrain region after 2 or 4 days of chronic i.c.v. morphine treatment. A decrease in the number of binding sites (B_{max}) with no change in the affinity (K_d) of the ligand for the adenosine A_1 receptor was observed. These results suggest that supraspinal morphine administration could cause the down-regulation of spinal adenosine A_1 receptors and this may play a role in the mechanism of morphine tolerance.

Keywords: Morphine; Antinociception; Tolerance; Adenosine receptor; Cyclohexyladenosine; (Rat)

1. Introduction

The phenomenom of morphine tolerance has been known for a long time. Because many complicating factors are involved, its mechanisms are still not well documented. One possible mechanism for the development of drug tolerance is a compensatory decrease in the number of binding sites or in the affinity to active receptors. However, previous studies with chronic morphine treatment gave controversial results in this respect (for review see Loh et al., 1988). Recent evidence indicates that uncoupling of opioid receptor and G protein (Christie et al., 1987; Wong et al., 1992; Tao et al., 1993a,b) or alteration of the properties of G protein (Vachon et al., 1985, 1987; Nestler et al., 1989; Van Vliet et al., 1993) may play an important role in opioid tolerance.

Besides the changes in receptor and signal transduction of opioid at the subcellular level, tolerance in the whole animal, such as analgesia, undoubtedly is mediated by multicellular networks. Evidence that tolerance might be based on such a collective response was reported by Christie et al. (1986, 1987). They found that hyperpolarization of the locus coeruleus neurons was induced after acute administration of opioids either to the locus coeruleus area in whole animals or to isolated brain slices. In animals chronically treated with morphine, a high degree of tolerance to this response was observed, but the tolerance was much less in isolated locus coeruleus. Apparently, connections of these cells with neurons of other regions are vital to the full development of tolerance.

Recent studies suggest that release of adenosine in the spinal cord may be a significant component of the spinal antinociceptive action of morphine (DeLander and Hopkins, 1986; Sweeney et al., 1987, 1991; DeLander and Wahl, 1989; Sawynok et al., 1991b; DeLander et al., 1992). Preliminary results from this laboratory (Tao and Liu, 1992) have shown that systemic chronic morphine treatment causes down-regulation of spinal, but not of cortical adenosine A_1 receptors in rats. The purpose of the present study was to further investigate at which level the down-regulation of

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adenosine A_1 receptors acts and whether this effect is related to morphine tolerance.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 250–300 g were used in this study. All animals were maintained on pellet food and water ad libitum in a room with a 12-h light/dark schedule. Each experimental group had at least six animals.

2.2. Chronic treatment of animals with intracerebroventricular (i.c.v.) morphine and the antinociceptive test

Animals were first anesthetized with sodium pentobarbital (50 mg/kg) and implanted with a stainless-steel cannula into the left lateral cerebroventricle (i.c.v.) according to the coordinates: P 1.0 mm, L 1.25 mm, V 4–5 mm using bregma as zero. Before the cannula was fixed on the skull with dental cement, a saline-filled polyethylene tube was put onto the cannula and tested for saline flow. After surgery, only those animals with patent saline flows were used following 3 or more days' recovery period. The animals were randomly divided into three groups, one control (group A) and two for chronic morphine treatment (group B, 2 days of morphine treatment; and group C, 4 days of morphine treatment). For groups B and C, before chronic treatment started, morphine (2 μl) was administered i.c.v. and the antinociceptive effect was determined with the heat-stimulated tail-flick test (D'Amour and Smith, 1941) after 20 min and the AD_{50} value was calculated according to the up-down method described by Dixon (1965). Briefly, a series of test levels was chosen with equal spacing between each log dose of morphine. Then a series of trials ($n > 5$) was carried out following the rule of a decrease in morphine dose after inhibition of the tail-flick response and an increase in morphine dose after no inhibition of the tail-flick response. Each rat received only one trial. The AD_{50} value was derived from the equation $AD_{50} = X_f + k \times d$, where X_f is the last dose administered, k is the tabular value outlined by Dixon, and d is the interval between doses. The control tail-flick latency was between 2.0 and 3.0 s. The mean and standard deviation (S.D.) of the control tail-flick latency of each group were calculated, and the animal responses were made quantal by defining a significant inhibition of tail-flick response (antinociception) as an increase in the individual reaction time greater than 3 S.D. of the control mean reaction time. After the tests, the amount of morphine administered to the animals was normalized to the initial dose (3 μg), the animals were then chronically treated with morphine twice a day. The dose schedule is shown in Table 1.

Table 1
Dose schedule for i.c.v. morphine treatment (μg)

	Day 1	Day 2	Day 3	Day 4
9:00 a.m.	3	9	18	54
5:00 p.m.	6	12	36	72

After chronic morphine treatment for 2 or 4 days, the antinociceptive AD_{50} values of morphine for the treated groups were determined next morning by the up-down method. Rats were then killed immediately after the tail-flick test (each rat received only one dose of a drug). For the control group (group A), rats were treated i.c.v. with saline instead of morphine, and the AD_{50} values of morphine were determined before killing.

2.3. Preparation of crude synaptosomal-mitochondrial membrane

Both control and chronic morphine-treated rats were decapitated. Brain and spinal cord were removed. Crude synaptosomal-mitochondrial membranes (P_2 membranes) were prepared according the method described previously (Tao and Liu, 1992). Spinal cord or midbrain was homogenized with a Kinematica polytron (setting 5, for 20 s) in 20 volumes of ice-cold 0.32 M sucrose buffered with 50 mM Tris-HCl, pH 7.4. Homogenates were centrifuged at $1000 \times g$ for 10 min at 4° C, and the P_1 pellet, which contains the nuclei, was removed. The supernatant was then collected and centrifuged at $22\,000 \times g$ for 20 min at 4° C. The resulting pellet (P_2 membrane) was suspended in 50 mM Tris buffer (20 mg original tissue weight/ml buffer solution) and recentrifuged at $22\,000 \times g$ for 20 min. The P_2 membrane fraction after the wash was resuspended in 50 mM Tris buffer containing 2 IU adenosine deaminase/ml and incubated at 37° C for 30 min to inactivate endogenous adenosine (Bruns et al., 1980). The membrane homogenates were recentrifuged and the final pellet was resuspended in 50 mM Tris buffer (about 1 mg protein/ml) and frozen at −70° C until assay.

2.4. Adenosine A_1 receptor binding studies

Adenosine A_1 receptor binding was measured in the P_2 membranes of rat spinal cord or midbrain according to the method described by Hutchison et al. (1990). All assays were carried out in triplicate in 13×100 mm disposable borosilicate glass test tubes. An aliquot of P_2 membranes (100–200 μg protein) was transferred to the incubation buffer (50 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.4) containing approximately 1 nM

[^{3}H]cyclohexyladenosine (specific activity 32 Ci/mmol) in a final volume of 1 ml. For the saturation binding studies, 0.1–10 nM [^{3}H]cyclohexyladenosine was used to determine the K_d and B_{max}. All assays were conducted at room temperature for 2 h. Non-specific binding was defined in the presence of 10 μM 2-chloroadenosine. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure. Filters were washed twice with 5 ml ice-cold buffer and placed in scintillation counting vials. After equilibration in 4 ml of 'Ready Safe' scintillation cocktail (Beckman), radioactivity was determined by conventional liquid scintillation spectrometry at an efficiency of 40–50%. The protein content in P_2 membranes used in each assay was determined by the method of Lowry et al. (1951).

2.5. Data analysis

The results are expressed as the means ± S.E.M. Student's *t*-test was used to analyze some simple comparisons between control and experimental groups. Analysis of variance and Duncan multirange tests were used to analyze the differences among binding data.

The saturation binding assays (Scatchard plots) were analyzed with the McPherson program (McPherson, 1983), which is a modification of the LIGAND progam originally written by Munson and Rodbard (1980). This progam utilizes a non-linear least-square curve-fitting algorithm to estimate the B_{max} (the maximum number of binding sites) and K_d (the equilibrium dissociation constant of the radioligand).

2.6. Materials

[^{3}H]Cyclohexyladenosine was purchased from Du Pont NEN, Boston, MA, USA. Morphine HCl was obtainted from the Narcotics Bureau of the National Health Administration, Taipei, Taiwan, ROC. All other chemicals were reagent grade and purchased from Sigma Chemical Company, St. Louis, MO, USA.

3. Results

3.1. Effect of chronic i.c.v. morphine treatment on tolerance to morphine-induced antinociception

As shown in Table 2, after multiple i.c.v. morphine treatment for 2 days, the AD_{50} of morphine increased from 2.95 ± 0.3 μg to 27.5 ± 2.1 μg, about a 9-fold degree of tolerance. When the chronic treatment was extended to 4 days, the AD_{50} of morphine increased further to 42.5 ± 3.2 μg, about a 14-fold degree of tolerance. It is apparent that under the current scheme of multiple i.c.v. morphine injections, tolerance to the antinociceptive effects of morphine had well developed.

Table 2
Alteration in antinociceptive potency of morphine after chronic i.c.v. morphine treatment

Treatments	AD_{50} (μg)	Degree of tolerance
Control	2.95 ± 0.3	
2 days	27.5 ± 2.1	9.3
4 days	42.5 ± 3.2	14.4

The up-down method of Dixon (1965) was used to determine the AD_{50} values (see text for details). Values are means ± S.E.M. Each group contained more than six rats. Degree of tolerance was calculated from the ratio (AD_{50} after treatment)/(AD_{50} before treatment).

3.2. Effect of chronic i.c.v. morphine treatment on tolerance to systemic cyclopentyladenosine-induced antinociception

In order to know the chronic effect of i.c.v. morphine on spinal adenosine systems, the AD_{50} of i.p. cyclopentyladenosine (CPA) was also measured in control and chronic i.c.v. morphine-treated rats. As shown in Table 3, the AD_{50} of CPA increased from 0.3 ± 0.06 mg/kg in group A (control group) to 1.1 ± 0.22 mg/kg in group B (2 days' morphine treatment group) and to 1.0 ± 0.25 mg/kg in group C (4 days' morphine treatment group). These results indicate that after 2 or 4 days of multiple i.c.v. morphine treatment, animals were tolerant not only to morphine but also to CPA, an adenosine A_1 analogue. However, the degree of tolerance to CPA was much less than that to morphine (3.5- versus 9–14-fold).

3.3. Effect of chronic i.c.v. morphine treatment on adenosine A_1 receptor binding activities

[^{3}H]Cyclohexyladenosine, a selective adenosine A_1 agonist (Bruns et al., 1980), was used to determine the adenosine A_1 receptor binding activities in P_2 membranes isolated from spinal cord and midbrain of rats after chronic morphine treatment. As shown in Table

Table 3
Alteration in antinociceptive potency of i.p. cyclopentyladenosine after chronic i.c.v. morphine treatment

Treatments	AD_{50} (mg/kg)	Degree of tolerance
Control	0.3 ± 0.06	
2 days	1.1 ± 0.22	3.7
4 days	1.0 ± 0.25	3.3

The up-down method of Dixon (1965) was used to determine the AD_{50} values (see text for details). Values are means ± S.E.M. Each group contained more than six rats. Degree of tolerance was calculated from the ratio (AD_{50} after treatment)/(AD_{50} before treatment).

Table 4
[^{3}H]Cyclohexyladenosine binding to P_2 membranes prepared from spinal cord and midbrain after chronic i.c.v. morphine treatment

Treatments	[^{3}H]Cyclohexyladenosine bound (fmol/mg protein)	
	Spinal cord	Midbrain
Control	227.6 ± 10.8	255.0 ± 7.4
2 days	178.4 ± 9.2 [a]	262.0 ± 7.4
4 days	198.2 ± 6.8 [a]	278.7 ± 10.2

Specific binding of 1 nM [^{3}H]cyclohexyladenosine to washed membranes from spinal cord or midbrain was assayed as described in the section on Materials and methods. Values are means ± S.E.M. from at least six animals. [a] $P < 0.05$ when compared with the control group by means of Duncan multirange tests.

4, a significant decrease ($P < 0.05$) in the amount of bound [^{3}H]cyclohexyladenosine was found only in the spinal cord but not in the midbrain region after 2 or 4 days of chronic i.c.v. morphine treatment. When Scatchard analyses were carried out on the results from 0.1–10 nM [^{3}H]cyclohexyladenosine, a decrease in the B_{max} value (about 12–18%) was observed in P_2 membranes from spinal cord of treated animals while no significant change in K_d value was noticed (Table 5). It is clear from these results that chronic i.c.v. morphine treatment reduced the number of binding sites in spinal adenosine A_1 receptors whereas the affinity of the ligand for receptors was not altered significantly.

4. Discussion

Opioid-stimulated descending spinal systems may mediate the antinociception in intact animals. Spinal serotonergic and noradrenergic pathways appear to be the primary antinociceptive systems activated by opioids (for review see Basbaum and Fields, 1984). Recently, DeLander and Hopkins (1986) have reported that antinociception produced by i.c.v. administration of morphine in mice was antagonized by i.t. injection of the adenosine receptor antagonists, such as theophylline, caffeine and isobutylmethylxanthine. Sawynok et al. (1991a) have also shown that antinociception produced by local application of morphine on the periaqueductal gray of rats was attenuated by i.t. injection of 8-phenyltheophylline, an adenosine receptor antagonist. Sweeney et al. (1987) have demonstrated that morphine (1–100 μM) produced a Ca^{2+}-dependent release of endogenous adenosine from synaptosomes and this effect was blocked by 1 μM naltrexone. Later on, they (Sweeney et al., 1991) further showed that i.c.v. morphine released adenosine in the spinal cord. These results suggest that adenosine released from the spinal cord may play an important role in the mediation of antinociception produced by morphine administered supraspinally.

Table 5
Scatchard analysis of spinal [^{3}H]cyclohexyladenosine binding after chronic i.c.v. morphine treatment

Treatments	K_d (nM)	B_{max} (fmol/mg protein)
Control	0.61 ± 0.021	343 ± 7.3
2 days	0.56 ± 0.004	283 ± 10.7 [a]
4 days	0.55 ± 0.007	303 ± 10.4 [a]

Values are means ± S.E.M. from three determinations with triplicates in each determination. [a] $P < 0.05$ when compared with the control group, using Duncan multirange tests.

In this report, we focused our attention on the role of the spinal adenosine system in the development of morphine tolerance. Since it has been shown that adenosine A_1 receptors were down-regulated following prolonged incubation of adipocytes with an adenosine A_1 receptor agonist, N^6-phenylisopropyl adenosine (PIA) (Green, 1987), we speculated that chronic morphine treatment may act on certain pathways to release adenosine consistently in the spinal cord, which may lead to down-regulation of the adenosine A_1 receptor. A preliminary report from this laboratory (Tao and Liu, 1992) has shown that multiple intraperitoneal injections of morphine down-regulated the spinal adenosine A_1 receptors in rats. In the present studies, we further investigated whether supraspinal administration of morphine will down-regulate the spinal adenosine A_1 receptors and whether this is related to the development of morphine tolerance.

Chronic i.c.v. morphine treatment for 2 days or 4 days induced an about 9- to 14-fold degree of tolerance to morphine (Table 2). These animals also showed tolerance to the adenosine A_1 analogue, CPA, although to a lesser degree (Table 3). When binding studies of adenosine A_1 receptors using [^{3}H]cyclohexyladenosine were carried out in P_2 membranes of spinal cord and midbrain, a significant decrease in the number of binding sites with no significant change in affinity (Table 5) was observed only in the spinal cord. These results indicate that chronic i.c.v. morphine may affect the adenosine system in the spinal cord.

Results from this study suggest that chronic i.c.v. morphine treatment causes the release of adenosine in the spinal cord and down-regulation of adenosine A_1 receptors in the spinal cord. This down-regulation of adenosine A_1 receptors may relate to the development of morphine tolerance.

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