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Activation of c-*fos* expression in the heart after morphine but not U-50,488H withdrawal

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1 In the present work we have studied in the heart the expression of Fos, the protein product of the c-*fos* proto-oncogene and the adaptive changes in noradrenergic neurons after naloxone or nor-binaltorphimine (nor-BNI) administration to morphine or U-50,488H pretreated rats.

2 Male rats were implanted with placebo (naïve) or morphine (tolerant/dependent) pellets for 7 days. On day 8 rats received saline s.c., naloxone (5 mg kg^{-1} s.c.) or nor-BNI (5 mg kg^{-1} i.p.). Other groups of rats were rendered tolerant/dependent on U-50,488H by injecting the drug twice daily (15 mg kg^{-1} i.p.) for 4 days. Control animals received saline. On day 5 the animals were injected with vehicle i.p. or nor-BNI (5 mg kg^{-1} i.p.).

3 Using immunohistochemical staining of Fos, present results indicate that morphine withdrawal induced marked Fos immunoreactivity (Fos-IR) within the cardiomyocyte nuclei. Moreover, Western blots analysis revealed a peak expression of c-*fos* in right and left ventricle after naloxone induced withdrawal in parallel with an increase in noradrenaline (NA) turnover.

4 However, after nor-BNI administration to rats chronically treated with U-50,488H, we found a decrease in the NA turnover. In addition, the administration of nor-BNI to rats chronically treated with U-50,488H or morphine did not induce modifications in the Fos-IR, in the heart.

5 These results demonstrated that morphine withdrawal induces the expression of Fos protein, as well as an enhancement of noradrenergic activity in the heart. In contrast to morphine U-50,488 withdrawal produces no changes in Fos-IR in parallel with a decrease in NA turnover, indicating that the kappa–opioid receptors are not involved in the molecular adaptive mechanisms responsible for the development of opioid dependence in the heart.

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Keywords: Heart; Fos; noradrenaline turnover; morphine withdrawal; U-50,488H withdrawal; immunohistochemical; western blot; chromatography.

Abbreviations: HPA, hypothalamopituitary-adrenocortical axis; IR, immunoreactivity; nor-BNI, nor-binaltorphimine.

Introduction

The induction of the immediate genes (IEG), c-*fos* and the expression of its protein product, Fos, in the central nervous system have been proposed to reflect second messengers activation, and hence serve as sensitive indicators of cellular responses induced by several kinds of stimuli including opioids (Hughes & Dragunow, 1995). The nuclear Fos protein probably translates transient synaptic events into long lasting changes in neuronal excitability and has been used as an anatomical marker for monitoring neuronal activity (Morgan & Curran, 1991; Hughes & Dragunow, 1995). Cardiac cells have similar excitation–depolarization characteristics to those of neuronal cells, suggesting that IEG may be markers for monitoring cardiac activity. Thus, the IEG such as c-*fos*, are rapidly induced in the cardiac cells in response to stimuli such as norepinephrine administration (Hannan *et al*., 1993), immobilization or emotional stress (Ueyama *et al*., 1996, 1999) or ischaemia-reperfusion (Mizukami & Yoshida, 1997).

As a result of repeated drug use, adaptations in neurons can result in a cascade of cellular events that may ultimately lead to the formation of drug addiction (Nestler *et al*., 1993; Nestler, 1994; Nestler & Aghajanian, 1997; Blendy & Maldonado, 1998). These adaptive responses in neurons occur as a result of changes in gene expression and may involve further alterations in the levels of receptors, ion channels, neurotransmitters or neuromodulators within neurons (Sheng & Greenberg, 1990). In this line it has been demonstrated that upon the development of tolerance to a kappa-opioid receptor agonist the response of the inositol 1,4,5-thrisphosfate (IP_3)/Ca^{2+} to kappa-opioid receptor stimulation were abolished in myocytes of rats had received chronic injection of U-50,488H (Sheng & Wong, 1996).

Previous finding of the present laboratory have demonstrated that morphine withdrawal induces a state of neuronal hyper-excitability in the brain (Milanés *et al*., 1998; Fuertes *et al*., 2000; Laorden *et al*., 2000b) and heart (Rabadán *et al*., 1997a; 1998; Milanés & Laorden, 2000; Milanés *et al*., 2000). Importantly in contrast to morphine, U-50,488H withdrawal produces no changes in the hypothalamus–pituitary–adenocortical (HPA) axis activity or hypothalamic NA turnover (Laorden & Milanés, 2000). In addition, we have

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observed a decrease in NA turnover in the heart after naloxone administration to U-50,488H dependent rats (Milanés & Laorden, 1998).

The transcription factor Fos, in particular, has been shown to be altered in several brain areas following morphine withdrawal (Beckmann *et al*., 1995; Frankel *et al*., 1999; Laorden *et al*., 2002). However, U-50,488H withdrawal was not accompanied by changes in Fos–IR in the central nervous system (unpublished data). Nowadays, the effects of morphine or U-50,488H withdrawal on the induction of Fos protein in the heart have not been established. Therefore, the purpose of the present study was to investigate the possible changes in c-*fos* expression after naloxone or norbinaltorphimine (nor-BNI)-precipitated withdrawal from morphine or U-50,488H, in the heart.

Methods

Male Sprague–Dawley rats (200–210 g at the beginning of the experiments) were housed four–five per cage under a 12 h light/dark cycle (light:8:00–20:00 h) in a room with controlled temperature ($22\pm2^{\circ}$C), humidity ($50\pm10\%$) and food and water available *ad libitum*. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 1986 (86/609/EEC). As stress can affect the expression of IEG and the catecholaminergic activity in the heart, the experimental design included efforts to reduce the potential effect of stress. For that, animals were handled daily for about 5 min (09:00–10:00 h) for at least 3 days before the experimental day in the experimental room to adapt them to manipulation and minimize non-specific stress responses. As the expression of IEG may vary diurnally, the experiments were also designed such as perfusion–fixation or heart removing for catecholamine analysis would be performed between 10:30 and 12:00 h.

Experimental procedure

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 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*., 1997b; 1998; Milanés & Laorden, 2000; Milanés *et al*., 2000). On day 8 the animals pretreated with morphine or placebo pellets were injected with saline s.c., naloxone (5 mg kg^{-1} s.c.) or nor-BNI (5 mg kg^{-1}, i.p.). Other groups of rats were rendered tolerant/dependent on U-50,488H by injecting the drug twice daily (15 mg kg^{-1}, i.p.) for 4 days. This procedure has been shown to induce tolerance/dependence to the different effects of the kappa agonist U-50,488H (Milanés & Laorden, 1998; Laorden & Milanés, 2000). Control animals received saline i.p. on the same time schedule. Groups of rats pretreated with saline or U-50,488H were injected on day 5 with vehicle (Milli-Q water) i.p. or the selective kappa-antagonist nor-BNI (5 mg kg^{-1}, i.p.). Rats were observed before and for 30 min after administration of naloxone, nor-BNI, saline or vehicle to determine the existence of withdrawal signs (wet-dog shakes, teeth chattering, salivation, lacrimation, locomotion, rhinorrhea ptosis and spontaneous jumping). Weight gain was checked during treatment to ensure that morphine or U-50,488H were correctly administered, since chronic opioid treatment induces a decrease in body weight gain as a result of lower caloric intake (Berhow *et al*., 1995). In addition, body weight was determined before and 30 min after opioid antagonists administration. The animals were killed after saline, vehicle, naloxone or nor-BNI administration. The nine experimental conditions were: placebo/saline, placebo/naloxone, morphine/saline, morphine/naloxone (withdrawal), morphine/nor-BNI (withdrawal), saline/vehicle, saline/nor-BNI, U-50,488H/saline, U-50,488H/nor-BNI (withdrawal).

Tissue preparation

Rats were sacrificed with an overdose of pentobarbitone (100 mg kg^{-1} i.p.) 90 min after administration of naloxone or saline. The delay of 90 min after administration of opioid antagonist, saline or vehicle injection was chosen since it was previously demonstrated that the peak effect of stimulated Fos is between 90 and 120 min (Morgan & Curran, 1991). 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 were removed, postfixed in the same fixative and stored at 4°C overnight. Free floating transversals sections (100 μm in thickness) of the right and left ventricle were obtained on a Vibratome.

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 as described previously (Laorden *et al*., 2000a). Briefly, the 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, Glostrup, 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; sc7202, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) 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 corresponding rat sequence. The bound primary antibody was then localized by biotynilated anti-rabbit IgG (dilution 1 : 200 in NSS–PBS, Vector, Burlingame, CA, U.S.A.), and subsequently with the avidin–biotin complex (ABC kits; Vector) at room temperature for 1 h each. Visualization of antigen–antibody reaction sites used 0.033% 3′,3′-diaminobenzidine (DAB; 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 gelatine, air dried, dehydrated through graded alcohols, cleared in xylene and cover-slipped with DPX.

Evidence of Fos-IR was examined under a light microscope (DMLB, Leica, Spain).

Western blot immunodetection

Animals were killed by decapitation under light ether anaesthesia 90 min after saline, naloxone, vehicle or nor-BNI. The hearts were rapidly removed, and the right and left ventricles were dissected and homogenized in phosphate buffered saline (PBS) with 1% sodium dodecylsulphate (SDS) with a Polytron-type homogenized (setting 4 for 50 s). Total cell lysates were boiled 5 m, and then centrifuged at 15.000 r.p.m., 4°C 20 min). Total protein content of the samples was determined by 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 to 100 μg) from duplicate samples of two different individuals from each experimental group. Equal amounts of protein (50 μ/lane) from each sample were loaded on a 10% sodium dodecyl sulphate-polyacrilamide gel (SDS–PAGE), electrophoresed, and transferred to a PVDF membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Lab., California, U.S.A.). Comparable loading and transfer was ascertained by cutting the lower portion of the blot and staining for total protein with Amido Black. Non-specific binding of antibodies was prevented by incubating membranes in 1% bovine serum albumin (BSA) in TBST buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 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, immunoreactivity was detected with an enhanced chemiluminescence Western blot detection system (ECL, Amersham-Pharmacia-Biotechnology, Madrid, Spain) and visualized by Amersham Hyperfilm-ECL. After film scanning, the integrated optical density of the bands was estimated (Scion Image software, Scion Corporation, Maryland, U.S.A.), and normalized 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 different gels for each individual sample were performed. Measurements were in the linear range.

Estimation of NA and its metabolite normetanephrine in the right and left ventricle

Rats were decapitated 30 min after saline (s.c.), naloxone (5 mg kg^{-1} s.c.), vehicle (i.p.) or nor-BNI (5 mg kg^{-1} i.p.) administration, the chest was opened with a midsternal incision and the right and left ventricle were dissected and stored immediately at −80°C. NA and its metabolite normetanephrine (NMN) 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 r.p.m., 4°C, 15 min), the supernatant layer was removed into a 1-ml-syringe and filtered through a 0.45 μm filter (Millipore Corp., Bedford, U.S.A.) and centrifuged (15,000 r.p.m., 4°C, 20 min) again through Ultrafree MC 0.2 (Millipore Corp). From each sample 10 μl was injected into a 5-μm C_{18} reverse phase column (Waters Corp., Milford, MA, U.S.A.) through a Rheodyne (Rheodyne Inc., Cotati, CA, U.S.A.) 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 Corp.). The mobile phase consisted of a 95:5 (v v^{-1}) 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^{-1}, and chromatographic data were analysed with Millenium 2010 Chromatography Manager (Millipore Corp.) Equipment NA and NMN were simultaneously detected by the described HPLC method at an elution time of 4.25 and 7.32 min, respectively. NA and NMN 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 NA and NMN in the left ventricle was expressed as n/g wet weight of tissue.

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). Naloxone HCl (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in sterile 0.9% NaCl (saline). Trans-3,4-dichloro-N-methyl-N-[2(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate (U-50,488H; a gift from Upjohn, Lalamazoo, MI, U.S.A.) and was also dissolved in saline. Nor-binaltorphimine (nor-BNI, Sigma) was dissolved in Milli-Q (Millipore Corp.) sterile water (vehicle). Sodium dodecylsulphate, polyacrylamide gel and the PVDF membrane were obtained from Bio-Rad Laboratory (Teknovas, Bilbao, Spain). All other chemicals were of reagent grade and obtained from Sigma-Aldrich (Spain). NA bitartrate and NMN (used as an HPLC standard) were purchased from Sigma (St. Louis. MO, U.S.A.). Naloxone HCl was prepared fresh every day, dissolved in saline. Other reagents were of analytical grade.

Statistical analysis

All values are expressed as means ± s.e.mean. Statistical comparison were done by one-way ANOVA 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.

Results

Weights were recorded on the day of morphine pellet implantation or U-50,488H administration and the day of decapitation before receiving any injection. In all the experiments groups, rats chronically treated with morphine

or U-50,488H showed significantly ($P<0.001$) lower body weight gain (21 ± 1.3 g, $n=20$ and 19 ± 1.5 g, $n=18$, respectively) than the groups pretreated with placebo (48 ± 2.2 g, $n=20$) or saline (53 ± 2.3 g, $n=18$).

Administration of naloxone (5 mg kg^{-1} s.c.) to rats did not significantly change the body weight when measured 30 min after the drug injection, as compared to control rats receiving saline s.c. (5.0 ± 0.9 g vs 4.1 ± 0.6 g, $n=11$). However, chronic morphine-treated animals showed a significant weight loss ($P<0.001$) 30 min after naloxone injection (16.5 ± 0.4 g, $n=11$) when compared with the placebo-pelleted group injected with naloxone or the morphine-pelleted group injected with saline (0.49 ± 0.7 g, $n=9$). The administration of naloxone to animals pretreated with morphine precipitated standard signs of withdrawal: wet-dog shakes, teeth chattering, tremor, piloerection, lacrimation, rhinorrea, ptosis and spontaneous jumping. However, these characteristic abstinence symptoms did not appear in the morphine-pelleted rats injected with saline.

Rats rendered dependent to U-50,488H receiving nor-BNI (5 mg kg^{-1} i.p.) on day 5 did not show any of the behavioural withdrawal signs seen during morphine-dependence. In addition, no significant change in body weight loss was observed after nor-BNI administration to U-50,488H-treated rats (5.5 ± 0.8 g, $n=9$) as compared to rats receiving saline (5.9 ± 1.1, $n=9$) or to naïve rats injected with nor-BNI (6.0 ± 0.5 g, $n=8$) or vehicle (4.8 ± 0.9 g, $n=9$).

c-fos *expression after naloxone or nor-binaltorphimine (nor-BNI)-induced withdrawal*

Fos-IR was unobservable in sections of control hearts but was greatly increased during morphine withdrawal. Fos-IR nuclei were of a size and distribution consistent with their being myocyte nuclei; no cytoplasmic staining was observed and it was also apparent that the majority of myocytes in the left and right ventricles contributed to this response.

No c-*fos* expression was observed in placebo pelleted rats injected acutely with saline or naloxone nor in morphine pelleted rats injected with saline (Figure 1). However, Fos-IR was strongly expressed after naloxone administration to morphine dependent rats. The distribution of Fos in the heart was not homogenous, c-*fos* was expressed in the left and right ventricular wall (Figure 2A–D). Strong signals were also observed in the ventricular septum (Figure 2F) and in the ventricular myocardium in the area surrounding the left ventricular cavity. At a regional level, Fos-IR was greatest in the left side of the heart, with high levels founds within the left ventricular (Figure 2A–C) and in the septum (Figure 2F). Signals were also observed in the right ventricular wall (Figure 2B,D).

In the Western blot immunoassays, performed in the right and left ventricle lysates, the Fos protein was detected in a band located at $\sim$62 kDa (Figure 3). In naïve animals injected with saline or naloxone only very low levels of c-*fos* could be detected. There was no statistically significant difference between c-*fos* expression in these two groups. In addition, c-*fos* expression was very low in morphine pelleted rats injected with saline. However, Western blot analysis revealed that naloxone administration to morphine-dependent rats produced a significant induction of c-*fos* expression in right and left ventricle versus the group of rats treated with saline ($P<0.01$, $P<0.001$) or naïve rats treated with naloxone ($P<0.01$, $P<0.001$) (Figure 3A,B).

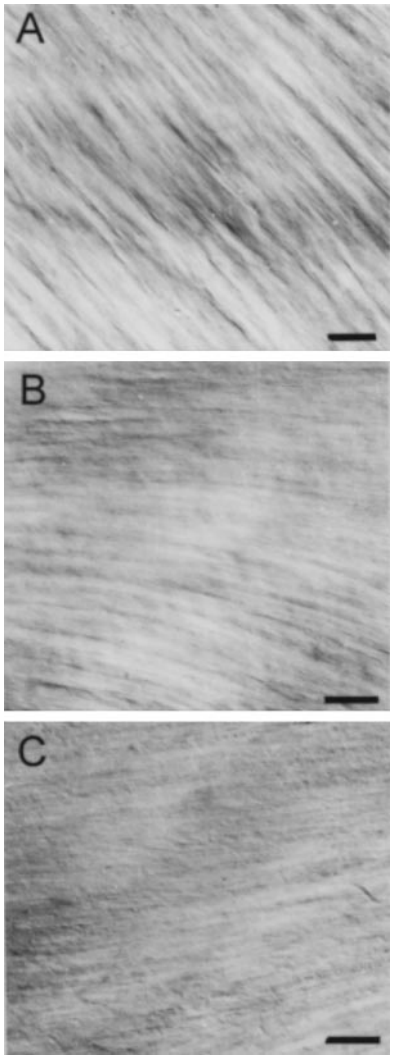

Figure 1 Photomicrographs of Fos immunoreactivity in the heart of rats pretreated with placebo pellets injected with saline (A) or naloxone (B) or rats pretreated with morphine pellets injected with saline (C). Results are representative of eight independent experiments. Normarski interference optics. *Scale bar*, 62 μm.

In contrast to morphine withdrawal the nor-BNI administration to rats chronically treated with morphine only induced a low expression of c-*fos* in the right or left ventricle, similar to that observed in placebo pelleted rats injected with naloxone or morphine pelleted rats injected with saline (Figure 3A,B). In addition, the nor-BNI administration to rats chronically treated with U-50,488H, induced no expression of c-*fos* in the right ventricle, left ventricle or septum (Figure 4). These results are confirmed with Western blot analysis, no signals of c-*fos* were detectable in U-50,488H-dependent rats injected with saline or nor-BNI (Figure 4).

Effects of morphine and U-50,488H withdrawal on NA turnover

Concentrations of NA, NMN, as well as NA turnover (as estimated by the NMN/NA ratio) were estimated in the right

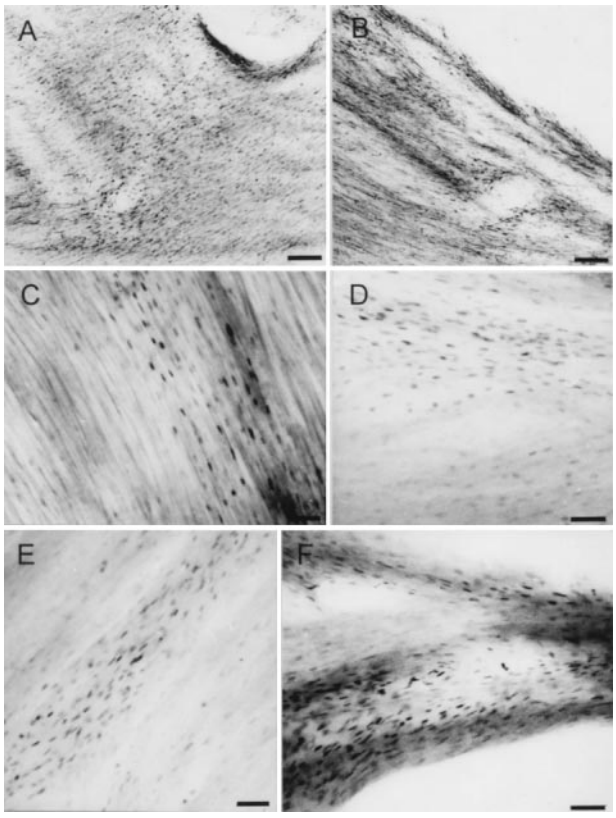

Figure 2 Photomicrographs of Fos immunoreactivity in the heart 90 min after naloxone (5 mg kg^{-1} s.c.) administration to rats pretreated with morphine pellets. Results are representative of eight independent experiments. (A and C) left ventricular wall; (B and D) right ventricular wall; (E) ventricular myocardium; (F) interventricular septum. Nomarski interference optics. *Scale bar*, 118 μm (A and B) 62 μm (C, D, E and F).

and left ventricle from rats dependent of morphine or U-50,488H. The NA turnover was not changed in naïve rats after naloxone administration when compared to naïve rats injected with saline in the right or left ventricle. However, in rats withdrawn from repeated morphine treatment by naloxone injection the NA turnover increased significantly ($P<0.001$) when compared with the naïve group injected with naloxone, or the morphine dependent group injected with saline (Figure 5A,B).

The administration of nor-BNI to rats treated chronically with U-50,488H induced a decrease ($P<0.05$) in the NA turnover in the right and left ventricle when compared to the naïve rats injected with nor-BNI, or the group chronically treated with U-50,488H injected with saline. There was no significant differences between naïve rats injected with nor-BNI versus naïve rats injected with saline (Figure 5C,D).

Discussion

Many workload-responsive IEG have been identified in cardiac cells both *in vivo* and *in vitro*, and their expression has been hypothesized to play an important role in the hypertrophic cardiac adaptation to increased mechanical stress (Komuro & Yazaki, 1993) the regulation of c-*fos* expression in the heart after morphine or U-50,488H withdrawal has not been investigated. Our immunohistochemical study of c-*fos* demonstrated, for the first time, that morphine withdrawal induces the expression of Fos protein within cardiomyocytes. This response was localized to the nucleus, which is the accepted site of action of Fos protein and other transcription factors. Fos-IR was not detected in any cell types of the heart of naïve animals injected with saline or naloxone, indicating that c-*fos* expression observed

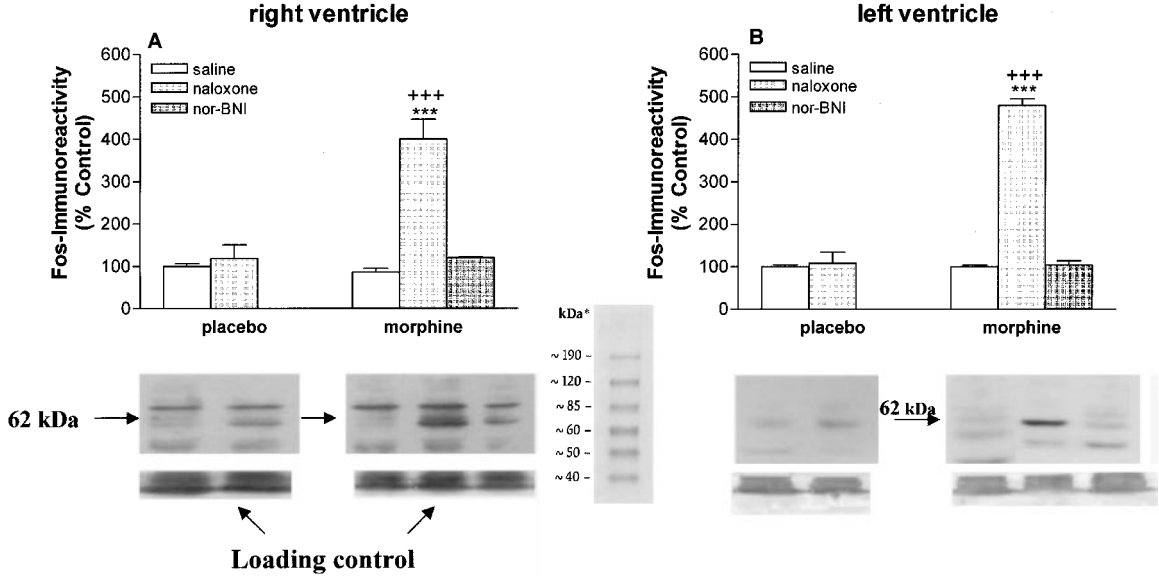

Figure 3 Western blotting analysis of Fos immunoreactivity levels in right (A) and left (B) ventricle from rats of the different experimental groups. Comparable loading and transfer was ascertained by cutting the lower portion of the blot and staining for total protein with Amido Black. Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100% value). Data are the mean ± s.e.mean, $n=3-6$ per group. Representative immunoblots directly scanned from the films, without any image manipulation, are shown. ***$P<0.001$ vs morphine + saline; + + + $P<0.001$ vs placebo + nx.

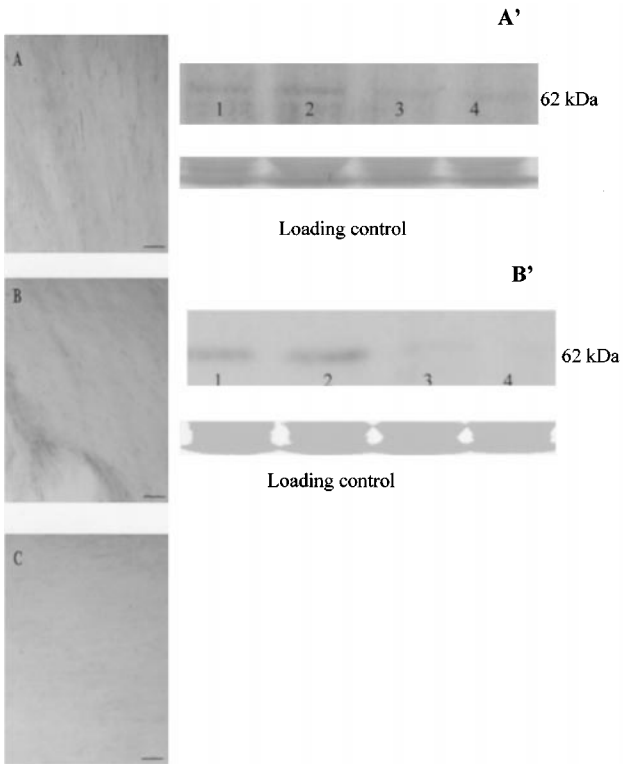

Figure 4 Photomicrographs of Fos immunoreactivity in the heart 90 min after nor-BNI (5 mg kg^{-1} i.p.) administration to rats pretreated with U-50,488H (i.p.). Results are representative of eight independent experiments. (A) right ventricle; (B) left ventricle; (C) interventricular septum. Nomarski interference optics. *Scale bar*, 114. Representative Western blots of Fos inmunoreactivity levels in right (A′) and left ventricle (B′) from rats of the different experimental groups. 1 = saline (i.p.) + saline (i.p.), 2 = saline (i.p.) + norBNI (5 mg kg^{-1}), 3 = U-50,488H (i.p.) + saline, 4 = U-50,488H (i.p.) + norBNI.

in this study is a consequence of naloxone administration to dependent tissues. Western blot analysis revealed low signals in the naïve rats, whereas the c-*fos* expression was strongly expressed after naloxone administration to morphine dependent rats. Accordingly, distribution of Fos-IR in the naïve was virtually not detected in the tissue section by immunohistochemical, whereas some specific immunoreactivity was found when applying Western blot. This discrepancy may be due to differences in the sensitivities between the two methods. Similar findings have been detected in previous work (Larsen *et al*., 1998).

The present study also demonstrated that morphine withdrawal leads to an increase in the right and left ventricle of the NMN/NA ratio (an index of NA turnover), and confirms previous results obtained in left atria (Rabadán *et al*., 1997a), right atria (Rabadán *et al*., 1998) and right ventricle (Milanés *et al*., 2000). These effects were dependent on adrenoceptor activation, which indicates that the catecholaminergic hyperactivity in the heart during morphine withdrawal is mediated *via* a stimulators noradrenergic pathway (Milanés & Laorden, 2000). In addition, our previous studies demonstrated a marked increase in the heart sympathetic activity 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.

Interestingly, the present data shows, for the first time, that in contrast to morphine, U-50,488H withdrawal was not accompanied by changes in Fos-IR in the right or left ventricle. Our immunohistochemical and Western blot studies detected no signals of c-*fos* in U-50,488H-dependent rats injected with saline or nor-BNI. In addition, the administration of nor-BNI to morphine-pelleted rats did not induce changes in Fos-IR. These data clearly indicate that kappa–opioid receptor is not involved in the molecular adaptive mechanisms responsible for the development of dependence at the heart level. In addition, our results also demonstrated that U-50,488H withdrawal was not accompanied by an increase in the heart catecholaminergic activity that was seen after morphine withdrawal (Milanés & Laorden, 1998). By contrast a decrease in the ventricular NMN content and in the NA turnover was observed after nor-BNI administration to U-50,488H-dependent rats, confirming previous data obtained in right ventricle (Milanés & Laorden, 1998). In agreement with previous studies (Milanés & Laorden, 1998; Laorden & Milanés, 2000), our results showed that, unlike morphine withdrawal, U-50,488H withdrawal induced neither behavioural or vegetative signs of physical dependence, suggesting that the kappa–opioid receptor is not involved in the development of physical dependence.

It is known that the repeated use of opioids induces adaptive changes in the central and peripheral nervous system leading to the development of tolerance and dependence. Induction of IEGs such as c-*fos* and c-*jun* is considered as one of these adaptive responses. The transcription factor Fos in particular has been shown to be altered in several brain areas following morphine withdrawal (Beckmann *et al*., 1995; Frankel *et al*., 1999; Laorden *et al*., 2002) and has been widely used as an indicator of cellular activity in the central nervous system (Morgan & Curran, 1991). Present results show that morphine withdrawal induces the expression of Fos protein within the cardiomyocytes. 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 & Karin, 1991). Elevation of Fos in the heart, as observed in our study, could contribute to the expression of target genes within the heart. Genes encoding neurotransmitter synthesizing enzymes which 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. In addition, the skeletal α-actin promoter was also shown to be trans-activated by co-expressed c-*fos* and c-*jun* in ventricular myocytes (Paradis *et al*., 1996).

On the other hand, possible mechanisms underlying the cardiac Fos response to morphine withdrawal are not known. However, the fact that heart receiving dense NA innervation and the hyperactivity of heart catecholaminergic neurons, observed during morphine withdrawal, may suggest that NA 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 yet

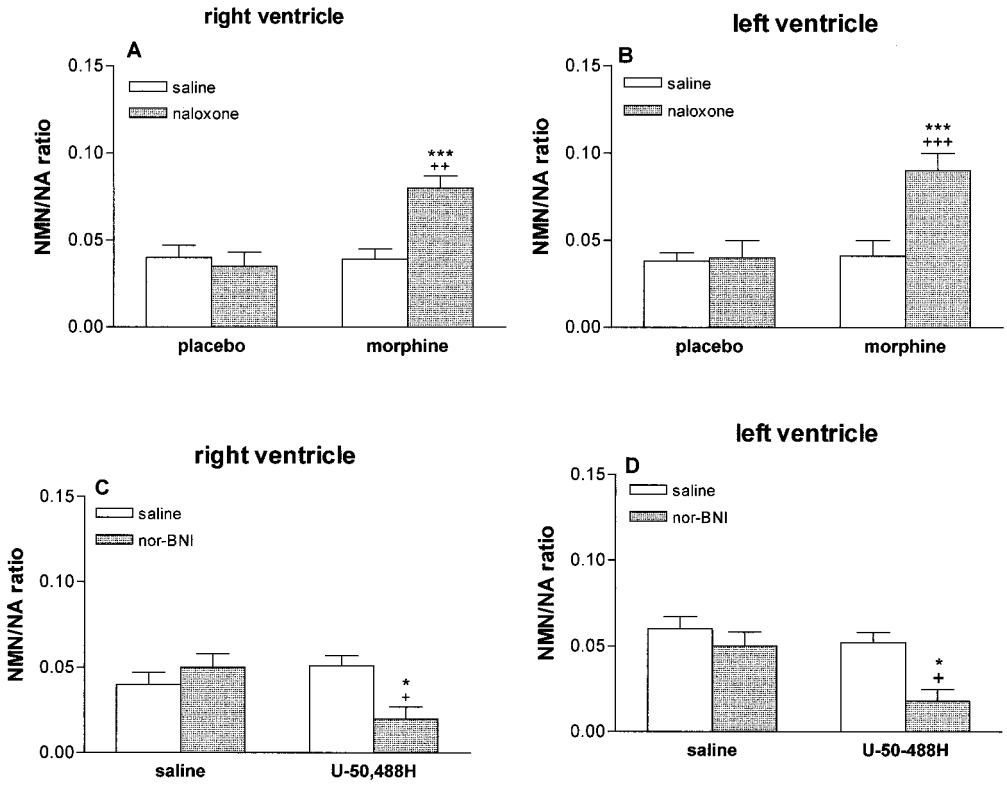

Figure 5 Ventricular NA turnover in placebo (naïve) and morphine-dependent rats (A, B) or in saline (naïve) and U-50,488H-dependent rats (C,D) 30 min after injection of saline, naloxone, vehicle or nor-BNI ($n=6-8$ per group; mean ± s.e.mean). $^{*}P<0.05$, $^{***}P<0.001$ vs morphine + saline; $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ vs placebo + naloxone.

determined, several studies have revealed that c-*fos* expression is induced rapidly after NA administration (Hannan & West, 1991), haemodynamic stress, such as pressure overload (Ogino *et al*., 1999), and immobilization or emotional stress (Ueyama *et al*., 1996; 1999).

In summary, the results of the present study revealed an enhanced noradrenergic activity in the heart after naloxone administration to morphine dependent rats in parallel with the expression of Fos protein, indicating a possible link between Fos induction and noradrenergic hyperactivity. By contrast to morphine U-50,488H withdrawal was not accompanied by increases in the noradrenergic activity or modifications in Fos expression. Therefore, we have demonstrated that kappa–opioid receptors are not involved in the molecular adaptive mechanisms responsible for the development of opioid dependence in the heart.

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