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Possible involvement of protein kinases in physical dependence on opioids: studies using protein kinase inhibitors, H-7 and H-8

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Abstract

Effects of a cAMP-dependent protein kinase and protein kinase C inhibitor, H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) and a cAMP-and cGMP-dependent protein kinase inhibitor, H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide), on the behavioral signs of naloxone (an opioid receptor antagonist)-precipitated withdrawal syndrome and effects of H-7 on the change of protein kinase C activity in the pons/medulla region induced by morphine (a μ-opioid receptor agonist) or butorphanol (a $\mu/\delta/\kappa$ mixed opioid receptor agonist) were investigated in this study. Rats were intracerebroventricularly (i.c.v.) infused with morphine (26 nmol/μl/h) or butorphanol (26 nmol/μl/h) through osmotic minipumps for 3 days. In some groups, either saline or drug-treated groups were concomitantly infused with H-7 (1 and 10 nmol/μl/h) or H-8 (10 nmol/μl/h). The expression of physical dependence produced by morphine or butorphanol, as evaluated by naloxone (5 mg/kg i.p.)-precipitated withdrawal signs, was reduced by concomitant infusion of H-7 or H-8. In the same condition, morphine and butorphanol chronic treatment enhanced (28.1% and 26.3% enhancement over the saline-treated group, respectively) cytosolic protein kinase C activity in the pons/medulla, but not in the membrane fraction. Furthermore, concomitant infusion of H-7 inhibited the enhancement of protein kinase C activity. These results indicate that various types of protein kinases may play an important role in the development and/or expression of physical dependence on opioids. Among them, the enhancement of cytosolic protein kinase C activity in the pons/medulla region seems to be one of the major underlying mechanisms in opioid physical dependence.

Keywords: Opioid; Physical dependence; Protein kinase; Morphine; Butorphanol; H-7; H-8

1. Introduction

The development of tolerance and dependence with repeated use is a characteristic feature of all the opioids represented by morphine, and the possibility of developing drug dependence is one of the major limitations of their clinical use. Although many studies regarding several neurotransmitters (Takemori, 1974) and receptors (Abdelhamid et al., 1991; Chang et al., 1983; Cowan et al., 1988; Gulya et al., 1988; Lahti et al., 1985; Miyamoto et al., 1993) have been conducted in an attempt to define the mechanism involved in the development of dependence on opioids, the definitive evidence has still not been presented. Recently, with increasing evidence that the phosphorylation of specific proteins may be involved in the production of various physiological functions, the implication of protein kinases on the development of dependence on opioids has been noted. Indeed, it has been reported that alterations of intracellular messenger proteins by cAMP-dependent protein kinase could be the key part of an overall mechanism underlying opioid addiction (Nestler et al., 1993), and that protein kinase C activation increases the rate and magnitude of agonist-induced opioid receptor down-regulation (Gucker and Bidlack, 1992). Accordingly, it is essential to determine the involvement of various protein kinases for elucidation of the mechanism of development and/or expression of dependence on opioids. The present study was designed to elucidate the involvement of protein kinases in opioid physical dependence; specifically, effects of a cAMP-dependent protein kinase and protein kinase C inhibitor, H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) (Garland et al., 1987; Hidaka et al.,

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1984) and a cAMP-and cGMP-dependent protein kinase inhibitor, H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide) (Hidaka et al., 1984), on the behavioral signs of naloxone (opioid receptor antagonist)-precipitated withdrawal syndrome in animals rendered dependent by continuous i.c.v. infusion of morphine or butorphanol. Furthermore, Narita et al. (1994a) have reported that a daily injection of rats with morphine leads to an enhancement of cytosolic protein kinase C activity in the pons/medulla. Protein kinase C, which is highly concentrated in the brain, is widely accepted to be a Ca^{2+}/phospholipid-dependent regulatory enzyme activated by diacylglycerol (Nishizuka, 1986,1992), and an important third messenger in the regulation of neuronal excitability, signal transduction, and synaptic plasticity (Kikkawa et al., 1982; Nishizuka, 1988). Hence, the effects of H-7 on the protein kinase C activity in the pons/medulla region in opioid-dependent animals were also examined. Interestingly, butorphanol, an opioid receptor agonist/antagonist, has been introduced as a potent analgesic for moderate to severe pain (Pircio et al., 1976), and has exhibited some pharmacological and biochemical differences as well as similarities to the prototype of a μ-opioid receptor agonist, morphine (Horan and Ho, 1989). As most studies of opioid dependence have focused on morphine, the use of butorphanol which possesses the affinity to μ-, δ-, and κ-opioid receptors (Horan and Ho, 1989) should be valuable in studies to evaluate the mechanism of development and/or expression of dependence on opioids.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 230–250 g (Charles River, Wilmington, MA, USA) were purchased and housed in a group of 3 or 4 animals in a cage. They were kept in a room maintained at 21 ± 2°C and a 12 h light-dark cycle with free access to food and tap water. After reaching 280–300 g, they were used for experiments.

2.2. Surgical procedures

Rats were anesthetized with Equithensin (4.25 g chloral hydrate, 2.23 g $MgSO_4 \cdot 7H_2O$, 0.972 g sodium pentobarbital, 44.4 ml propylene glycol, 10 ml 95% ethanol, and distilled water to make a final volume of 100 ml), 0.3 ml/100 g body weight, i.p., and then placed in a stereotaxic instrument. An indwelling stainless steel guide cannula (26 gauge, 10 mm long) was implanted into the right lateral cerebral ventricle (AP: −0.5 mm, LAT: +1.3 mm, and DV: −4.5 mm) with the bregma chosen as the stereotaxic reference point (Paxinos and Watson, 1986). Dental acrylic cement (Lang Dental MFG Co., Wheeling, IL, USA) was applied to the surface of the skull, and a protective cap was placed around the cannula. After the acrylic had hardened, the animal was removed from the stereotaxic frame. A stylet (32 gauge stainless steel tubing) was placed into the guide cannula to maintain patency. The presence of cerebrospinal fluid in the guide cannula was examined to assure proper placement. After surgery, rats were given 300 000 units of procaine penicillin G (Pfizerpen, Pfizer Corp., New York, NY, USA), s.c., to prevent infection and were allowed at least one week to recover before commencing the infusion of morphine-HCl (Sigma Chemical Corp., St. Louis, MO, USA), butorphanol-tartrate (17-cyclobutylmethyl-3,14-dihydroxy morphinan; a generous gift from Bristol-Myers-Squibb Corp., Evansville, IN, USA), and/or H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine; Research Biomedicals International, Natick, MA, USA) or H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; Research Biomedicals International, Natick, MA, USA).

2.3. Administration schedule and induction of morphine and butorphanol dependence

Animals were infused i.c.v. continuously with saline (1 μl/h), morphine (26 nmol/μl/h), or butorphanol (26 nmol/μl/h) for 3 days through osmotic minipumps (Alzet 2001, Alza Corp., Palo Alto, CA, USA). Some of these animals were concomitantly infused with H-7 (1 or 10 nmol/μl/h) or H-8 (10 nmol/μl/h) for the same period of time. This infusion period and dose paradigm were determined to be optimal from our previous experiments (Jaw et al., 1993a,b). In the case of concomitant infusion of opioid and H-7 or H-8, they were mixed in the same pump. Under ether anesthesia, animals were implanted s.c. with minipumps between the scapulae. A 4 cm piece of Tygon tubing (0.38 mm inner diameter, Cole-Palmer, Chicago, IL, USA) was applied to connect the minipump to a piece of L-shaped stainless steel injector tubing (32 gauge, 30 mm long) with one end having the same length as the guide cannula. All drug solutions were passed through a 0.2 mm sterile Acrodisk filter (Gelman Sciences, Ann Arbor, MI, USA) before being introduced into the pumps, and the delivery apparatus was assembled under sterile conditions. Minipumps were primed overnight at room temperature in normal saline so that an optimal flow rate (1 μl/h) was obtained. Rats were injected with naloxone (Sigma Chemical Corp., St. Louis, MO, USA), 5 mg/kg i.p., 2 h after the termination of drug infusion conducted by cutting the tubing.

2.4. Measurement of behavioral signs during morphine and butorphanol withdrawal

Ten distinct behaviors (escape behavior, wet dog shakes, teeth chattering, rearing, locomotion, stretching, scratching, salivation, penis-licking, and ptosis) were scored during a 30 min period following naloxone injection as behavioral signs of withdrawal. The reactions of each animal were evaluated by an independent investigator who did not have prior knowledge of the nature of the treatment received. Loss of body weight (number of animals exhibiting > 3% body weight loss) was measured before and 1 h after the administration of naloxone.

2.5. Preparation of cytosolic and membrane fractions

The method described by Otani et al. (1993) was slightly modified. Briefly, 6 h after the termination of drug infusion without naloxone challenge, rats were killed by decapitation, and their brains were rapidly removed. The pons/medulla region was separated according to the method of Glowinski and Iversen (1966), and the tissue was homogenized in 15 volumes (w/v) of buffer A containing 50 mM Tris-HCl (pH 7.5), 5 mM ethylenediaminetetra-acetate (EDTA), 10 mM ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetra-acetate (EGTA), 0.3% (w/v) β-mercaptoethanol and 1 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at $100000 \times g$ for 30 min to yield supernatant and pellet fractions. The supernatant contained the cytosolic fraction. The pellet was resuspended in buffer A containing 0.1% Triton X-100 (w/v), stirred for 30 min at 4°C, and centrifuged for 10 min at $20000 \times g$. The supernatant contained solubilized membranes. This membrane fraction and the previously saved cytosolic fraction were diluted with 50 mM Tris-HCl (pH 7.5) and assayed for protein kinase C activity.

2.6. Measurement of protein kinase C activity

Protein kinase C activity was measured by the transfer of phosphate from [γ-^{32}P]ATP (5000 Ci/mmol; Amersham, Arlington Heights, IL, USA) to the threonine group on a specific synthetic peptide (Amersham Protein Kinase C enzyme assay system RPN 77, Arlington Heights, IL, USA). This assay is a modification of a mixed micelle assay, in which the enzyme is made active by phorbol 12-myristate 13-acetate (PMA). Samples (cytosolic or solubilized membrane fraction containing about 0.6 μg of protein) were incubated for 15 min at 25°C in a mixture (75 μl total volume) containing 50 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM Ca^{2+}, 75 μM synthetic peptide, 3.125 μl of mixed micelles (8 mol% L-phosphatidylserine, 24 mg/ml PMA, 50 μM [γ-^{32}P]ATP (0.2 μCi/assay), and 15 mM $MgCl_2$). The reaction was stopped by adding 100 μl of 75 mM orthophosphoric acid. A 125 μl aliquot was spotted onto a phosphocellulose paper. The paper was washed twice with 75 mM orthophosphoric acid (at least 10 ml per paper), transferred to vials, and counted in a Packard liquid scintillation analyzer (2200 CA, Grove, IL, USA). All assays were conducted in duplicate. Results were expressed as pmol ^{32}P incorporated into peptide/min/μg protein.

Protein concentrations were determined by the microassay method of Bradford (1976) (Bio-Rad Protein Assay, Hercules, CA, USA) using γ-globulin as a standard.

2.7. Statistics

Quantal (all or none) data from the behavioral studies on the experimental groups and saline controls

Table 1
Effects of concomitant administration of H-7 or H-8 on naloxone-precipitated withdrawal signs in morphine-dependent rats

Withdrawal signs	Saline	H-7 (nmol/μl/h for 3 days)		H-8 (nmol/μl/h for 3 days)
		1	10	10
Escape behavior	7/14 #	2/7	0/7	0/7
Wet dog shakes	14/14	7/7	5/7	5/7
Teeth chattering	14/14	4/7 [a]	2/7 [b]	1/7 [b]
Rearing	14/14	4/7 [a]	2/7 [b]	2/7 [b]
Locomotion	11/14	5/7	3/7	2/7 [a]
Stretching	8/14	1/7	1/7	3/7
Scratching	13/14	5/7	3/7 [a]	3/7 [a]
Salivation	8/14	1/7	0/7 [a]	1/7
Penis-licking	11/14	5/7	2/7 [a]	1/7 [a]
Ptosis	9/14	3/7	2/7	1/7
Weight loss (> 3%)	13/14	4/7	1/7 [b]	0/7 [b]

Rats received i.c.v. infusion of morphine (26 nmol/μl/h) for 3 days and were challenged with naloxone (5 mg/kg i.p.) 2 h after the termination of drug infusion. # Numbers denote the number of rats showing positive signs over the total number of rats tested. [a] $P < 0.05$, [b] $P < 0.01$, values are significantly lower than the control values as determined by the chi-square test.

were compared by the chi-square test. In the case of the measurement of protein kinase C activity, the data were presented as the mean ± S.E.M. The Newman-Keuls multiple comparison test was used for the statistical analysis of the data. A difference was considered significant at $P < 0.05$.

3. Results

3.1. Withdrawal behavioral studies

Characteristic abnormal behavioral signs (escape behavior, wet dog shakes, teeth chattering, rearing, locomotion, stretching, scratching, salivation, penis-licking, and ptosis) and body weight loss showing the expression of withdrawal syndrome were induced by i.p. injection of naloxone, 5 mg/kg, 2 h after the termination of i.c.v. infusion of morphine (26 nmol/μl/h) or butorphanol (26 nmol/μl/h) for 3 days. Concomitant treatment with H-7 or H-8 blocked the expression of these behavioral signs (Table 1 and Table 2). In detail, H-7 significantly inhibited teeth chattering (1 and 10 nmol/μl/h), rearing (1 and 10 nmol/μl/h), scratching (10 nmol/μl/h), salivation (10 nmol/μl/h), penis-licking (10 nmol/μl/h), and body weight loss (10 nmol/μl/h) over the morphine-treated control group. Furthermore, teeth chattering, rearing, locomotion, scratching, penis-licking, and body weight loss were also significantly blocked by H-8 at the dose of 10 nmol/μl/h in morphine-dependent rats. Other behavioral signs except wet dog shakes were tended to be reduced by H-7 and H-8 (Table 1). In butorphanol-infused animals, teeth chattering (1 and 10 nmol/μl/h), rearing (10 nmol/μl/h), stretching (1 nmol/μl/h), and penis-licking (10 nmol/μl/h) were significantly blocked by H-7. H-8 at 10 nmol/μl/h also inhibited teeth chattering, rearing, penis-licking, and body weight loss. Other signs except wet dog shakes were also tended to be reduced by these inhibitors (Table 2). On the other hand, in animals which received H-7 or H-8 infusion alone, behaviors were not altered after naloxone injection (data not shown).

3.2. Protein kinase C assay studies

The cytosolic protein kinase C activity in the pons/medulla region was significantly increased by i.c.v. infusion of morphine and butorphanol (28.1% and 26.3% increase, $P < 0.05$ and $P < 0.01$ over the saline-treated group, respectively). On the other hand, both drugs produced no changes in the protein kinase C activity in the membrane fraction (Fig. 1 and Fig. 2).

As also shown in Fig. 2, the enhancement of cytosolic protein kinase C activity in the pons/medulla region by continuous infusion of morphine or butorphanol was completely prevented by their combination with H-7 (10 nmol/μl/h). Meanwhile, i.c.v. infusion of H-7 alone did not affect protein kinase C activity in this region (Fig. 2).

4. Discussion

In our behavioral studies, concomitant infusion of H-7 or H-8 with morphine or butorphanol blocked the expression of withdrawal syndrome evaluated by several abnormal behaviors except wet dog shakes, suggesting that various types of protein kinases are closely involved in the expression of physical dependence on opioids. It has been known that H-7 inhibits cAMP-dependent protein kinase and protein kinase C (Garland et al., 1987; Hidaka et al., 1984) and H-8 inhibits cAMP-and cGMP-dependent protein kinase but with

Table 2
Effects of concomitant administration of H-7 or H-8 on naloxone-precipitated withdrawal signs in butorphanol-dependent rats

Withdrawal signs	Saline	H-7 (nmol/μl/h for 3 days)		H-8 (nmol/μl/h for 3 days)
		1	10	10
Escape behavior	4/14 #	1/7	0/7	1/7
Wet dog shakes	14/14	6/7	6/7	5/7
Teeth chattering	11/14	2/7 [a]	2/7 [a]	2/7 [a]
Rearing	11/14	4/7	1/7 [a]	2/7 [a]
Locomotion	9/14	6/7	2/7	1/7
Stretching	8/14	0/7 [a]	2/7	2/7
Scratching	12/14	3/7	3/7	3/7
Salivation	5/14	0/7	0/7	1/7
Penis-licking	10/14	4/7	1/7 [a]	1/7 [a]
Ptosis	9/14	2/7	1/7	1/7
Weight loss (> 3%)	8/14	1/7	1/7	0/7 [a]

Rats received i.c.v. infusion of butorphanol (26 nmol/μl/h) for 3 days and were challenged with naloxone (5 mg/kg i.p.) 2 h after the termination of drug infusion. # Numbers denote the number of rats showing positive signs over the total number of rats tested. [a] $P < 0.05$, values are significantly lower than the control values as determined by the chi-square test.

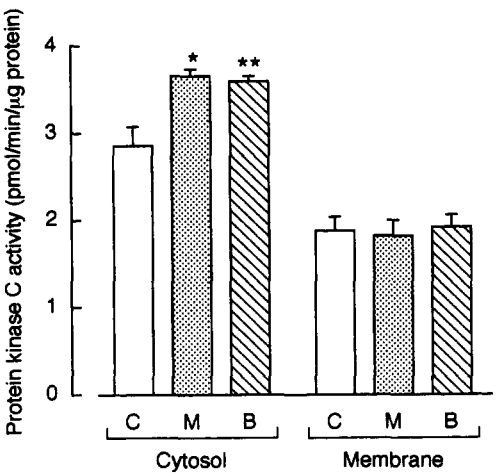

Fig. 1. Protein kinase C activity in cytosolic and membrane fractions in the pons/medulla region of rats continuously infused with morphine or butorphanol. Animals were treated with i.c.v. infusion of morphine (M, 26 nmol/μl/h), butorphanol (B, 26 nmol/μl/h) for 3 days, and killed 6 h after the termination of the drug infusion. Control group (C) was infused with saline (1 μl/h) instead of drugs. Values are the means ± S.E.M. of the data obtained from 6–8 different pooled samples assayed in duplicate. * $P < 0.05$, ** $P < 0.01$, compared with the saline-treated group (Newman-Keuls multiple comparison test).

much less inhibition of protein kinase C activity (Hidaka et al., 1984). These results suggest that the induction of wet dog shakes may not be mediated through a protein kinase-implicated mechanism. At the present time, it is difficult to differentiate which steps are inhibited by these protein kinase inhibitors, i.e. the primary effect of opioids, the development of dependence and/or the expression of physical dependence, under this experimental condition. However, one of these problems could be best solved by the findings, in which concomitant infusion of H-7 inhibited the development of tolerance to the antinociception of morphine and butorphanol (Narita et al., 1994b). The evidence indicates that H-7, at least, does not influence the primary effect of opioids. This finding, together with that of the present study, strongly suggest that the changes in the activities of protein kinases plays a key role not only in the expression of but also in the development of physical dependence on opioids.

The present results have confirmed our recent findings (Narita et al., 1994a) that upregulation of cytosolic protein kinase C in a specific area may contribute to morphine tolerance/dependence induced by daily i.p. injection of the drug. Thus, cytosolic protein kinase C activity in the pons/medulla region, but not membrane fraction, is increased by chronic treatment with morphine, while the dependence on morphine is developed by the same treatment of rats with this drug. We have further demonstrated that continuous i.c.v. infusion of butorphanol which produced significant withdrawal symptoms after the naloxone challenge also enhanced cytosolic protein kinase C activity in the same region. The earlier study from our laboratory (Narita et al., 1994a) did not show a significant increase in the protein kinase C activity following daily injection of butorphanol (20 mg/kg/day i.p., for 7 days). With this daily injection regimen, the signs of physical dependence on butorphanol were also not been observed (data not shown). In addition, H-7, a protein kinase inhibitor, completely blocked the expression of physical dependence, and H-7 inhibited the enhancement of protein kinase C activity in i.c.v. morphine- or butorphanol-infused animals. These findings provide further evidence that the enhancement of cytosolic protein kinase C activity in the pons/medulla region is closely related to the development and/or expression of physical dependence on these opioids.

Butorphanol is known to act on μ-, δ-, and κ-opioid receptors (Horan and Ho, 1989). The development of dependence on butorphanol has been shown to be mediated through these opioid receptors (Horan and Ho, 1991; Jaw et al., 1993a,b; Oh et al., 1992). Butorphanol also exhibits pharmacological and biochemical differences as well as similarities to the prototype of μ-opioid agonist morphine (Horan and Ho, 1989). The present study indicates that both morphine and butorphanol exhibit similar effects on the protein kinase-mediated system. The interpretation of these results is discussed in the following sections.

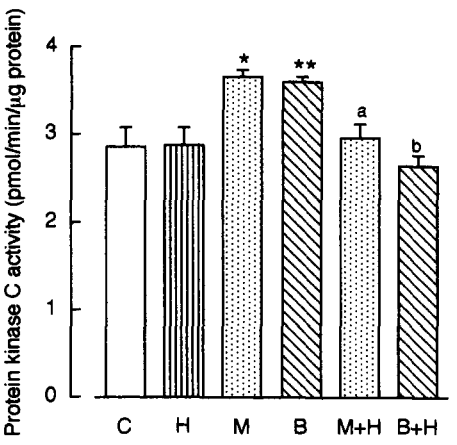

Fig. 2. Effects of H-7 on the enhancement of cytosolic protein kinase C activity in the pons/medulla region of rats continuously infused with morphine or butorphanol. Animals were treated with i.c.v. infusion of morphine (M, 26 nmol/μl/h), butorphanol (B, 26 nmol/μl/h) and/or H-7 (H, 10 nmol/μl/h) for 3 days, and were killed 6 h after the termination of the drug infusion. Control group (C) was infused with saline (1 μl/h) instead of drugs. Values are the means ± S.E.M. of the data obtained from 6–8 different pooled samples assayed in duplicate. * $P < 0.05$, ** $P < 0.01$, compared with saline-treated group, [a] $P < 0.01$, compared with the morphine-treated group, [b] $P < 0.01$, compared with the butorphanol-treated group (Newman-Keuls multiple comparison test).

We have shown that continuous infusion of morphine or butorphanol increases cytosolic protein kinase C activity in the pons/medulla region. However, it is not certain whether the enhancement of protein kinase C activity is due to an increase in the quantity of enzymes in the cytosolic fraction or other changes such that the added activator significantly enhances the activity, since protein kinase C activity was being measured as the quantity of in vitro substrate phosphorylation as stimulated by the addition of an activator such as phorbol ester. Hence, it is still unclear if the mechanism mediated through the increase in cytosolic protein kinase C activity is directly related to the development and/or expression of physical dependence on opioids.

The enhancement of protein kinase C activity through the activation of guanine nucleotide-binding proteins coupled with phospholipase C and/or through activation of the enhancement of cAMP-dependent protein kinase activity could be induced by continuous opioid infusion. In fact, Nestler and Tallman (1988) have reported that chronic treatment of rats with morphine increases protein kinase A activity in the locus coeruleus, which is located in the pons/medulla region, and have suggested that the observed increase in cAMP-dependent protein kinase activity in this area may contribute to the biochemical basis of opioid addiction. Furthermore, it has been reported that cAMP-dependent phosphorylation enhances phosphoinositide synthesis such as inositol 1,4,5-triphosphate and diacylglycerol which are well known protein kinase C activators of phosphatidylinositol and 4-phosphate kinase (Kato et al., 1989). Indeed, in this study, concomitant treatment with a cAMP-dependent protein kinase and protein kinase C inhibitor, H-7, or a cAMP- and cGMP-dependent protein kinase inhibitor, H-8, completely blocked the expression of physical dependence. Furthermore, H-7 inhibited the enhancement of cytosolic protein kinase C activity in the pons/medulla region produced by continuous infusion of opioids. Taken together, the enhancement of cAMP-dependent protein kinase activity in the locus coeruleus following continuous opioid infusion may lead to a compensatory upregulation of protein kinase C activity in the pons/medulla which includes the locus coeruleus.

It is widely believed that the activation of protein kinase C by second messengers involves the translocation of this enzyme, in which elevations of diacylglycerol or applications of phorbol esters cause a shift in the protein kinase C population from the cytosolic to the membrane-bound form (Bell, 1986; Kraft and Anderson, 1983; Kraft et al., 1982). However, the enhancement of membrane-associated protein kinase C activity was not detected in the present study. It is possible that the development of dependence on opioids may not be accompanied by protein kinase C translocation to membranes in the brain and that chronic opioid treatment may lead to the enhancement of cytosolic protein kinase C subspecies.

In conclusion, the findings of the study indicate that various types of protein kinases may play an important role in the development and/or expression of physical dependence on opioids. Among them, the enhancement of cytosolic protein kinase C activity in the pons/medulla region seems to be one of the major underlying mechanisms in opioid physical dependence.

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