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Morphine withdrawal-induced c-*fos* expression in the heart: a peripheral mechanism

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Abstract

We previously demonstrated that hyperactivity of cardiac noradrenergic pathways observed during morphine withdrawal is mediated by peripheral mechanisms. In the present study, naloxone methiodide (quaternary derivative of naloxone that does not cross the blood–brain barrier) and naloxone were administered to morphine-dependent rats and Fos immunostaining was used as a reflection of neuronal activity. Dependence on morphine was induced by 7-day chronic subcutaneous (s.c.) implantation of six morphine pellets (75 mg). Morphine withdrawal was precipitated by administration of naloxone methiodide (5 mg/kg, s.c.) or naloxone (5 mg/kg, s.c.) on day 8. Using immunohistochemical staining of Fos, present results indicate that the administration of naloxone methiodide or naloxone to morphine-dependent rats induced marked Fos immunoreactivity within the cardiomyocyte nuclei. Moreover, Western blot analysis revealed a peak expression of c-*fos* in the right and left ventricles after naloxone methiodide- or naloxone-precipitated withdrawal. In addition, in the hypothalamic paraventricular nucleus (PVN), Fos expression was increased after naloxone—but not after naloxone methiodide—administration to morphine-dependent rats. These results suggest that the activation of c-*fos* expression observed during morphine withdrawal in the heart is due to intrinsic mechanisms outside the central nervous system (CNS).

Keywords: Morphine withdrawal; Fos; Heart; Hypothalamic paraventricular nucleus; Naloxone; Naloxone methiodide

1. Introduction

Opioid peptides are known to be involved in the control of cardiac function through reflex mechanisms involving the central nervous system (CNS), or by modulation of neurotransmitter release from neurons located within the heart (Holaday, 1983). The discovery that mammalian myocardial cells possess opioid receptors (Jin et al., 1995; Witter et al., 1996) has led to studies aimed at investigating direct myocardial effects due to opioid receptor stimulation and identifying possible intracellular opioid effects.

The repeated use of opioid induces adaptive changes in the central and peripheral nervous system, leading to the development of tolerance and dependence. The effects of opioid and the physical aspects of withdrawal have been associated with changes in noradrenergic transmission in the brain (Nestler, 1992; Maldonado, 1997; Milanés et al., 1998; Fuertes et al., 2000). In addition, previous studies from our laboratory demonstrated that morphine withdrawal increases the turnover of noradrenaline in the heart (Milanés et al., 2000; Rabadán et al., 1998), suggesting that catecholaminergic pathways are involved in the hyperactivity of the autonomic nervous system associated with morphine withdrawal. Moreover, our previous studies (Milanés et al., 2001) demonstrated a marked increase in heart sympathetic activity after naloxone methiodide (a quaternary derivate of naloxone) administration to morphine-dependent rats, suggesting that the changes in the heart observed during morphine withdrawal are mediated by peripheral mechanisms.

On the other hand, morphine dependence exerts long-lasting effects on gene expression in the CNS (Nestler and Aghajanian, 1997; Nestler, 1992; Blendy and Maldonado, 1998). The transcription factor Fos has been altered in several brain areas following morphine withdrawal (Curran et al., 1996; Laorden et al., 2000, 2002) and has been widely used as an indicator of cellular activity (Morgan and Curran, 1991). Cardiac cells have similar excitation–depolarization charac-

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teristics to those of neuronal cells, indicating that the immediate genes may be markers for monitoring cardiac activity. Thus, the immediate genes such as c-*fos* are rapidly induced in the cardiac cells in response to stimuli such as noradrenaline administration (Hannan et al., 1993), immobilization or emotional stress (Ueyama et al., 1996), or ischemia–reperfusion (Mizukami and Yoshida, 1997). Moreover, we previously reported that morphine withdrawal induces the expression of Fos protein in the heart (González-Cuello et al., 2003). Studies involving Fos expression have not established whether the adaptive cardiac changes observed during morphine withdrawal are mediated peripherally or centrally. The administration of quaternary derivatives of opiates or their antagonists has been used to distinguish between central and peripheral actions of opiates (Milne et al., 1990).

The purpose of the present study was to determine whether the changes in c-*fos* expression observed after naloxone-induced withdrawal are mediated via a central or peripheral locus of action. To demonstrate the role of central or peripheral opioid receptor pathways, we used naloxone methiodide (a quaternary derivate of naloxone), which possesses a positively charged nitrogen and does not cross the blood–brain barrier (Milne et al., 1990).

2. Materials and methods

2.1. Animals and treatments

Male Sprague–Dawley rats (220–240 g at the start of the experiments) were housed four to five per cage under a 12-h light/dark cycle (L: 0800–2000 h) in a room with controlled temperature (22 ± 2 °C) and humidity (50 ± 10%), and food and water available ad libitum, and prehandled for several days preceding the experiment to minimize stress, as previously described (Laorden et al., 2000). All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and those of the local committee.

Rats were rendered tolerant/dependent on morphine by subcutaneous (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 of 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., 1998; Milanés and Laorden, 2000; Milanés et al., 2000). On day 8, animals pretreated with morphine or placebo pellets were injected with saline (s.c.), naloxone methiodide (5 mg/kg, s.c.), or naloxone (5 mg/kg, s.c.). Body weight gain was checked during treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (Berhow et al., 1995). Furthermore, body weight loss was determined as the difference between the weight determined immediately before saline, naloxone methiodide, or naloxone injection and a second determination performed 90 min later.

2.2. Tissue preparation

Rats were sacrificed with an overdose of pentobarbital (100 mg/kg, i.p.) 90 min after administration of saline, naloxone methiodide, or naloxone. Following anaesthesia, rats were perfused through the descending aorta with 300 ml of phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of cold 4% paraformaldehyde in PBS (pH 7.4). Following perfusion, hearts and brains were removed, postfixed in the same fixative, and stored at 4 °C overnight. Free-floating transversals sections (100 μm thick) of the right and left ventricles and free-floating coronal brain sections (50 μm) throughout the rostrocaudal extent of the hypothalamus were obtained using a vibratomo. The hypothalamic sections correspond to plates 25–26 in the atlas of Palkovits and Brownstein (1988), which contain the hypothalamic paraventricular nucleus (PVN; plane of sections posterior to bregma 1.8–2.12 mm) (Paxinos and Watson, 1998).

2.3. Immunohistochemistry

Expression of Fos protein was examined in free sections, which were shaken in PBS for 30 min to remove the fixative and processed for immunohistochemistry (Laorden et al., 2000). Briefly, sections were preincubated for 20 min in absolute methanol plus 30% H_2O_2 to block endogenous peroxidase activity. They were rinsed in PBS twice (15 min each) and treated with NSS–PBS [PBS containing 1% normal swine serum (Dako, Gostrup, Denmark) and 0.5% Triton X-100] for 30 min. All sections were incubated in the primary polyclonal Fos antibody (dilution 1:3000 in NSS–PBS, sc-7202; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 36 h at 4 °C. The antibody was raised in rabbits against a peptide corresponding to amino acids 210–335 mapping at the carboxy terminus of Fos of human origin, which is identical to its corresponding rat sequence. The bound primary antibody was then localized by biotinylated antirabbit immunoglobulin G (IgG) (dilution 1:200 in NSS–PBS; Vector, Burlingame, CA USA), and subsequently with the avidin–biotin complex (ABC kits; Vector) for 1 h each at room temperature. Visualization of antigen–antibody reaction sites used 0.033% 3′ ,3′ -diaminobenzidine (Sigma) and 0.014% H_2O_2 in 0.05 M Tris–HCl buffer for 7 min. The reaction was stopped in PBS. The sections were mounted onto glass slides coated with gelatin, air-dried, dehydrated through graded alcohols, cleared in xylene, and cover-slipped with dibutylphthalate (DPX).

2.4. Quantification of Fos immunoreactivity

Evidence of Fos immunoreactivity in the heart or brain was examined under a light microscope (DMLB; Leica,

Madrid, Spain). Since the distribution of Fos in the heart was not homogeneous, it was impossible to quantify the levels of Fos. The density of Fos-like immunopositive nuclei in the paraventricular nucleus was determined using a computer-assisted image analysis system, Q500 MC (Leica). This system consists of a light microscope (DMB; Leica) connected to a videocamera (Sony 151-AP; Sony, Madrid, Spain) and the image analysis computer. Sections showing a discernible level of Fos immunoreactivity were selected for quantitative image analysis. Based on orientation criteria, the medial parvocellular neurosecretory portion of the paraventricular nucleus was defined once as having identified adjacent boundaries of the posterior magnocellular and periventricular parts (Swanson and Simmons, 1989), and the parvocellular zone was used for estimating Fos immunoreactivity. A square field (129-μm side) was superimposed upon the captured image (× 40 magnification) to be used as a reference area. The area of Fos immunolabelling included in this square was used for estimating immunoreactivity, and the percentage of Fos immunoreactivity was evaluated by means of Fos immunoreactivity area per reference area, which is a square field (129-μm side). The percentage of Fos immunoreactivity of both right and left sides of five to six correlative sections was averaged per animal.

2.5. Tissue preparation for Western blotting analyses

Animals were killed by decapitation under light ether anaesthesia 90 min after naloxone methiodide, naloxone, or saline. Hearts and brains were rapidly removed and fresh-frozen. The right and left ventricles were dissected and both ventricles and the brain were stored immediately at − 80 °C until use. The hypothalamic tissue containing the paraventricular nucleus was dissected according to the technique of Palkovits (1973) and the paraventricular nucleus corresponds to those in plates 25 and 26 in the atlas of Palkovits and Brownstein (1988).

2.6. Western blotting

Paraventricular nucleus and heart samples were placed in homogenization buffer [PBS, 2% sodium dodecyl sulfate (SDS) plus protease inhibitors; Boehringer Mannheim, Germany] and homogenized for 50 s prior to centrifugation at 15,000 rpm for 20 min at 4 °C. The supernatant was boiled (5 m) and total protein concentrations were determined spectrophotometrically using the method of bicinchoninic acid (Wiechelman et al., 1988).The optimal amount of protein to be loaded was determined in preliminary experiments by loading gels with increasing protein contents (25–100 μg) from duplicate samples of two different individuals from each experimental group. Paraventricular nucleus samples containing 30 μg of protein or heart samples containing 50 μg of protein for Fos analysis were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrophoresed, and transferred onto poly(vinylidene difluoride) (PVDF) membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, CA, USA). Similar loading and transfer were ascertained by cutting the lower portion of the blot and staining for total protein with Amido Black. Nonspecific binding of antibodies was prevented by incubating membranes in 1% bovine serum albumin in TBST buffer (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20). The membranes were incubated overnight, at 4 °C, with polyclonal primary antibody c-*fos* (sc-7202; Santa Cruz Biotechnology) at 1:2000 in TBST with BSA. After extensive washings with TBST, the membranes were incubated for 1 h, at room temperature, with peroxidase-labelled secondary antibody (sc-2004; Santa Cruz Biotechnology) at 1:5000 dilution. After washing, immunoreactiviy was detected with an enhanced chemiluminescence (ECL) Western blot detection system (Amersham-Pharmacia Biotechnology, Madrid, Spain) and visualised by Amersham Hyperfilm-ECL. After film scanning, the integrated optical density of the bands was estimated (Scion Image software; Scion, MD, USA) and normalised to the background values. Relative variations between the bands of the problem samples and the control samples were calculated in the same image. Duplicate measurements in three or four different gels for each individual sample were performed. Measurements were in the linear range.

2.7. Drugs

Pellets of morphine base (Alcaliber Laboratories, Madrid, Spain) or lactose were prepared by the Department of Pharmacy and Pharmaceutic Technology (School of Pharmacy, Granada, Spain); naloxone HCl and naloxone methiodide were purchased from Sigma and RBI, respectively. Naloxone and naloxone methiodide were dissolved in sterile 0.9% NaCl (saline). All drugs were administered in volumes of 0.10 ml/100 g body weight.

2.8. Statistical analysis

The mean ± S.E.M. was expressed for values obtained from a minimum of four rats per group. Data were analysed by analysis of variance (ANOVA) followed by the Newman–Keul's post-hoc test. Body weight gain and loss in naive and morphine-dependent rats were analysed by unpaired Student's *t* test. Behaviour patterns were quantified as the ratio between the number of animals exhibiting the sign and the total number of animals observed, and data were analysed nonparametrically using the χ^2 test. Differences with a P value less than 0.05 were considered significant.

3. Results

Before performing the immunodetection assays, we assessed the efficacy of chronic treatment with morphine by mean pellets implantation. For this purpose, the weight of

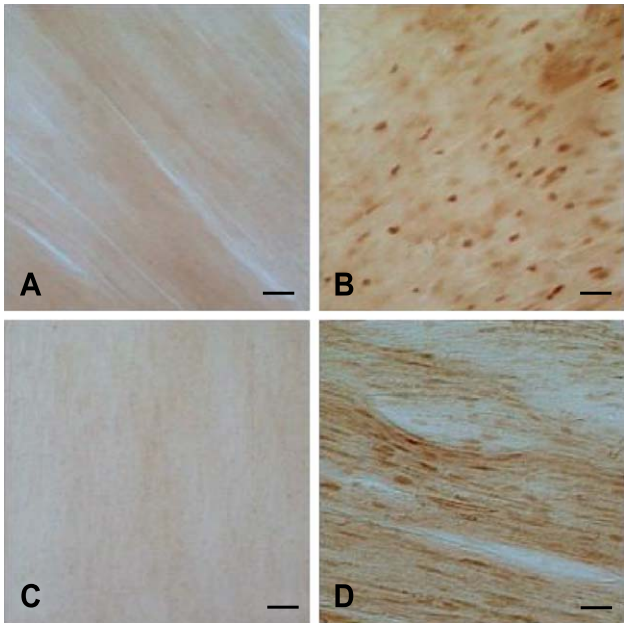

Fig. 1. Photomicrograph of Fos immunoreactivity in the heart (left ventricular wall) 90 min after naloxone methiodide (5 mg/kg, s.c.) or naloxone (5 mg/kg, s.c.) administration to placebo (A and C, respectively) or morphine-dependent rats (B and D, respectively). Results are representative of seven independent experiments. Scale bar, 35 μm.

the animals was recorded on the days of pellets implantation and the day of killing (day 8), before receiving any injection. Rats treated with morphine showed a significant lower ($P<0.001$; t test) body weight gain (17.08 ± 1.1 g, $n=60$) than animals receiving placebo pellets (40.35 ± 1.8 g, $n=68$). Administration of naloxone methiodide or naloxone to control rats resulted in no significant changes in body weight when measured 90 min after drug injection, as compared to control rats receiving saline. However, chronically morphine-treated rats injected with naloxone methiodide or naloxone showed an important weight loss (11.20 ± 0.6 g, $n=20$ and 19.80 ± 1.2, $n=21$, respectively; $P<0.001$) when compared with the placebo-pelleted group receiving NxM or Nx (3.63 ± 0.28 g, $n=23$ and 5.15 ± 0.3 g, $n=23$, respectively) or morphine-pelleted groups injected with saline (4.33 ± 0.7 g, $n=19$). In addition, naloxone caused characteristic abnormal behaviour signs, such as teeth chattering, tremor, piloerection, chromodiacriorrhea, rhinorrhoea, ptosis, wet dog shakes, lacrimation, and spontaneous jumping. Significant ($P<0.001$) total suppression of seven of the nine signs (tremor, piloerection, chromodiacriorrhea, rhinorrhoea, ptosis, wet shakes, and lacrimation) and reduction ($P<0.01$) of spontaneous jumping and teeth chattering were noted in the morphine-dependent group treated with naloxone methiodide.

3.1. c-fos expression after naloxone methiodide- or naloxone-induced withdrawal in the heart

Fos immunoreactivity was examined by immunohistochemistry and Western blot 90 min after s.c. injection of saline, naloxone methiodide (5 mg/kg, s.c.), or naloxone (5 mg/kg, s.c.) to control rats and to animals considered dependent on morphine. The delay of 90 min after drug injection was chosen because it is well known that the peak effect of stimulated Fos is 90–120 min (Morgan and Curran, 1991). The administration of naloxone methiodide to morphine-dependent rats induced expression of Fos in the

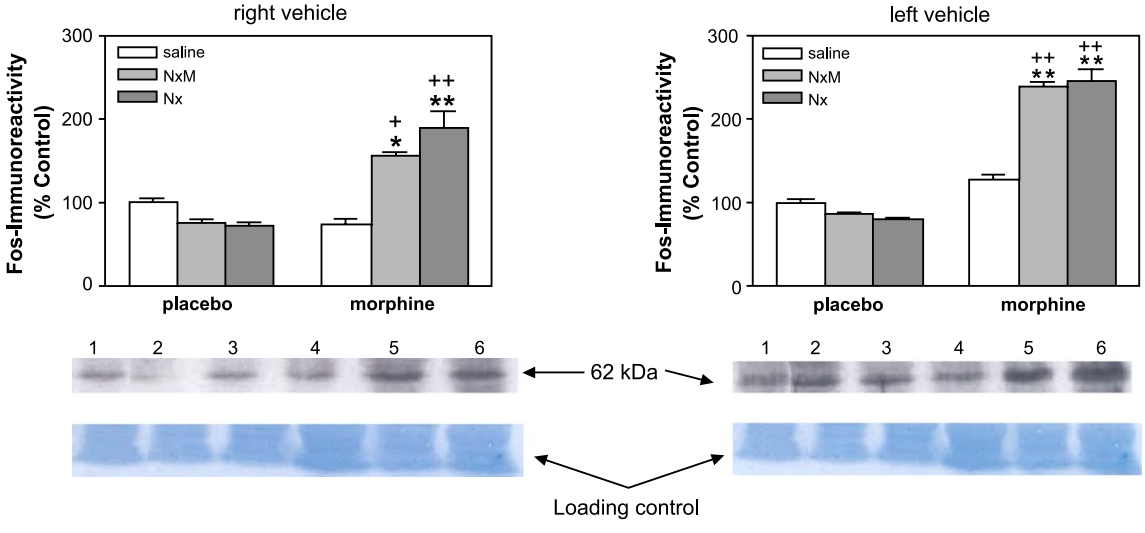

Fig. 2. Western blotting analysis of Fos immunoreactivity levels in the right and left ventricles from rats of the different experimental groups. Comparable loading and transfer were ascertained by cutting the lower portion of the blot and staining for total proteins with Amido Black. Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100% value). Data are expressed as mean ± S.E.M. ($n=4$–5 per group). NxM (naloxone methiodide, 5 mg/kg, s.c.), Nx (naloxone, 5 mg/kg, s.c.). (1) Placebo + saline; (2) placebo + NxM; (3) placebo + Nx; (4) morphine + saline; (5) morphine + NxM; (6) morphine + Nx. *$P<0.05$, **$P<0.01$ versus morphine + saline; $^{+}P<0.05$, $^{++}P<0.01$ versus their respective control groups (placebo + NxM or placebo + Nx).

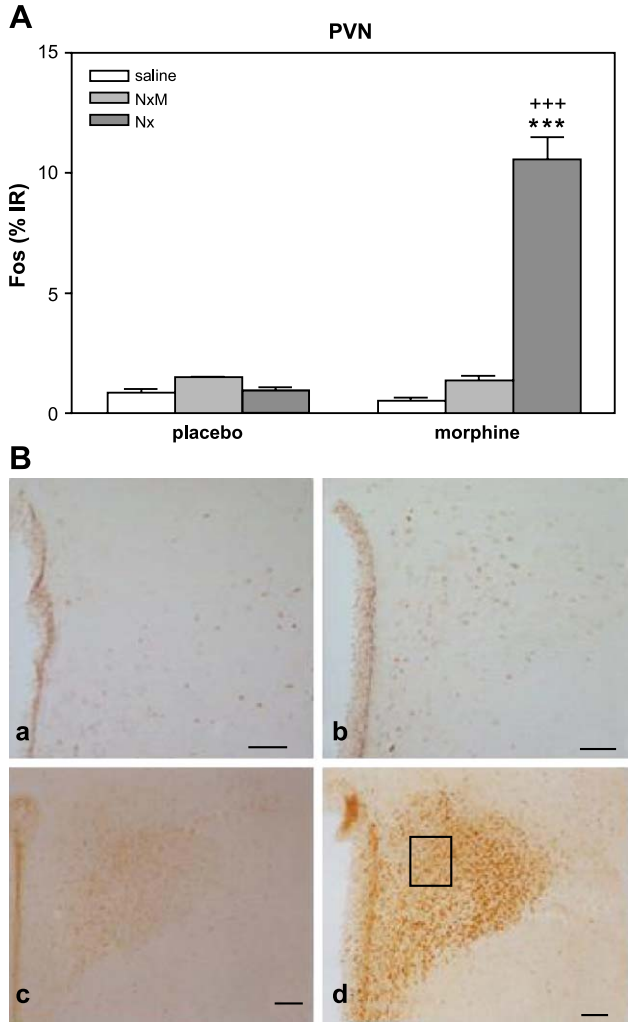

Fig. 3. Quantitative analysis of Fos immunoreactivity (Fos-IR) levels in the hypothalamic PVN of the different experimental groups. (A) Data are expressed as mean ± S.E.M. ($n = 5–6$ per group). NxM (naloxone methiodide, Nx (naloxone). ***$P < 0.001$ versus morphine + saline; $^{+++}P < 0.001$ versus placebo + Nx. (B) Photomicrograph of Fos-IR in the PVN in sections from placebo control rats injected with NxM (a) or Nx (c), or from morphine-dependent rats injected with NxM (b) or Nx (d). Scale bar, 58 μm (a and b) and 100 μm (c and d).

ventricle wall (Fig. 1B). Strong signals were also observed in the ventricular septum and ventricular myocardium. The distribution of Fos in the heart was not homogenous; c-*fos* expression was greatest in the left side of the heart. Similarly, rats rendered dependent on morphine and given naloxone instead of naloxone methiodide showed Fos immunoreactivity in the ventricular wall (Fig. 1D). Fos immunoreactivity was also detected in the ventricular septum and ventricular myocardium in the area surrounding the left ventricular cavity. No c-*fos* expression was observed in placebo-pelleted rats injected acutely with saline, naloxone methiodide (Fig. 1A), or naloxone (Fig. 1C), nor in morphine-pelleted rats injected with saline (data not shown).

In the Western blot analysis performed in the right and left ventricle lysates, the Fos protein was detected in a band located at ~ 62 kDa (Fig. 2). In naive animals injected with saline, naloxone methiodide, or naloxone only, very low levels of Fos immunoreactivity could be detected. In addition, c-*fos* expression was very low in morphine-pelleted rats injected with saline. However, Western blot analysis revealed that naloxone methiodide administration to morphine-dependent rats produced a significant induction of c-*fos* expression in the right ($P < 0.05$) and left ($P < 0.01$) ventricles versus the group of rats treated with saline or placebo rats treated with naloxone methiodide ($P < 0.05$ and $P < 0.01$, respectively). In addition, Western blot analysis showed that administration of naloxone instead of naloxone methiodide also induced a significant expression of c-*fos* in the right and left ventricles versus the morphine-dependent rats injected with saline ($P < 0.01$) or placebo rats injected with Nx ($P < 0.01$) (Fig. 2).

3.2. c-fos expression after naloxone methiodide- or naloxone-induced withdrawal in the PVN

To confirm that naloxone methiodide did not enter in CNS, we investigated the effects of this peripheral opioid antagonist on c-*fos* expression during withdrawal in the PVN by immunohistochemistry and Western blot analyses. Low but detectable levels of Fos immunoreactivity were observed in control groups that received saline, naloxone methiodide, or naloxone (Fig. 3A and B). As shown in Fig. 3A, rats dependent on morphine and given naloxone

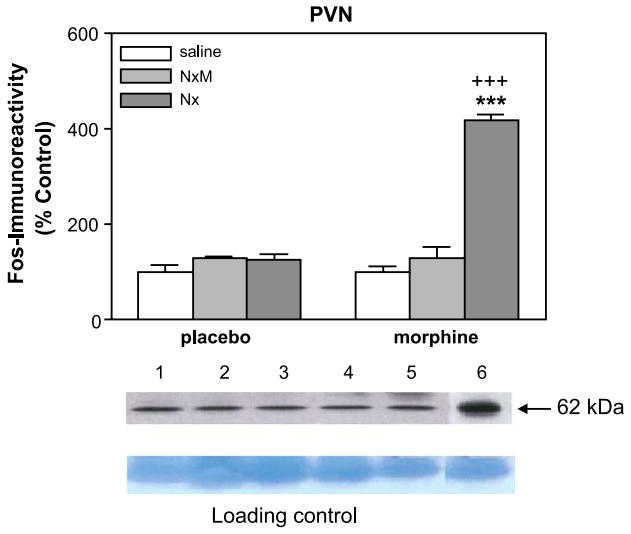

Fig. 4. Western blotting analysis of Fos immunoreactivity levels in the hypothalamic PVN from rats of the different experimental groups. Comparable loading and transfer were ascertained by cutting the lower portion of the blot and staining for total proteins with Amido Black. Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100% value). Data are expressed as mean ± S.E.M. ($n = 4–5$ per group). NxM (naloxone methiodide, 5 mg/kg, s.c.), Nx (naloxone, 5 mg/kg, s.c.). (1) Placebo + saline; (2) placebo + NxM; (3) placebo + Nx; (4) morphine + saline; (5) morphine + NxM; (6) morphine + Nx. ***$P < 0.01$ versus morphine + saline; $^{+++}P < 0.001$ versus placebo + Nx.

methiodide did not show significant changes in Fos immunoreactivity compared with saline administration or placebo rats injected with naloxone methiodide. However, in animals rendered dependent on morphine, significant ($P<0.001$) increases in the levels of Fos were observed after naloxone administration to morphine-dependent rats versus placebo-pelleted rats injected with naloxone or morphine-dependent rats injected with saline (Fig. 3A). Representative photomicrographs are depicted in Fig. 3B.

As shown in Fig. 4, when Fos was determined by Western blot analysis, similar results were obtained. Thus, rats dependent on morphine given naloxone also showed significant ($P<0.001$) induction of c-*fos* expression, compared with the control group also receiving naloxone or with the morphine-dependent group injected with saline. However, c-*fos* expression was not modified after naloxone methiodide administration to morphine-dependent rats.

The administration of higher dose of naloxone methiodide (9 mg/kg, s.c.) to morphine-dependent rats did not induce Fos expression in the PVN.

4. Discussion

As expected, chronic treatment with morphine produced physical dependence, as shown by the behavioural abstinence sings precipitated by the opioid antagonist, naloxone. The present results demonstrated that naloxone methiodide suppressed tremor, piloerection, chromodiacriorrhea, rhinorrhoea, ptosis, lacrimation, and wet dog shakes, and reduced spontaneous jumping, teeth chattering, and loss of weight observed after administration of naloxone (Lookingland et al., 1991). These data agree with previous data from our laboratory (Milanés et al., 2001) and suggest that a central mechanism seems to be important in the mediation of physical opiate dependence in rats. Indeed, previous results have demonstrated the participation of multiple brain sites in physical morphine withdrawal syndrome (Kerr and Pozuelo, 1971; Calvino et al., 1979; Maldonado et al., 1992; Maldonado and Koob, 1993).

The regulation of cellular events through altered expression of proteins signalled by chronic activation of opioid receptors is critical for understanding tolerance and dependence to opioids (Williams et al., 2001). As a first step in studying the effects of morphine dependence on the heart, we examined the expression of Fos protein in the right and left ventricles. Recent studies from our laboratory (González-Cuello et al., 2003) showed that morphine withdrawal induced Fos expression within cardiomyocytes. In agreement with these data, present results showed that the administration of naloxone to morphine-dependent rats induced Fos expression (as revealed by immunohistochemistry and Western blot analysis) in the heart. Despite these reports showing a marked increase in Fos expression in the heart after morphine withdrawal, previous studies have not established whether the adaptive changes observed in the heart during morphine withdrawal were of central or peripheral origin. To determine a possible peripheral component of these adaptive changes, naloxone methiodide was tested. Peripherally administered naloxone methiodide in doses similar to those used in the present study have been shown to produce a significant increase in noradrenaline turnover in the heart during morphine withdrawal (Milanés et al., 2001) and have demonstrated to antagonise several peripheral effects of opioid agonists such as morphine (Milne et al., 1990; Russel et al., 1982). In addition, the same doses of naloxone methiodide (5 mg/kg, s.c.) were used to determine Fos expression in the CNS in morphine-dependent rats (Hamlin et al., 2001).

Our present results show that Fos immunoreactivity was seen in the cardiomyocytes after naloxone methiodide or naloxone administration to morphine-dependent rats. This response was localized in the nucleus, which is the accepted site of action of Fos protein and other transcription factors. Fos immunoreactivity was not detected in any cell types of the heart of naive animals injected with saline, naloxone methiodide, or naloxone, indicating that c-*fos* expression observed in this study is a consequence of naloxone methiodide or naloxone administration to dependent tissues. Western blot analysis revealed low signals in the naive rats, whereas the c-*fos* expression was strongly expressed after naloxone methiodide or naloxone administration to morphine-dependent rats. Accordingly, distribution of Fos immunoreactivity in the naive rats was not virtually detected in the tissue section by immunohistochemistry, whereas some specific immunoreactivity was found when Western blot was applied. This discrepancy may be due to differences in the sensitivity between the two methods. Similar finding have been reported in previous investigations (Larsen et al., 1998).

Recent studies from our laboratory (Laorden et al., 2000) showed that naloxone administration to morphine-dependent rats induced the neuronal expression of Fos protein within neurons of the paraventricular nucleus. In agreement with these data, our present results show that, under identical experimental conditions, a significant increase in the Fos immunoreactivity was seen in the paraventricular nucleus (the major integrative and regulatory centre for many behavioural, autonomic, and neuroendocrine processes). In contrast, administration of naloxone methiodide (quaternary compounds) to morphine-dependent rats did not induce significant changes in the Fos expression. In agreement with these data, a previous study shows that Fos can be induced in the paraventricular nucleus by naloxone—but not naloxone methiodide (5 mg/kg, s.c.)—administration to morphine-dependent rats (Hamlin et al., 2001). Together, all these data suggest that enhancement of Fos levels in the heart observed after naloxone methiodide or naloxone administration to morphine-dependent rats could be mediated by a peripheral mechanism. Consistent with the hypothesis that the cardiac adaptive changes induced by morphine withdrawal are mediated by peripheral mechanisms, our previous data have shown an increase in the noradrenaline turnover in the heart

after naloxone methiodide or levallorphan administration to morphine-dependent rats (Milanés et al., 2001). Other studies have demonstrated that the blockade of opiate receptors outside the CNS by quaternary derivatives of opioid antagonists blocks the infarct limitation of ischemic preconditioning (Chien et al., 1999; Schultz et al., 1997) and also reduces the incidence of ischemia-induced arrhythmias (Murphy and Murphy, 1999). In addition, intrinsic cardiac neurons can function independently of central neuronal input as a ''little brain'' in the heart (Armour, 1991). The activity generated by intrinsic cardiac neurons can be modified by different substances applied locally (Armour et al., 1993; Huang et al., 1993). Taken together, these studies and our results support the hypothesis that CNS participation is not required for the increase in the levels of Fos in the heart observed during morphine withdrawal.

It is known that the repeated use of opioid induces adaptive changes in the central and peripheral nervous system, leading to the development of tolerance and dependence. As Fos protein functions as a transcription factor, which binds to the AP-1 site of various target genes, its induction may modify the expression of other genes and has been implicated in a diverse range of cellular processes (Angel and Karin, 1991). The elevation of Fos in the heart observed in our study could contribute to the expression of target genes within the heart. Genes encoding neurotransmitter-synthesizing enzymes that contain AP-1 sites in their promoter regions (e.g., tyrosine hydroxylase) could be putative targets of c-*fos*-mediated gene expression in the heart induced by morphine withdrawal.

On the other hand, possible mechanisms underlying the cardiac Fos response to morphine withdrawal are not known. However, the fact that heart receiving dense noradenaline innervation and the hyperactivity of heart catecholaminergic neurons observed during morphine withdrawal (Milanés et al., 2000, 2001; González-Cuello et al., 2003) may suggest that noradrenaline could trigger the induction of the transcription factor gene product, Fos. Although the element that mediates c-*fos* induction after morphine withdrawal has not been determined yet, several studies have revealed that c-*fos* expression is induced rapidly after noradrenaline administration (Hannan and West, 1991).

In conclusion, our results demonstrate that naloxone methiodide or naloxone administration to rats treated chronically with morphine enhances Fos expression in the heart and suggest that these changes were due to intrinsic mechanisms outside the CNS. These data may be important for understanding the adaptive changes induced in the heart in subjects dependent on opioids and perhaps allow new perspectives for the therapy of morphine withdrawal syndrome.

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