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Ca^{2+} channel blocker, diltiazem, prevents physical dependence and the enhancement of protein kinase C activity by opioid infusion in rats

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Abstract

The influence of an L-type Ca^{2+} channel blocker, diltiazem {(2*S-cis*)-3-(acetyloxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one}, on the behavioral signs of naloxone (opioid receptor antagonist)-precipitated withdrawal syndrome and the enhancement of protein kinase C activity in the pons/medulla regions of rats rendered dependent on morphine (μ-opioid receptor agonist) or butorphanol ($\mu/\delta/\kappa$ mixed opioid receptor agonist) was investigated. The expression of physical dependence produced by continuous intracerebroventricular (i.c.v.) infusion of morphine (26 nmol/μl per h) or butorphanol (26 nmol/μl per h) for 3 days, as evaluated by naloxone (5 mg/kg, i.p.)-precipitated withdrawal signs, was dose dependently attenuated by concomitant infusion of diltiazem (10 and 100 nmol/μl per h). Furthermore, diltiazem (100 nmol/μl per h) completely inhibited the enhancement of cytosolic protein kinase C activity in the pons/medulla regions in rats rendered dependent by continuous infusion with morphine or butorphanol. These results suggest that the augmentation of intracellular Ca^{2+} concentration mediated through L-type Ca^{2+} channels during continuous opioid infusion leads to the enhancement of cytosolic protein kinase C activity in the pons/medulla region which is intimately involved in the development and/or expression of physical dependence on opioids.

Keywords: Opioid dependence; Central nervous system; Ca^{2+} channel; Diltiazem; Protein kinase C; Morphine; Butorphanol

1. Introduction

Several studies have shown that changes in neuronal Ca^{2+} availability may be involved in opioid dependence since Ca^{2+} and inorganic Ca^{2+} blockers can modulate the withdrawal syndrome from opioids (Bhargava, 1978; Harris et al., 1977; Sanghvi and Gershon, 1976). Presently, Ca^{2+} channels are classified into three electrophysiologically and pharmacologically different types: L-, T-, and N-type (Miller, 1987; Nowycky et al., 1985; Tsien et al., 1988). It has been noted that L-type Ca^{2+} channel plays an important role in the expression of the withdrawal syndrome from various centrally acting drugs, such as ethanol (Little et al., 1986; Littleton et al., 1990; Watson and Little, 1994), benzodiazepines (Dolin et al., 1990), clonidine (Barrios et al., 1993), and opioids (Baeyens et al., 1987; Bongianni et al., 1986). In addition, this channel also participates in opioid withdrawal expression in the isolated ileum (Barrios and Baeyens, 1988; Valeri et al., 1990). Recently, Narita et al. (1994) have reported that daily injection of rats with morphine (μ-opioid receptor agonist) leads to the enhancement of cytosolic protein kinase C in the pons/medulla region, but not membrane fraction, and suggested that upregulation of cytosolic protein kinase C in specific area may contribute to morphine tolerance/dependence. Furthermore, we have confirmed that protein kinase C activity in the same area is also increased in rats rendered dependent by continuous intracerebroventricular (i.c.v.) infusion with morphine or butorphanol ($\mu/\delta/\kappa$ mixed opioid receptor agonist) (manuscript in preparation). It is widely accepted that protein kinase C, which is highly concentrated in the brain, is a Ca^{2+} dependent regulatory enzyme activated by diacylglycerol (Nishizuka, 1986, 1992), and is an important third

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messenger in the regulation of neuronal excitability, signal transduction, and synaptic plasticity (Kikkawa et al., 1982; Nishizuka, 1988). Accordingly, it is important to examine the influence of Ca^{2+} channel blocker on the enhancement of protein kinase C activity by continuous infusion of opioids in order to elucidate the mechanism of the development and/or expression of physical dependence on opioids. Interestingly, butorphanol has multiple actions on the opioid receptor systems, including μ-, δ-, and κ-opioid receptors (Horan and Ho, 1989, 1991; Jaw et al., 1993a,b; Oh et al., 1992; Pircio et al., 1976). As most studies of opioid dependence have focused on morphine, the use of butorphanol should be valuable in studies to evaluate the mechanism of the development and/or expression of physical dependence on opioids. Therefore, the present study is designed to elucidate the relationship of Ca^{2+} and protein kinase C activity in the development of dependence on opioids. The effects of concomitant infusion of diltiazem {an L-type Ca^{2+} channel blocker; (2*S-cis*)-3-(acetyloxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one} and opioid on the behavioral signs of naloxone (opioid receptor antagonist) precipitated withdrawal syndrome and the enhancement of protein kinase C activity in animals rendered dependent on morphine or butorphanol by continuous i.c.v. infusion with the opioid were investigated.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 230–250 g (Charles River, Wilmington, MA) were purchased and housed in a group of three or four 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) 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-AS, Pfizer Corp., New York, NY), s.c., to prevent infection and were allowed at least 1 week to recover before commencing the infusion of morphine-HCl (Sigma Chemical Corp., St. Louis, MO), butorphanol-tartrate (17-cyclobutylmethyl-3,14-dihydroxy morphinan; a generous gift from Bristol-Myers-Squibb Corp., Evansville, IN) and/or diltiazem-HCl {(2*S-cis*)-3-(acetyloxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one; Sigma Chemical Corp., St. Louis, MO}.

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 per h), butorphanol (26 nmol/μl per h), and/or diltiazem (10 or 100 nmol/μl per h) for 3 days through osmotic minipumps (Alzet 2001, Alza Corp., Palo Alto, CA). 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 opioids and diltiazem, 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) 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) 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), 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 fraction

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 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 100 000 × g for 30 min to yield supernatant and pellet fractions. The supernatant contained the cytosolic fraction. This cytosolic fraction was 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]adenosine 5'-triphosphate (ATP, 5000 Ci/mmol; Amersham, Arlington Heights, IL) to the threonine group on a specific synthetic peptide (Amersham Protein Kinase C enzyme assay system RPN 77, Arlington Heights, IL). 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 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/paper), transferred to vials, and counted in a Packard liquid scintillation analyzer (2200 CA, Grove, IL). All assays were conducted in duplicate. Results were expressed as pmol ^{32}P incorporated into peptide/min per μg protein.

Protein concentrations were determined by the microassay method of Bradford (1976) (Bio-Rad Protein Assay, Hercules, CA) 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 were compared by the chi-square test. In the case of measurement of protein kinase C activity, the data were presented as means ± 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

Table 1 and Table 2 demonstrated that the continuous i.c.v. infusion of morphine (26 nmol/μl per h) or butorphanol (26 nmol/μl per h) for 3 days induced physical dependence, manifested as withdrawal syndrome (escape behavior, wet dog shakes, teeth chattering, rearing, locomotion, stretching, scratching, salivation, penis-licking, ptosis, and body weight loss) when naloxone, 5 mg/kg, was injected i.p. 2 h after the termination of drug infusion. In saline or diltiazem continuously infused control animals, naloxone did not precipitate withdrawal syndrome. Without the injection of naloxone, no obvious withdrawal syndrome was ob-

Table 1
Effects of diltiazem on naloxone-precipitated withdrawal signs in morphine dependent rats

Withdrawal signs	Treatment with diltiazem (nmol/μl per 3 days)		
	0	10	100
Escape behavior	7/14 *	1/7	0/7
Wet dog shakes	14/14	5/7	3/7 [a]
Teeth chattering	14/14	2/7 [b]	0/7 [b]
Rearing	14/14	6/7	4/7 [a]
Locomotion	11/14	5/7	3/7
Stretching	8/14	1/7	0/7 [a]
Scratching	13/14	5/7	1/7 [b]
Salivation	8/14	2/7	0/7 [a]
Penis-licking	11/14	2/7 [a]	0/7 [b]
Ptosis	9/14	3/7	0/7 [a]
Weight loss (> 3%)	13/14	2/7 [a]	0/7 [b]

Rats were treated with i.c.v. infusion of morphine (26 nmol/μl per h) for 3 days and 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.

served in morphine or butorphanol dependent rats (data not shown).

Concomitant treatment with diltiazem significantly inhibited wet dog shakes (100 nmol/μl per h), teeth chattering (10 and 100 nmol/μl per h), rearing (100 nmol/μl per h), stretching (100 nmol/μl per h), scratching (100 nmol/μl per h), salivation (100 nmol/μl per h), penis-licking (10 and 100 nmol/μl per h), ptosis (100 nmol/μl per h), and body weight loss (10 and 100 nmol/μl per h) as compared with that of morphine infused group. Escape behavior and locomotion were tended to be reduced by diltiazem (Table 1). In butorphanol infused animals, wet dog shakes (100 nmol/μl per h), teeth chattering (10 and 100 nmol/μl per h), rearing (100 nmol/μl per h), stretching (100 nmol/μl per h), scratching (100 nmol/μl per h), penis-licking (100 nmol/μl per h), and ptosis (100 nmol/μl per h) were also significantly blocked by concomitant infusion of diltiazem and other signs were also tended to be reduced by diltiazem (Table 2).

3.2. Protein kinase C assay studies

As shown in Fig. 1, the cytosolic protein kinase C activity in the pons/medulla region was significantly increased by i.c.v. infusion of morphine or butorphanol (34.1% and 25.4% increase, $P < 0.05$ over the saline treated group). The enhancement of cytosolic protein kinase C activity in the pons/medulla induced by continuous infusion of morphine or butorphanol was completely prevented by its combination with diltiazem (100 nmol/μl per h). Meanwhile, i.c.v. infusion of diltiazem alone did not affect the protein kinase C activity in this region.

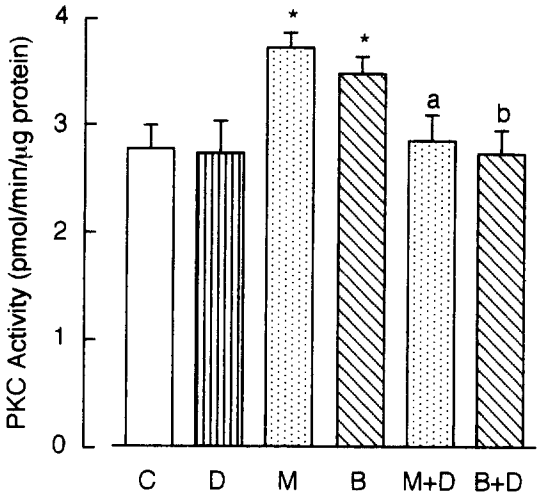

Fig. 1. Effects of diltiazem on the enhancement of cytosolic protein kinase C activity in the pons/medulla regions of rats continuously infused with morphine or butorphanol. Rats were treated with i.c.v. infusion of morphine (M, 26 nmol/μl per h), butorphanol (B, 26 nmol/μl per h) and/or diltiazem (D, 100 nmol/μl per h) for 3 days, and were killed 6 h after the termination of 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 six to eight different pooled samples assayed in duplicate. $^{*}P < 0.05$, compared with saline treated group, $^{a}P < 0.05$, compared with the morphine treated group, $^{b}P < 0.01$, compared with the butorphanol treated group (Newman-Keuls multiple comparison test).

Table 2
Effects of diltiazem on naloxone-precipitated withdrawal signs in butorphanol dependent rats

Withdrawal signs	Treatment with diltiazem (nmol/μl per 3 days)		
	0	10	100
Escape behavior	4/14 *	0/7	0/7
Wet dog shakes	14/14	5/7	4/7 [a]
Teeth chattering	11/14	2/7 [b]	0/7 [b]
Rearing	11/14	4/7	1/7 [a]
Locomotion	9/14	4/7	2/7
Stretching	8/14	1/7	0/7 [a]
Scratching	12/14	4/7	1/7 [b]
Salivation	5/14	2/7	0/7
Penis-licking	10/14	4/7	1/7 [a]
Ptosis	9/14	2/7	0/7 [a]
Weight loss (> 3%)	8/14	2/7	1/7

Rats were treated with i.c.v. infusion of butorphanol (26 nmol/μl per h) for 3 days and 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.

4. Discussion

The results obtained in this study indicate that a Ca^{2+} channel blocker, diltiazem, inhibits the expression of physical dependence and prevents the enhancement of protein kinase C activity in the pons/medulla regions in rats rendered dependent on morphine or butorphanol by continuous i.c.v. infusion of the drug. In our behavioral study, the appearance of almost all withdrawal signs was significantly blocked by diltiazem. However, some of the withdrawal signs, e.g., escape behavior and locomotion in morphine dependent rats, or escape behavior, locomotion, salivation, and body weight loss in butorphanol dependent rats were not attenuated significantly by diltiazem. This lack of significance is probably due to a low degree of withdrawal behaviors seen in the morphine or butorphanol continuously infused groups.

It is well known that voltage dependent neuronal Ca^{2+} channels are divided into three different types, according to their activation and inactivation characteristics in cultured cells (Nowycky et al., 1985), or different electrophysiological characteristics and pharmaco-

logical sensitivities (Miller, 1987; Tsien et al., 1988). Diltiazem has been shown to suppress Ca^{2+} currents in neurons (Nishi et al., 1983), and belongs to the L-type Ca^{2+} channel blocker (Akaike et al., 1989; Tytgat et al., 1988). Consequently, the antagonism by concomitant continuous infusion of diltiazem with morphine or butorphanol on naloxone-precipitated opioid withdrawal suggests that L-type Ca^{2+} channel activation is involved in the expression of physical dependence on opioids. To the best of our knowledge, it has not been reported that concomitant infusion of Ca^{2+} blocker with opioids blocks withdrawal syndrome from opioids, although it has been reported that withdrawal syndrome is inhibited by single injection of Ca^{2+} blocker right before naloxone challenge (Baeyens et al., 1987; Barrios and Baeyens, 1991; Bongianni et al., 1986). These findings, together with those of the present study, strongly suggest that L-type Ca^{2+} channel activation plays a key role not only in the expression of but also in the development of physical dependence on opioids. Further study is warranted to differentiate between the development and expression of physical dependence on 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). In the present study, diltiazem inhibited morphine or butorphanol withdrawal to a similar degree, suggesting that the development and/or expression of physical dependence on both opioids is mediated through the mutual mechanism which activates the L-type Ca^{2+} channel.

Narita et al. (1994) have reported that daily injection of rats with morphine results in the enhancement of cytosolic protein kinase C activity in the pons/medulla region. Recently, we have further demonstrated that continuous i.c.v. infusion of morphine or butorphanol which produces significant withdrawal symptoms after the naloxone challenge also enhanced the cytosolic protein kinase C activity, but not membrane fraction, in the same region (manuscript in preparation). Accordingly, in this study we focused on the changes of cytosolic protein kinase C activity in the pons/medulla region. It is now well recognized that synergistic interaction between the protein kinase C and Ca^{2+} pathways underlies a variety of cellular responses to external stimuli (Nishizuka, 1986, 1992). Indeed, an L-type Ca^{2+} channel blocker, diltiazem, blocked the enhancement of protein kinase C activity in morphine or butorphanol i.c.v. infused animals. These findings provide evidence that the enhancement of cytosolic protein kinase C activity in the pons/medulla region could result from the influx of extracellular Ca^{2+} through L-type Ca^{2+} channel after continuous infusion of these opioids.

We measured protein kinase C activity as the amount of in vitro substrate phosphorylation stimulated by externally applied activators. Under these conditions, it is not certain that whether the changes we detected are due to an increase in the amount of enzymes in the cytosolic fraction or other changes which lead to an increased activation by added activators. Hence, it is still unclear as to which system in cellular signal transduction is activated by extracellular Ca^{2+} during the development and/or expression of physical dependence on opioids. However, it can be postulated that the enhancement of protein kinase C activity may be mediated through the activation of guanine nucleotide binding proteins coupled with phospholipase C and/or through the activation of protein kinase A activity in which Ca^{2+} is required. Therefore, it is concluded that extracellular Ca^{2+} influx may play an important role in activating cytosolic protein kinase C activity in the pons/medulla region.

It has been reported that μ-opioid potentiates glutamate response mediated through the NMDA receptor by activating protein kinase C in dorsal horn neurons in the spinal cord (Chen and Huang, 1991). In addition, Trujillo and Akil (1991) have reported that the development of tolerance to and dependence on morphine is inhibited by a non-competitive NMDA receptor antagonist, MK-801, which acts on Ca^{2+} channel. Taken together, sustained potentiation of NMDA receptor-responses mediated through the activation of protein kinase C by extracellular Ca^{2+} influx exhibits a significant role in the development of tolerance to and dependence on opioids.

Earlier study from our laboratory (Zhang et al., 1994) has shown that naloxone-precipitated morphine withdrawal increases glutamate release in locus coeruleus which locates in the pons/medulla region. Furthermore, it has been reported that chronic treatment of rats with morphine increases protein kinase A activity in locus coeruleus (Nestler and Tallman, 1988), suggesting that the observed increase in protein kinase A activity in this area may contribute to the biochemical basis of opioid addiction. Accordingly, it is now worthwhile to examine the involvement of protein kinase C and/or intracellular Ca^{2+} on the release of glutamate in locus coeruleus using opioid dependent animals.

In conclusion, the results of the present study suggest that the activation of L-type Ca^{2+} channel is involved in the development and/or expression of physical dependence on opioids. Further, the augmentation of intracellular Ca^{2+} concentration in neurones of the pons/medulla region during continuous opioid

infusion would lead to the enhancement of cytosolic protein kinase C activity which closely related to the development and/or expression of physical dependence on opioids.

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