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Lack of cyclic AMP-specific phosphodiesterase 4 activation during naloxone-precipitated morphine withdrawal in rats

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Abstract

Intracellular cyclic AMP regulation systems play an important role in the mechanisms of morphine dependence and withdrawal. In the present study, to clarify the involvement of phosphodiesterase (PDE) 4, degradation enzyme of cyclic AMP in morphine dependence and withdrawal we investigated the activities of PDE4 after naloxone-precipitation in single morphine treatment and repeated morphine treatment (morphine-dependence) rats. Naloxone (5 mg/kg, s.c.) challenge caused a significant withdrawal signs such as jumping in morphine-dependent rats following repeated treatment with morphine (4, 8, 12, and 16 mg/kg, twice a day for 4 days), but not in single morphine-treated rats (16 mg/kg, single). Naloxone challenge caused an increase in PDE4 activities in the brain of rats treated with single morphine in connection with the elevation of brain cyclic AMP. In contrast, increase in the PDE4 activities was not caused by naloxone challenge in all brain regions of morphine-dependent rats, although brain cyclic AMP was significantly increased. These results suggest that the lack of PDE4 activation leading to remarkable elevation of cyclic AMP is involved in naloxone-precipitated morphine withdrawal symptoms.

Keywords: Cyclic AMP-specific phosphodiesterase 4; Cyclic AMP; Morphine withdrawal

Repeated administration of morphine produces tolerance and dependence in humans and animals. Cessation of morphine treatment precipitates withdrawal symptoms that have been linked to alteration in a number of second messenger systems and neurotransmitters [11,15]. Morphine dependence and withdrawal has been demonstrated to be associated with alterations in the cyclic AMP systems in numerous in vivo and in vitro experiments. Acute opiate exposure inhibits the cyclic AMP pathway in many types of neurons in the brain [3], whereas chronic opiate exposure leads to a compensatory up-regulation of the cyclic AMP pathway in at least a subset of these neurons. This up-regulation of cyclic AMP involves increased activity of adenylate cyclase, cyclic AMP-dependent protein kinase A (PKA) and other signaling pathways [15]. G protein-mediated adenylate cyclase activity and cyclic AMP levels are enhanced in the striatum on chronic treatment by morphine pellet implantation to mice and mice in withdrawal [10]. Also, implantation of morphine pellets for 5 days in rat increases the level of basal, GTP- and forskolin-stimulated adenylate cyclase in the locus coeruleus, but not in the frontal cortex and neostriatum [5]. However, to the best of our knowledge, there has been no study investigating the alterations of the cyclic AMP degradation system such as phosphodiesterase (PDE) activity in morphine-dependent and withdrawal rats.

Intracellular levels of cyclic AMP are regulated by the adenylate cyclases, which synthesize and are caused by the PDEs, which degrade this second messenger. The type IV, cyclic AMP-specific phosphodiesterases (PDE4) comprise the largest of 11 known classes of mammalian 3′,5′-cyclic nucleotide PDEs. The PDE4 enzymes are characterized by high affinity and specificity for cyclic AMP, low Km, insensitivity to Ca^{2+}, and sensitivity to the selective inhibitor rolipram [4]. PDE4 plays an important role in homeostatic regulation of intracellular cyclic AMP, which involves up-regulation of PDE4 activity in response to increases in intracellular cyclic AMP [4,6,7,14]. Also, repeated administration of rolipram, a PDE4 inhibitor, with morphine for 5 days has been reported to attenuate the withdrawal syndrome and the increment of cyclic AMP levels after administration of naloxone [12]. These reports suggest that PDE4 is involved in morphine dependence and withdrawal.

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In the present study, we investigated the effects of naloxone precipitation on PDE4 activity in the rat brain following single and repeated morphine treatment to clarify the role of cyclic AMP degradation system in morphine dependence and withdrawal. Three brain regions of frontal cortex, hippocampus and striatum relating to an emotional, memory and movement, respectively, were investigated to acquire the dynamic effects of cyclic AMP system.

Male Sprague-Dawley rats (7–8-weeks-old, Clea Japan, Tokyo, Japan) were used. The animals were housed in groups of five or less per cage in an aluminum cage (W × D × H; 400 mm × 500 mm × 200 mm) under the conditions of 23 ± 2 °C and a relative humidity of 55 ± 15% with food and water freely available. The room was set for a 12-h light/12-h dark schedule (turned on at 08:00:turned off at 20:00) and ventilated over 10 times h^{-1} with 100% fresh air. All procedures performed on the animals in the present study were conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals and were approved by the Shionogi Animal Care and Use Committee. Morphine hydrochloride (Shionogi and Co., Ltd., Osaka, Japan) and naloxone hydrochloride (Sigma Co. St. Louis, MO, USA) were dissolved in physiological saline.

Rats were subcutaneously (s.c.) administered morphine hydrochloride twice daily for 4 days with escalating doses of 4, 8, 12, and 16 mg/kg, respectively, on each day. On the 5th day, naloxone (5 mg/kg, s.c.) was administered 1 h after the morphine (16 mg/kg) treatment to induce morphine withdrawal. For acute morphine-precipitation, naloxone (5 mg/kg, s.c.) was administered 1 h after a single administration of morphine hydrochloride (16 mg/kg). Immediately after the naloxone challenge, each rat was placed in the chamber, and the jumping was counted for 30 min after naloxone injection.

For measurement of cyclic AMP levels in the brain, rats receiving single or chronic doses of morphine were decapitated 1 h after the treatment with morphine hydrochloride (16 mg/kg, s.c.) on the 5th day. Rats subjected to precipitated withdrawal were decapitated 2 h after naloxone (5 mg/kg, s.c.) challenge. The brains of these animals were removed rapidly, and the frontal cortex, hippocampus and striatum were dissected on ice and frozen with liquid nitrogen. All brain tissues were stored at −80 °C until assayed. Each tissue was homogenized in cold (6 w/v%) trichloroacetic acid at 2–8 °C to give a (10 w/v%) homogenate, and centrifuged at 2000 × *g* for 15 min at 4 °C. The supernatant was washed three times with 5 volumes of water-saturated diethyl ether. The upper ether layer was discarded after each washing. The aqueous extraction remaining was dried under a stream of nitrogen at 60 °C. The dried extract was dissolved in 5 ml of assay buffer, and the amounts of cyclic AMP were determined using a non-acetylation enzyme immunoassay kit (Amersham Biosciences Corp., Piscataway, NJ, USA).

The PDE activity was assayed according to the method previously described [2]. The brain tissues were obtained as described above. The cytosolic fractions were prepared by homogenizing the frozen tissues in 10 volumes of 50 mM Tris–HCl (pH 7.5) and centrifuging at 100,000 × *g* for 30 min at 4 °C. The cytosolic fractions (10 μg of protein) were preincubated with reaction buffer containing 50 mM Tris–HCl, 10 mM $MgCl_2$ and 1 mM DTT in the absence or presence of rolipram (50 μM) for 60 min at 4 °C. PDE4 activity has been reported to be approximately 20% of the total PDE activity in the whole brain [16], and we confirmed that 50 μM of rolipram could inhibit approximately 20–30% of the PDE activity in each tissue. The assay (200 μl of total volume) was initiated by adding 200 nM [^{3}H]cyclic AMP in 10 μM cyclic AMP solution and incubating for 10 min at 30 °C. Next, the assay tube was placed in boiling water for 1 min to stop the reaction. To decompose the 5′-AMP to adenosine, 50 μg of snake venom (4 °C) was added to the tube followed by incubation for 20 min at 30 °C. This reaction was terminated by soaking the tube in boiling water for 1 min, and then 1 ml of 20% Dowex AG1-X8 suspension (200–400 mesh, Cl-form, Bio-Rad Laboratories, Inc., Hercules, CA, USA) was added three times with vigorous mixing to remove both cyclic AMP and 5′-AMP. The mixture was centrifuged at 1000 × *g* for 5 min at 4 °C and 250 μl of the supernatant was collected. The radioactivity was measured with a liquid scintillation counter. The values of PDE4 activity were calculated by subtracting the radioactivity level in the presence of rolipram from the level in its absence.

[^{3}H]Rolipram binding was performed as previously described [19,20] with some modifications. The brain tissues were obtained as above, and the cytosolic fraction was prepared according to the method described above. The binding reaction was performed in a final volume of 100 μl containing 100 μg protein of the cytosolic fraction, 50 mM Tris–HCl (pH 7.4), 6 mM $MgCl_2$ and 3 nM [methyl-^{3}H]rolipram (Amersham Biosciences Corp., specific activity, 86.0 Ci/mmol). The reaction was started with the addition of the protein for 60 min at 4 °C, and terminated by adding ice-cold 50 mM Tris–HCl (pH 7.4) followed by rapid filtration through Whatman GF/C glass filters. After washing twice with ice-cold 50 mM Tris–HCl (pH 7.4), the amount of radioactivity was determined using a scintillation counter. Non-specific binding was determined as the amount of radioactivity in the presence of 1 μM rolipram (Tocris Cookson, Bristol, UK).

The values are expressed as the percent of control rats and represented as mean ± S.E. The statistical significance of differences was determined using unpaired Student's *t*-test or Dunnett's multiple comparison test. *P* values of less than 0.05 were considered to be statistically significant.

Naloxone challenge produced a significant increase in jumping in rats injected with chronic morphine (45.1 ± 11.0; $P < 0.01$) as compared with that of rats given single morphine (2.1 ± 0.55).

The cyclic AMP levels were 55.3, 38.0 and 23.0 pmol/mg protein on the frontal cortex, hippocampus and striatum in the control animal, respectively. In single morphine treatment rats, naloxone challenge significantly increased the cyclic AMP levels in the frontal cortex and hippocampus ($P < 0.05$), but not in the striatum compared with single morphine + saline (Fig. 1). The cyclic AMP levels of the frontal cortex, hippocampus and striatum were significantly increased by naloxone challenge in chronic morphine-dependent rats ($P < 0.01$).

The PDE4 activities were 147.0, 100.2 and 67.7 pmol/min/mg protein on the frontal cortex, hippocampus and striatum in the control animal, respectively. In single morphine treatment