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Implication of the signal transduction pathways in the enhancement of noradrenaline turnover induced by morphine withdrawal in the heart

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Abstract

Our previous studies have shown an enhanced activity of the noradrenergic system in the heart in rats withdrawn from morphine. In the current study, we examined the role of protein kinase A, protein kinase C and Ca^{2+} entry through L-type Ca^{2+} channels in naloxone-precipitated increase turnover of noradrenaline in the right and left ventricle. Chronic pretreatment for 7 days with the selective protein kinase A inhibitor, HA-1004 (*N*-(2′ guanidinoethyl)-5-isoquinolinesulfonamide) concomitantly with morphine significantly antagonized the increase in normetanephrine/noradrenaline ratio (an index of noradrenaline turnover) observed in morphine withdrawn rats. However, the infusion of calphostin C (2-(12-(2-(benzoyloxy)propyl)-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl)-1 methylethy carbonic acid 4-hydroxyphenyl ester, a selective protein kinase C inhibitor) did not modify the morphine withdrawal-induced increase in noradrenaline turnover. In addition, when the selective L-type Ca^{2+} channel antagonist, nimodipine, was infused it diminished the increased in noradrenaline turnover observed after naloxone administration to morphine dependent rats. Taken together, these data might indicate that protein kinase A activity is necessary for the enhancement of noradrenaline turnover during morphine withdrawal and that an up-regulated Ca^{2+} system might contribute to the increase of noradrenaline turnover. The present finding suggests that protein kinase A and Ca^{2+} influx through L-type Ca^{2+} channels might contribute to the activation of noradrenergic system in the heart observed during morphine withdrawal.

Keywords: Morphine withdrawal; Protein kinase A; Protein kinase C; Ca^{2+} channel; Noradrenaline turnover; Heart

1. Introduction

The repeated use of opioids induces adaptive changes in the central and peripheral nervous system leading to the development of tolerance and dependence. Previous studies in our laboratory have demonstrated that the administration of naloxone to morphine-dependent rats produced an increase in the normetanephrine/noradrenaline ratio in the heart (Rabadán et al., 1997a,b, 1998; Milanés et al., 2000). These effects were dependent on α_2-adrenoceptor activation, located at presynaptic sites in the heart (Milanés and Laorden, 2000). In addition, our previous studies demonstrated a marked increase in the heart noradrenergic activity after naloxone-methiodide and *N*-methyl levallorphan administration to morphine-dependent rats suggesting that these adaptive changes could be due to intrinsic mechanisms outside the central nervous system (Milanés et al., 2001).

On the other hand, opioid receptor are coupled by G_i/G_o to intracellular signalling responses by acting on effector molecules such as adenylate cyclase or phospholipase or regulating ion channel function. The protein kinase pathway is now well known as a major pathway for signal transduction from cell surface opioid receptors to nuclear transcriptional activation. Although the μ opioid receptor is negatively coupled to the adenylate cyclase/cAMP-dependent protein kinase A pathway upon acute stimulation (Childers, 1991), the protein kinase A pathway has been shown to be up-regulated in several brain areas with chronic morphine treatment (Nestler, 1992). In addition, previous studies in our laboratory have demonstrated that naloxone administration to morphine-dependent rats leads to an enhancement of cAMP levels in the heart (Milanés et al., 2000). Therefore, up-regulation of the adenylate cyclase/cAMP transduction system is currently the best-characterized potential mechanism for opioid tolerance and dependence. Recently, a number of intracellular pathways have been suggested to

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play a role in opioid tolerance/dependence. The chronic adaptive molecular mechanisms in these phenomena involve protein kinases A, C and the mitogen-activated protein (MAP) kinase and intracellular calcium, which are relevant for a wide variety of cellular regulatory and signalling processes involving protein phosphorylation and gene expression (Nestler and Aghajanian, 1997; Nestler, 1992; Schulz and Höllt, 1998; Van Haasteren et al., 1999).

The present experiments examined whether alterations of protein kinase A, protein kinase C and/or Ca^{2+} influx through L-type Ca^{2+} channels underlie the changes in heart associated with chronic morphine treatment and withdrawal. This was assessed by chronically infusing specific inhibitors of protein kinase A, protein kinase C and selective antagonist of L-type Ca^{2+} channels. We reported the results of a series in an attempt to elucidate the signalling pathways of morphine withdrawal, which lead to an activation of noradrenergic pathways in the heart.

2. Methods

2.1. Animals and experimental procedure

Male Sprague–Dawley rats (200–210 g at the beginning of the experiments) were housed four to five per cage under a 12-h light/dark cycle (L: 08:00–20:00 h) in a room with controlled temperature (22 ± 2 °C), humidity (50 ± 10%) and food and water available ad libitum. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Local Committee.

Rats were rendered tolerant/dependent on morphine by s.c. implantation of morphine base pellets (75 mg), one on day 1, two on day 3 and three on day 5, under light ether anaesthesia. Control animals were implanted with placebo pellets containing lactose instead morphine on the same time schedule. These procedures have repeatedly been shown to induce both tolerance and dependence as measured behaviourally and biochemically (Rabadán et al., 1997a,b, 1998; Milanés and Laorden, 2000; Milanés et al., 2000). Animals were co-treated for 7 days with Milli-Q water (vehicle), via s.c. implantation of osmotic minipumps (Alzet mod. 2001, 1 μl/h). On day 8, the animals pretreated with morphine or placebo pellets were injected with saline s.c. or naloxone (5 mg/kg s.c.). Rats were observed before and for 30 min after administration of naloxone or saline to determine the existence of withdrawal signs (wet-dog shakes, teeth chattering, salivation, lacrimation, locomotion, rhinorrhea, ptosis and spontaneous jumping). Body weight was determined before and 30 min after opioid receptor antagonist administration. The animals were killed after saline or naloxone administration.

In order to determine the effect of inhibiting protein phosphorylation and to test the involvement of Ca^{2+} in the morphine withdrawal induced an enhancement of noradrenaline turnover in the heart, noradrenaline and its metabolite normetanephrine were determined in tolerant/dependent and naive rats pretreated with inhibitors of protein kinase A, protein kinase C and L-type Ca^{2+} channels, and compared with that observed in tolerant/dependent and naive animals that had not been so treated. Briefly, animals were continuously infused for 7 days, via s.c. osmotic minipumps (Alzet mod 2001, 1 μl/h), with HA-1004 (*N*-(2′ guanidinoethyl)-5-isoquinolinesulfonamide, 40 nmol/day), a protein kinase A selective inhibitor, calphostin C (2-(12-(2-(benzoyloxy)-propyl)-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy -3,10-dioxo-1-perylenyl)-1 methylethy carbonic acid 4-hydroxyphenyl ester (a protein kinase C selective inhibitor; 40 pmol/day) or nimodipine (a specific L-type Ca^{2+} channels antagonist; 48 μg/day). The selection of the drug doses used in this study was based on the IC_{50} value observed for protein kinase A and protein kinase C (Hidaka et al., 1984; Kobayashi et al., 1989) and on the dose that has been demonstrated to be effective in inhibiting some biochemical changes induced during morphine withdrawal (Vargas et al., 1997; Martínez et al., 2001; Cerezo et al., 2002). Minipumps were implanted simultaneously with the chronic morphine or placebo pellets. Pumps were primed for 5 h before implantation at 37 °C in sterile saline in order to obtain an optimal flow rate (1 μl/h). On day 8, a withdrawal syndrome was induced by s.c. injection of naloxone (5 mg/kg).

2.2. Estimation of noradrenaline and its metabolite normetanephrine in the right and left ventricle

Rats were decapitated 30 min after saline (s.c.) or naloxone (5 mg/kg s.c.) administration, the chest was opened with a midsternal incision and the right and left ventricle were dissected and stored immediately at − 80 °C. Noradrenaline and its metabolite normetanephrine were determined by high-performance liquid chromatography (HPLC) with electrochemical detection. Each tissue was weighed, placed in a dry-cooled propylene vial and homogenized with a Polytron-type homogenizer (setting 4 for 50 s) in 1.5 ml perchloric acid (0.1 M). The homogenates were then centrifuged (20,000 rpm, 4 °C, 15 min), the supernatant layer was removed into a 1-ml syringe and filtered through a 0.45-μm filter (Millipore, Bedford, MA) and centrifuged (15,000 rpm, 4 °C, 20 min) again through Ultrafree MC 0.2 (Millipore). From each sample, 10 μl was injected into a 5-μm C_{18} reverse phase column (Waters, Milford, MA) through a Rheodyne (Rheodyne, Cotati, CA) syringe-loading injector 200-μl loop. Electrochemical detection was accomplished with a glass carbon electrode set at a potential of + 0.65 with respect to the Ag/AgCl reference electrode (Waters). The mobile phase consisted of a 95:5 (vol/vol) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), 1-octyl-sodium sulfonate (3.75 mM), di-*n*-butylamine (1 mM), and EDTA (0.135 mM), adjusted to pH 4.3. The flow rate was 0.9 ml/min, and chromatographic data were analysed with Millenium 2010 Chromatography Manager

(Millipore) Equipment. Noradrenaline and normetanephrine were simultaneously detected by the described HPLC method at an elution time of 4.25 and 7.32 min, respectively. Noradrenaline and normetanephrine were quantified by reference to calibration curves run at the beginning and the end of each series of assays. Linear relationships were observed between the amount of standard injected and the peak height measured. The content of noradrenaline and normetanephrine in the right and left ventricle was expressed as n/g wet weight of tissue.

2.3. Drugs and chemicals

Pellets of morphine base (Alcaliber Labs., Madrid, Spain) or lactose were prepared by the Department of Pharmacy and Pharmaceutic Technology (School of Pharmacy, Granada, Spain); noradrenaline bitartrate, normetanephrine (used as a HPLC standards) and naloxone were purchased from Sigma (St. Louis, MO, USA). Naloxone HCl was, dissolved in sterile 0.9% NaCl (saline) and given in a volume of 0.1 ml/100 g). Nimodipine(isopropyl-(2-methoxyenthyl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate) was purchased from Sigma and diluted in polyethylene glycol 400 (PEG); all manipulations were carried under our sodium light. HA-1004 HCl, a protein kinase A-selective inhibitor (Hidaka et al., 1984), was purchased from Sigma and dissolved in Milli-Q (Millipore) sterile water. Calphostin, selective protein kinase C inhibitor (Kobayashi et al., 1989), was purchased from RBI (Natick, MA), dissolved in dimethyl sulfoxide (DMSO) and serially diluted in Milli-Q-water (final concentration of DMSO was 0.06%). Aliquots of the stock solutions were stored at −30 °C until used for experimentation, and those of nimodipine were protected from light at all times. The chronic delivery of nimodipine, HA-1004 and calphostin C was achieved by means of Alzet 2001 osmotic minipumps (Alza, Palo Alto, CA), which

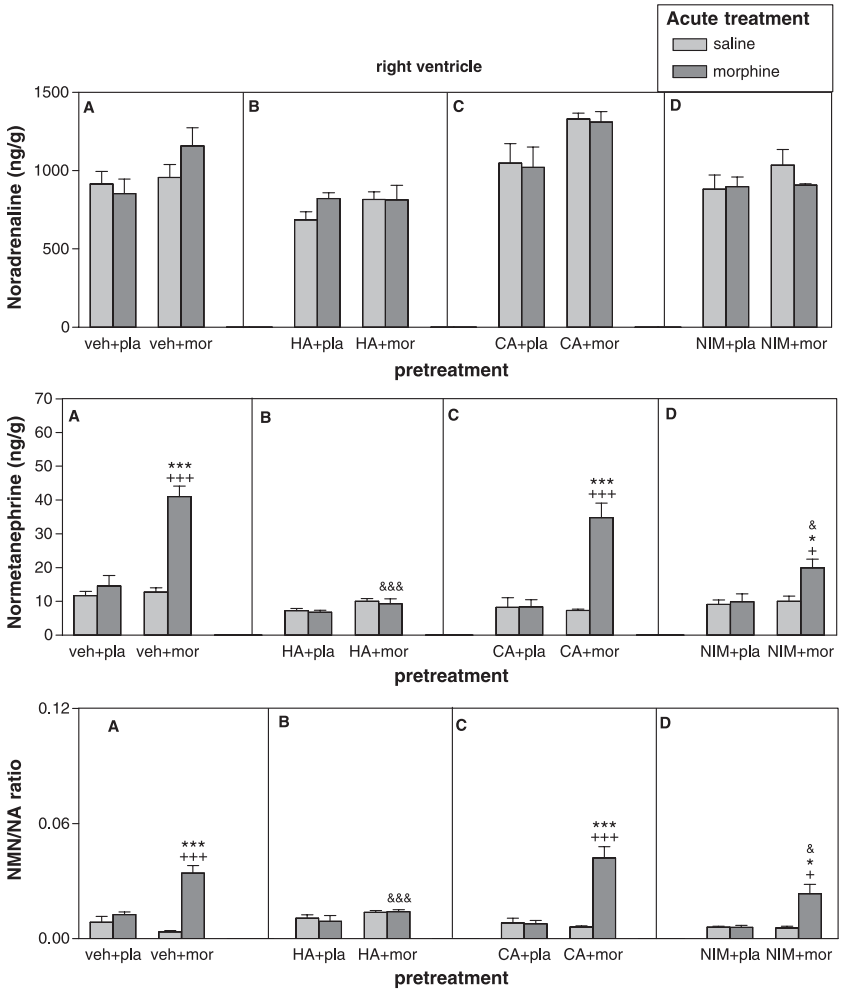

Fig. 1. Content of noradrenaline, normetanephrine and normetanephrine (NMN)/noradrenaline (NA) ratio in the right ventricle after naloxone-precipitated withdrawal in vehicle (veh)-infused rats (A) and in animals chronically administered with HA-1004 (HA) (B),calphostin C (CA) (C) or nimodipine (NIM) (D). Animals received s.c. implantation of placebo (pla) or morphine (mor, 75 mg) pellets for 7 days and concomitantly were infused with veh, HA, 40 nmol/day, CA, 40 pmol/day or NIM, 48 μg/day. On day 8, rats were injected with saline s.c. or naloxone (5 mg/kg, s.c.) and were decapitated 30 min later. Data are the means ± S.E.M. ($n = 5–8$). *$P < 0.05$, ***$P < 0.001$ versus their respective control groups receiving saline instead naloxone; +$P < 0.05$, +++$P < 0.001$ versus group pretreated with placebo instead morphine; &$P < 0.05$, &&&$P < 0.001$ versus animals treated with veh plus mor plus naloxone.

deliver at a rate of 1 μl/h. Drugs were prepare fresh every day. Other reagents were of analytical grade.

2.4. Statistical analysis

All values are expressed as means ± S.E.M. Statistical comparison were done by one-way ANOVA (analysis of variance) followed by the Newman–Keuls test. Student's *t*-test was used when comparing the means of body weight change. Differences with a P value less than 0.05 were considered significant.

3. Results

The weight of each animal was recorded on the day of implantation and on the day of decapitation, before receiving any injection. In all experimental groups, rats treated with morphine showed significantly ($P<0.001$) lower (22.3 ± 2.6 g) body weight gain than animals receiving placebo pellets (52.0 ± 3.1 g). Administration of naloxone (5 mg/kg s.c.) to chronically morphine-treated rats resulted in an important weight loss (16.5 ± 0.4 g, $P<0.001$) when compared with placebo-pelleted groups injected with naloxone (5.0 ± 0.9 g) or saline (4.1 ± 0.6 g). All the animals undergoing morphine withdrawal displayed characteristic abstinence symptoms: wet-dog, shakes, teeth chattering, tremor, piloerection, lacrimation, rhinorrhea, ptosis and spontaneous jumping.

The injection of naloxone in rats chronic pretreated with nimodipine or selective protein kinase A or protein kinase C inhibitors concomitantly with morphine induced a weight loss, similar to that described in the group chronically pretreated with vehicle plus morphine (data not shown).

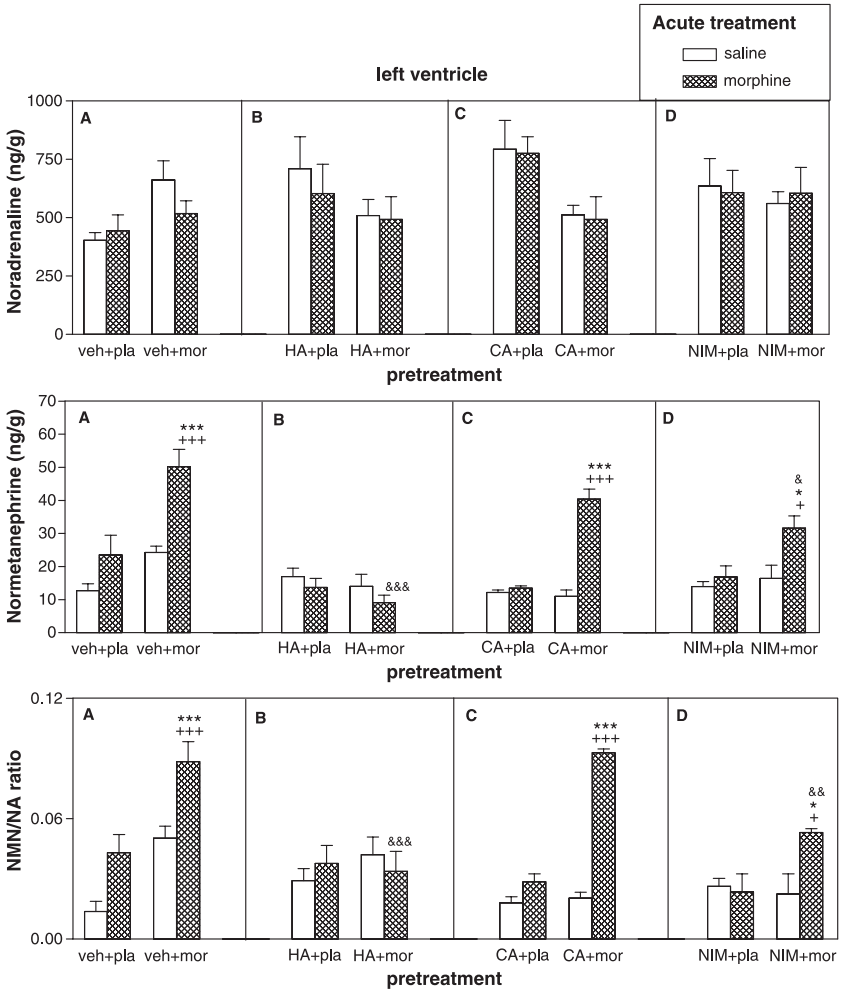

Fig. 2. Content of noradrenaline, normetanephrine and normetanephrine (NMN)/noradrenaline (NA) ratio in the left ventricle after naloxone-precipitated withdrawal in vehicle (veh)-infused rats (A) and in animals chronically administered with HA-1004 (HA) (B), calphostin C (CA) (C) or nimodipine (NIM) (D). Animals received s.c. implantation of placebo (pla) or morphine (mor, 75 mg) pellets for 7 days and concomitantly were infused with veh or HA, 40 nmol/day, CA, 40 pmol/day or NIM, 48 μg/day. On day 8, rats were injected with saline s.c. or naloxone (5 mg/kg, s.c.) and were decapitated 30 min later. Data are the means ± S.E.M. ($n=5–8$). *$P<0.05$, ***$P<0.001$ versus their respective control groups receiving saline instead naloxone; +$P<0.05$, +++$P<0.001$ versus group pretreated with placebo instead of morphine; &$P<0.05$, &&$P<0.01$, &&&$P<0.001$ versus animals treated with veh plus mor plus naloxone.

3.1. Effects of protein kinase A and protein kinase C inhibitors on noradrenaline turnover in the heart

Present data shown that the administration of naloxone to morphine-dependent rats pretreated with vehicle did not induce modifications in the noradrenaline content but the normetanephrine content and the normetanephrine/noradrenaline ratio (an index of noradrenaline turnover) were increased ($P<0.001$) in the right (Fig. 1A) and left ventricle (Fig. 2A) when compared with dependent rats receiving saline instead naloxone or naive rats injected with naloxone. Chronic pretreatment with HA-1004 concomitantly with morphine antagonized ($P<0.001$) the increase in the normetanephrine content and noradrenaline turnover observed during morphine withdrawal in the right (Fig. 1B) and left ventricle (Fig. 2B). The administration of naloxone or saline to placebo rats infused with HA-1004 did not modify the normetanephrine content or the noradrenaline turnover (Figs. 1B and 2B).

Having established that protein kinase A inhibitor antagonized the increased in noradrenaline turnover observed during morphine withdrawal in the heart, we then sought to determine whether increased protein kinase C activity was also responsible for the naloxone-induced increases of noradrenaline turnover in morphine withdrawn rats. For this purpose, the selective protein kinase C inhibitor calphostin C was coadministered with morphine for 7 days. The administration of protein kinase C inhibitor did not alter the morphine withdrawal-induced noradrenaline turnover increase. As shown in Figs. 1C and 2C, the injection of naloxone in morphine dependent rats produced an increase ($P< 0.001$) in normetanephrine content and noradrenaline turnover in rats pretreated with calphostin C in the right and left ventricle compared with those receiving placebo plus naloxone or morphine plus saline. Neither normetanephrine content nor noradrenaline turnover showed any significant modifications in the placebo groups when calphostin C was administered concomitantly with morphine (Figs. 1C and 2C).

3.2. Involvement of Ca^{2+} entry through L-type Ca^{2+} channels on the increased noradrenaline turnover during morphine withdrawal

To determine whether the blockaded of Ca^{2+} can attenuate the increase of noradrenaline turnover in the heart observed in morphine withdrawn rats, we tested the effect of the selective L-type Ca^{2+} channel blocker, nimodipine. The administration of naloxone to rats pretreated with nimodipine concomitantly with morphine induced an increase in the normetanephrine content and noradrenaline turnover in the right ($P<0.05$) and left ventricle ($P<0.05$) when compared to that in the dependent group injected with saline instead naloxone or in placebo rats injected with naloxone. However, the administration of naloxone to rats treated with nimodipine concomitantly with morphine decrease normetanephrine content and noradrenaline turnover in the right ($P<0.05$) and left ($P<0.05$, $P<0.01$) ventricle versus the dependent group chronically infused with vehicle instead nimodipine (Figs. 1A,D and 2A,D). These results indicate that L-type Ca^{2+} channels play a prominent role in the enhancement of noradrenaline turnover in the heart during morphine withdrawal.

4. Discussion

This study was designed to investigate if alteration in the protein kinase pathways and/or Ca^{2+} influx through L-type Ca^{2+} channels underlie the changes in heart associated with chronic morphine treatment and withdrawal. As expected, chronic morphine treatment produced physical dependence, as shown by naloxone-precipitated weight loss and behavioural signs. Additionally, and consistent with previous results obtained in left atria (Rabadán et al., 1997b), right atria (Rabadán et al., 1998) and right ventricle (Milanés et al., 2000), the present study shows that morphine withdrawal leads to an increase in the right and left ventricle of the normetanephrine/noradrenaline ratio (an index of noradrenaline turnover). Importantly, these effects were dependent on adrenoceptor activation: administration of α_2-adrenoceptor blocker antagonized the effects of morphine withdrawal on noradrenaline turnover, which indicates that the increase in the normetanephrine/noradrenaline ratio observed in the heart during morphine withdrawal is mediated by α_2-adrenoreceptor activation (Milanés and Laorden, 2000). On the other hand, our group have demonstrated an increase in the cardiac normetanephrine/noradrenaline ratio after naloxone-methiodide and *N*-methyl levallorphan administration to morphine-dependent rats (Milanés et al., 2001). Because quaternary compounds do not readily cross the blood–brain barrier (Milner et al., 1990), these data suggest that the changes in the heart observed during morphine withdrawal are mediated by peripheral mechanisms.

On the other hand, protein phosphorylation represents one of the major intracellular regulatory mechanisms. Protein kinases are primary targets of intracellular second messengers in most signal cascades. These effectors are involved in the regulation of different cellular processes, including gene expression (Taylor et al., 1991). Alterations of both protein kinase A and protein kinase C pathways have been suggested as one of the molecular mechanisms of opioid tolerance and dependence (Nestler and Aghajanian, 1997; Tokuyama et al., 1995). Thus, up-regulation of protein kinase A after chronic use of morphine has been suggested as one of the molecular mechanisms of opioid tolerance and addiction (Nestler and Aghajanian, 1997). In addition, recent reports have demonstrated that chronic treatment with morphine enhances protein kinase C activity (Tokuyama et al., 1995). Despite substantial evidence that protein kinase A and protein kinase C in the central nervous system are involved in opioid tolerance/dependence, no data are available on the character-

istics of the functional disturbances of the heart protein kinases after chronic morphine treatment and upon drug withdrawal.

The present data indicate that chronic inhibition of protein kinase A (with the selective protein kinase A inhibitor, HA-1004) concurrently with morphine treatment significantly blocks the enhancement of noradrenaline turnover during morphine withdrawal in both the right and left ventricle. Previous study in our laboratory have demonstrated that withdrawal from morphine is associated with a marked increase in the ventricular levels of cAMP and suggest that the up-regulation of cAMP is critical to the development and expression of physical dependence on opioids (Milanés et al., 2000). In addition, it has been demonstrated that tyrosine hydroxylase is directly phosphorylated by protein kinase A and that phosphorylation regulates tyrosine hydroxylase activity. The enzyme phosphorylated has a higher affinity for the pteridine cofactor accelerating the synthesis of noradrenaline (Kumer and Vrana, 1996). On the other hand, it is known that protein kinase A is up-regulated in the central nervous system during chronic morphine (Nestler and Aghajanian, 1997). Our findings are consistent with previous reports and suggest that protein kinase A activity is necessary for the enhancement of noradrenaline turnover observed during morphine withdrawal in the heart.

Moreover, the results of the present study strongly suggest that the expression of morphine dependence for cardiac increase of noradrenaline turnover involves protein kinase A but not protein kinase C signalling mechanisms. Thus, the inhibition of protein kinase C activity with calphostin C did not modify the increased in normetanephrine/noradrenaline turnover observed during morphine withdrawal in the right or left ventricle. These data do not support a role for protein kinase C-mediated phosphorylation in the morphine withdrawal-induced enhancement of noradrenaline turnover in the heart. However, the present results do not preclude a role for this kinase in other sequelae of morphine dependence. Indeed, several studies have indicated that protein kinase C is involved in opioid tolerance and dependence (Narita et al., 1994; Smart and Lambert, 1996). It is possible that the protein kinase C is differentially affected by morphine withdrawal in the central nervous system and in the peripheral nervous system or that noradrenaline turnover is not a target of protein kinase C during morphine withdrawal at a heart level.

In the present study, we further examined the possible requirement for Ca^{2+} entry in the increase of noradrenaline turnover in morphine withdrawn rats. Ca^{2+} not only regulates neurotransmitter release but also acts as a key mediator of adaptive responses observed during morphine withdrawal. The present results indicate that the increase in noradrenaline turnover in the right and left ventricle seen after naloxone-induced withdrawal was significantly attenuated by chronic pretreatment with the L-type Ca^{2+} channel blocker nimodipine, suggesting a role for Ca^{2+} influx in the expression of morphine dependence, as estimated by noradrenaline turnover. Our data agree with the finding that chronic administration of Ca^{2+} channel blockers can attenuate the expression of dependence (Kochuvelikakam and Levine, 1997; Martínez et al., 2001). Recent reports have shown that activation of opioid receptors can augment several components of neuronal Ca^{2+} signalling pathways and, as a consequence, enhance the intracellular Ca^{2+} signal, which can contribute to the long-term effects of opioids (Przewlocki et al., 1999).

In summary, present data suggest that the increase in the cardiac noradrenaline turnover observed during morphine withdrawal might be under the control of protein kinase A and indicate that changes in protein kinase A activity are closely related to the expression of dependence on morphine in the heart. We further showed that the enhancement of noradrenaline turnover after naloxone-precipitated morphine withdrawal also appears to require Ca^{2+} influx through L-type Ca^{2+} channels.

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