EFFECT OF NMDA RECEPTOR ANTAGONISTS ON PROTEIN KINASE ACTIVATED BY CHRONIC MORPHINE TREATMENT

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ABSTRACT — In a previous study we indicated the involvement of the N-methyl-D-aspartate (NMDA) receptor in the development of morphine dependence as assessed by naloxone-induced rise in norepinephrine release in chronically morphine-treated rats. In the present experiments, we studied (1) the possible role of protein kinases in the increased norepinephrine release occurring after naloxone injection and (2) the effects of NMDA receptor antagonists on chronic morphine exposure-induced changes in protein kinase activity.

The naloxone-induced rise in norepinephrine release was attenuated by concomitant administration of a protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride (H-7) or an NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]-cyclohepten-5,10-imine hydrogen maleate (dizocilpine, MK-801) with morphine.

Both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), which mediate neurotransmitter release, were clearly activated in the cytosol of the pons/medulla, but not in that of the hippocampus, in chronically morphine-treated rats. This activation of PKA and PKC by chronic morphine treatment was inhibited by infusion of dizocilpine or D(-)-2-amino-5-phosphonopentanoic acid (AP-5), an ionotropic glutamate receptor antagonist, together with morphine.

These results suggest that NMDA receptor antagonists inhibit the increase in protein kinase activity produced by chronic morphine treatment, thus suppressing the naloxone-induced rise in norepinephrine release.

KEY WORDS : Morphine dependence, Protein kinase, Pons/medulla, Hippocampus, Norepinephrine release, NMDA antagonist

INTRODUCTION

Repeated administration of narcotic drugs for pain relief leads to the development of tolerance to and physical dependence upon them, and has resulted in their clinical utilization being limited. Tolerance refers to the reduction in potency of a drug on repeated administration, while dependence refers to the physical and/or psychological symptoms that occur when

administration of the drug is suddenly stopped or its antagonist is administered.

Recently, the mechanism by which chronic opiate treatment induces a state of tolerance and dependence in target neurons has been investigated. Chronic treatment with opiates has been shown to result in tolerance to the inhibitory effect on neuronal activity in the locus coeruleus (LC), while withdrawal of chronic opiate treatment is associated with an increase in electrical (Akaoka and Aston-Jones, 1991; Rasmussen et al., 1990) and metabolic activity (Kimes et al.,

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1990) in noradrenergic neurons. However, the mechanisms by which chronic opiate treatment induces a state of tolerance and dependence in target neurons remains largely unknown, in spite of extensive interest in the underlying neuronal intracellular processes.

Recent studies have indicated the involvement of the N-methyl-D-aspartate (NMDA) receptor in opiate tolerance (Marek *et al.*, 1991, Trujillo and Akil, 1991), dependence (Trujillo and Akil, 1991), and other phenomena involving plasticity in the nervous system (Linden *et al.*, 1988; Mao *et al.*, 1992). We have previously demonstrated that naloxone induces an immediate and long-lasting increase in hippocampal norepinephrine release in chronically morphine-treated rats, and that concurrent administration of dizocilpine, an NMDA receptor antagonist, attenuates this naloxone-induced rise in norepinephrine release (Makimura *et al.*, 1996). Therefore, it is considered that this sustained rise in hippocampal norepinephrine following naloxone injection is due to the enhancement of subcellular components such as cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC), which mediate release of neurotransmitters. So we examined the effect of chronic treatment with morphine or/and NMDA receptor antagonists on these protein kinases.

The aims of the present study were to elucidate the role of intracellular protein kinases in the sustained naloxone-induced rise in norepinephrine release and to investigate the mechanism underlying the inhibitory effect of an NMDA receptor antagonist on the increase in norepinephrine release in chronically morphine-treated rats.

MATERIALS AND METHODS

Animals

Seven-week-old male Sprague-Dawley rats (210–250 g) were purchased from Sankyo Laboratory Service, Inc. (Shizuoka, Japan). The animals were housed in a room maintained at $21 \pm 2^\circ\text{C}$ with a 12 hr light-dark cycle and had free access to food and water.

Chemicals

Morphine hydrochloride was purchased

from Sankyo Co. Ltd. (Tokyo, Japan), naloxone hydrochloride, kemptide (a phosphate acceptor peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly), leupeptin, benzamidine, phenylmethylsulfonyl fluoride (PMSF), adenosine triphosphate hydrochloride (ATP) and cyclic adenosine monophosphate (cyclic AMP) from Sigma Chemical Co. Ltd. (St Louis, MO, USA), and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride (H-7), (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]-cyclohepten-5,10-imine hydrogen maleate (dizocilpine, MK-801) and D(-)-2-amino-5-phosphonopentanoic acid (AP-5) from Research Biochemicals International (Natick, MA, USA). Protein kinase C enzyme assay system RPN 77A and [γ - ^{32}P]ATP (5000 Ci/mmol) were purchased from Amersham, Arlington Heights, IL, USA. Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All reagents were of analytical grade.

Procedures for surgery, drug administration, and norepinephrine analysis

Surgery for cannula implantation, drug administration and norepinephrine analysis were carried out as described in a previous report (Makimura, *et al.*, 1996), except that the dosage of morphine was 25 nmol/ $\mu\text{l/hr}$. Briefly, an indwelling stainless steel guide cannula was stereotactically implanted into the lateral cerebral ventricle according to a brain map (Paxinos and Watson, 1986) and was used to infuse morphine. In some experiments, an additional guide cannula through which a microdialysis probe was inserted was implanted into the ventral hippocampus and cemented in place. After surgery, the rat was allowed at least 4 days to recover before the morphine infusion began. Saline or 25 nmol/ μl morphine hydrochloride was infused into the intracerebral ventricle (i.c.v.) of each animal at a rate of 1 $\mu\text{l/hr}$ for 72 hr via an osmotic minipump (Alzet 2001; Alza Corp., Palo Alto, CA, USA). The rats with only the infusion cannula were subjected to enzyme assay, while those in which an additional cannula had been implanted in the hippocampus underwent norepinephrine analysis.

For the norepinephrine analysis, a 3 mm concentric dialysis probe was inserted into the hippocampus 2 hr before the termination of the

morphine or saline infusion. This was continuously perfused (2 μ l/min) with Ringer's solution. Successive 40 μ l samples were collected at 20-min intervals and were analyzed immediately for norepinephrine using high-performance liquid chromatography with an electrochemical detection system.

Enzyme preparation

The rats were anesthetized with ether and decapitated 5 hr after termination of the drug infusions. The brains were removed rapidly and cooled immediately in ice-cold 50 mM Tris-HCl buffer (pH 7.5) including 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 0.3 % (w/v) β -mercaptoethanol, 0.28 mM PMSF and 10 mM benzamidine (Buffer A). Coronal sections, 3 mm thick, were obtained at the level of the LC (excluding the cerebellum) and the hippocampus. The isolated brain regions were homogenized in the same buffer using a Polytron and centrifuged at 100,000 $\times$ g for 30 min. The resulting supernatants were used as the cytosolic fractions of the tissue specimens after dilution with 50 mM Tris-HCl (pH 7.5). The remaining pellets were sonicated (0.5 mg tissue/100 μ l) in 20 mM Tris-HCl buffer (pH 7.4) including 2 mM EDTA, 1 mM dithiothreitol and 10 μ g/ml leupeptin (Buffer B) and were used as the membrane fractions of the tissue specimens after further dilution with Buffer B. Both the membrane and cytosolic fractions were then assayed to determine protein kinase activity.

Protein kinase assays

The PKA assay used a modification of the method described by Nestler and Tallman (1988). Aliquots of the cytosolic or membrane fractions were incubated for 5 min at 25°C in a final volume of 50 μ l of phosphorylation assay buffer (final concentrations: 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.05% Nonidet P-40, 10 mM dithiothreitol) containing 0.2 mM kemptide and 50 μ M [γ -³²P]ATP (5000 Ci/mmol, Amersham) in the absence or presence of cyclic AMP (5 μ M). Following each incubation period, the phosphorylation reactions were terminated by adding 50

μ l of 75 mM orthophosphoric acid. The phosphorylation mixture was blotted onto 2 $\times$ 2 cm squares of P81 phosphocellulose filter paper (Whatman). Each filter paper square was washed 4 times with 10 ml of 75 mM orthophosphoric acid and air-dried. The ³²P contained in the filter paper squares was then quantified by liquid scintillation spectrophotometry. PKA activity was evaluated after subtracting the amount of phosphorylation obtained in the absence of cyclic AMP from that produced in its presence.

PKC activity was measured using a specific enzyme assay system (Amersham). This assay is a modification of a mixed micelle assay, in which the enzyme is activated by phorbol 12-myristate 13-acetate (PMA). Samples (cytosolic or membrane fraction containing ~1 μ g protein) were incubated for 15 min at 25°C in a mixture (37.5 μ l total volume) containing 50 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM Ca²⁺, 75 μ M synthetic peptide, 3.125 μ l of mixed micelles (8 mol % L-phosphatidylserine, 24 μ g/ml PMA), 50 μ M [γ -³²P]ATP (0.2 μ Ci/assay) and 15 mM MgCl₂. The reaction was stopped by adding 50 μ l of 75 mM orthophosphoric acid. A 62.5 μ l aliquot was then spotted onto a phosphocellulose paper. The paper was washed with 75 mM orthophosphoric acid and counted as for the PKA assay. The results were expressed as pmol of ³²P incorporated into the peptide/min/ μ g protein. Protein concentrations were determined by the microassay method of Bradford (Bio-Rad Protein Assay, Hercules, CA, USA) using γ -globulin as a standard (Bradford, 1976).

It was confirmed that the PKA and PKC activities were inhibited dose-dependently by the protein kinase inhibitor added to each reaction mixture. Data are expressed as mean $\pm$ S.E.M. Student's *t* test or Newman-Keuls multiple comparison test was used for the statistical analysis of the data.

RESULTS

Effect of H-7 on naloxone-induced rise in norepinephrine release

Morphine hydrochloride (25 nmol/ μ l/hr) was continuously infused into the intracerebral

ventricle (i.c.v.) via an osmotic minipump for 72 hr. Mean basal level of hippocampal norepinephrine in the morphine-treated rats was 1.42 ± 0.04 pg/40 μ l sample of dialysate, which was similar to that in the chronically saline-treated rats (1.36 ± 0.03 pg/40 μ l sample). Figure 1 illustrates that injection of naloxone hydrochloride (3 mg/kg, s.c.) induced an immediate and long-lasting (> 120 min) increase in norepinephrine output from the hippocampus in the morphine-treated rats (2.94 ± 0.23 pg/40 μ l sample). However, norepinephrine output in the hippocampus of chronically saline-treated (1 μ l/hr) rats was not affected by naloxone administration (Fig. 1).

In order to clarify the influence of protein kinases on the sustained rise in norepinephrine release after naloxone challenge, H-7 (5 or 10 nmol/ μ l/hr), a protein kinase inhibitor, was infused together with morphine for 72 hr. There was no subsequent increase in the release of norepinephrine after naloxone challenge. Dizocilpine (1 nmol/ μ l/hr) also reduced the naloxone-induced rise in norepinephrine release when it was infused together with morphine for

72 hr. Continuous administration of dizocilpine or H-7 alone had no effect on norepinephrine release following naloxone injection.

Effect of various drugs on protein kinases

The activities of PKA and PKC were measured in cytosolic and membrane fractions prepared from the pons/medulla and hippocampus of rats. Although no difference in PKA activity was observed between the cytosolic and membrane fractions in either the pons/medulla or the hippocampus, PKC activity was higher in the cytosolic than in the membrane fraction, especially in the hippocampal region (Fig. 2).

The effects of chronic morphine treatment on the activities of both PKA and PKC were examined. As shown in Fig. 2, cytosolic PKA and PKC activities in the pons/medulla region increased significantly (approximately 20-40 % increase, $p < 0.05$ vs. saline-treated group) after morphine (25 nmol/ μ l/hr) was infused (i.c.v.) for 72 hr. In contrast, the cytosolic PKA and PKC activities in the hippocampus were not affected by chronic morphine treatment. Chronic morphine treatment also produced no change in the

Fig. 1. Effect of H-7 or dizocilpine administered during morphine infusion on naloxone-induced rise in norepinephrine release in the hippocampus of morphine-treated rats.

Either H-7 or dizocilpine was infused concomitantly with morphine into the intracerebral ventricle (i.c.v.) of each rat for 72 hr through an osmotic minipump. Each point represents the mean $\pm$ S.E.M. obtained from 3-6 rats. * $p < 0.05$, ** $p < 0.01$, significantly different from saline-treated group (Student's *t* test). # $p < 0.05$, ## $p < 0.01$, significant different from morphine-treated group (Student's *t* test). ○, saline (1 μ l/hr); ●, morphine (25 nmol/ μ l/hr); ■, morphine (25 nmol/ μ l/hr) plus dizocilpine (1 nmol/ μ l/hr); ▲, morphine (25 nmol/ μ l/hr) plus H-7 (10 nmol/ μ l/hr).

PKA and PKC activities in the membrane fractions in either the pons/medulla or the hippocampus.

In order to determine whether opioid receptors were involved in the activation of these protein kinases by chronic morphine treatment, naloxone hydrochloride was infused (i.c.v.) together with morphine for 72 hr (Fig. 3). The

enhancement of cytosolic PKA and PKC activity in the pons/medulla produced by chronic morphine treatment was blocked by combination with naloxone. The dosages of naloxone required to block the activation of PKA and PKC were 5 and 10 nmol/ μ l/hr, respectively. However, chronic naloxone treatment alone had no effect on cytosolic PKA and PKC activity in

Fig. 2. PKA and PKC activities in cytosolic and membrane fractions prepared from the pons/medulla and hippocampus of rats.

Morphine (25 nmol/ μ l/hr) was infused into the i.c.v. of rats for 72 hr. Control rats received i.c.v. infusion of saline (1 μ l/hr) over the same period. Rats were killed 5 hr after termination of the infusion and PKA and PKC activities were measured in the cytosolic and membrane fractions of both brain regions. Each column represents results obtained from 4 to 5 rats. Values are expressed as pmol/min/ μ g protein (mean $\pm$ S.E.M.). * $p < 0.05$, significantly different from the control group (Student's *t* test). A, PKA activity; B, PKC activity; $\square$, control group; $\square$, morphine group.

Fig. 3. Reversible effect of naloxone on the enhancement of cytosolic PKA and PKC activity in the pons/medulla region following chronic morphine treatment.

Chronic administration of morphine and/or naloxone was achieved by infusion into the i.c.v. (saline, morphine: 25 nmol, naloxone: 5 or 10 nmol/ μ l/hr) as described for Fig. 2. Results were obtained for four to five independent sets of experiments. Values are expressed as pmol/min/ μ g protein (mean $\pm$ S.E.M.). * $p < 0.05$, significantly different from the control group (Newman-Keuls multiple comparison test). A, PKA activity; B, PKC activity; $\square$, saline (1 μ l/hr); $\square$, morphine (25 nmol/ μ l/hr), $\square$, naloxone (5 nmol/ μ l/hr for PKA, 10 nmol/ μ l/hr for PKC); $\blacksquare$, morphine (25 nmol/ μ l/hr) plus naloxone (5 nmol/ μ l/hr for PKA, 10 nmol/ μ l/hr for PKC).

the pons/medulla.

In order to elucidate the mechanism by which dizocilpine inhibited the naloxone-induced rise in norepinephrine release, its effects were compared with those of AP-5, a competitive NMDA receptor antagonist. Both dizocilpine and AP-5 inhibited the activation of cytosolic PKA and PKC when administered together with morphine (Fig. 4). However, continuous infusion of each antagonist alone for 72 hr did not affect the activity of cytosolic PKA or PKC in the pons/medulla.

DISCUSSION

In the present study, we investigated the possible role of protein kinases in the rise in norepinephrine release following naloxone injection and the effects of NMDA receptor antagonists on chronic morphine exposure-induced protein kinase changes.

Concomitant administration of H-7, a protein kinase inhibitor, with morphine decreased the level of hippocampal norepinephrine release after naloxone challenge (Fig. 1). This finding suggests that the marked and long-lasting increase in hippocampal norepinephrine output after naloxone injection is due to the enhancement of subcellular components such as cyclic

AMP-dependent protein kinase (PKA) and protein kinase C (PKC).

Protein kinases have been reported to be involved in intracellular responses following activation of opioid receptors and the transduction mechanism (Childers, 1991). These enzymes have also been suggested to play an important role in various neuronal adaptive processes such as long-term potentiation (Malenka *et al.*, 1989), learning (Alkon and Nelson, 1990) and drug addiction (Nestler *et al.*, 1993). Furthermore, it has been described that activation of protein kinases is associated with translocation; for example, PKC is translocated from the cytosolic fraction to the membrane fraction after having been converted to the active form by activators (Ron *et al.*, 1994). In chronically morphine-treated rats, the activities of both PKA and PKC in cytosolic fraction of the pons/medulla region increased significantly compared with the activities in the chronically saline-treated groups, when these activities were measured in terms of the amount of *in vitro* substrate phosphorylation stimulated by externally applied activators (Figs. 2-4). However, we found no change of PKA and PKC activities in the membrane fraction. One possible explanation of the findings is that the enzymes which increase following the chronic morphine treat-

Fig. 4. Inhibitory effects of dizocilpine and AP-5 on the enhancement of PKA and PKC activity in the pons/medulla of chronically morphine-treated rats.

Experiments were carried out under the same conditions as shown in Fig. 3, except that each NMDA receptor antagonist was infused together with morphine. A, PKA activity; B, PKC activity;

□, saline (1 μ l/hr); ▨, morphine (25 nmol/ μ l/hr); ▩, dizocilpine (5 nmol/ μ l/hr for PKA, 1 nmol/ μ l/hr for PKC); ▤, AP-5 (5 nmol/ μ l/hr); ▥, morphine (25 nmol/ μ l/hr) plus dizocilpine (5 nmol/ μ l/hr for PKA, 1 nmol/ μ l/hr for PKC); ▦, morphine (25 nmol/ μ l/hr) plus AP-5 (5 nmol/ μ l/hr).

ment are present in the cytosolic fraction as inactive forms until they are required for the subsequent physiological responses.

Regulation of protein kinase activity by chronic morphine treatment appears to be due to morphine action at opioid receptors, since concomitant administration of naloxone with morphine blocked this effect (Fig. 3). The finding that enhancement of both PKA and PKC activity is produced by chronic morphine treatment is consistent with previous reports (Narita et al., 1994; Nestler and Tallman, 1988). Such enhancement of PKA and PKC activity was found in the pons/medulla, but not in the hippocampus, in which norepinephrine release increased after naloxone challenge. The pons/medulla contains the LC, which is the origin of the central noradrenergic system (Curet and de Montigny, 1988; Foote et al., 1983) and is involved in opioid actions (Akaoka and Aston-Jones, 1991; Kimes et al., 1990; Rasmussen et al., 1990). Although it is not considered that the whole enhancement of both protein kinase activities in the pons/medulla is due to the enhancement of them in the LC, activation of PKA and PKC in the noradrenergic neurons of the LC, which project to the hippocampus (Curet and de Montigny, 1988; Foote et al., 1983), may contribute to the naloxone-induced rise in norepinephrine release in the hippocampus of chronically morphine-treated rats, although no enhancement of protein kinase activity was observed in the hippocampus.

In order to clarify the mechanism underlying the inhibitory action of dizocilpine on the naloxone-induced rise in norepinephrine release in morphine-treated rats, we examined the effects of NMDA receptor antagonists on protein kinases. Interestingly, concurrent administration of dizocilpine or AP-5, an ionotropic glutamate receptor antagonist, with morphine inhibited not only activation of PKC, but also that of PKA. With regard to interactions between opiates, the NMDA receptor and PKC, our results are consistent with those of an earlier study which found that the NMDA receptor antagonist suppresses the increase in PKC produced by chronic morphine treatment (Mao et al., 1995), although we can not explain why the enhancement of both PKA and PKC activities produced

through opioid receptors was blocked by NMDA receptor antagonists. However, since the exact interactions between opiates, the NMDA receptor and PKA are unknown, NMDA receptor-mediated intracellular mechanisms (such as PKA) underlying morphine tolerance/dependence need to be further investigated.

In summary, the present experiments demonstrated an increase in cytosolic PKA and PKC activity in the pons/medulla of chronically morphine-treated rats. This activation of PKA and PKC was blocked by concurrent administration of dizocilpine or AP-5 with morphine, and naloxone failed to sustain the rise in norepinephrine release. These results may provide evidence for the involvement of the NMDA receptor, via the interaction of intracellular components as reflected by the naloxone-induced rise in norepinephrine release, in the expression of morphine dependence.

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