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RESEARCH PAPER

The PKs PKA and ERK 1/2 are involved in phosphorylation of TH at Serine 40 and 31 during morphine withdrawal in rat hearts

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Background and purpose: Our previous studies have shown that morphine withdrawal induced hyperactivity of cardiac noradrenergic pathways. The purpose of the present study was to evaluate the effects of morphine withdrawal on site-specific phosphorylation of TH in the heart.
Experimental approach: Dependence on morphine was induced by a 7-day s.c. implantation of morphine pellets in rats. Morphine withdrawal was precipitated on day 8 by an injection of naloxone (2 mg kg^{-1}). TH phosphorylation was determined by quantitative blot immunolabelling using phosphorylation state-specific antibodies.
Key results: Naloxone-induced morphine withdrawal induced phosphorylation of TH at serine (Ser)40 and Ser31 in the right ventricle, associated with both an increase in total TH levels and an enhancement of TH activity. When HA-1004 (PK A inhibitor) was infused, concomitantly with morphine, it diminished the increase in noradrenaline turnover, total TH levels and TH phosphorylation at Ser40 in morphine-withdrawn rats. In contrast, the infusion of calphostin C (PKC inhibitor), did not modify the morphine withdrawal-induced increase in noradrenaline turnover and total TH levels. In addition, we show that the ability of morphine withdrawal to stimulate phosphorylation at Ser31 was reduced by SL327, an inhibitor of ERK 1/2 activation.
Conclusions and implications: The present findings demonstrate that the enhancement of total TH levels and the increased phosphorylation state of TH during morphine withdrawal were dependent on PKA and ERK activities and suggest that these transduction pathways might contribute to the activation of the cardiac catecholaminergic neurons in response to morphine withdrawal.
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Keywords: heart; morphine withdrawal; noradrenaline turnover; PKA; PKC; ERK; TH phosphorylation

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated extracellular kinase; PKA, cyclic AMP-dependent PK; SDS-PAGE, SDS-polyacrylamide gel; TH, tyrosine hydroxylase

Introduction

There is growing evidence that the cellular and molecular adaptation following long-term opioid exposure results from the phosphorylation of opioid receptor proteins, their coupled G proteins and several related effector proteins. The enzymes producing these changes include second messenger-dependent PKs (PKC), cyclic AMP-dependent PK (PKA) and mitogen-activated PKs, which play important roles in the regulation of opioid signal transduction (Wegener and Kummer, 1994; Liu and Anand, 2001). Alterations in the PKA pathway have been suggested as one of the molecular mechanisms of opioid tolerance and dependence (Nestler and Aghajanian, 1997). Although the μ opioid receptor is negatively coupled to the adenylate cyclase/cAMP-dependent PKA pathway upon acute stimulation (Childers, 1991), the pathway is upregulated in several brain areas with chronic morphine treatment (Nestler, 1992; Tokuyama *et al*., 1995). Furthermore, it has been demonstrated that this pathway is important in the sympathetic regulation of the cardiovascular system. Cardiac inotropic activity is strongly regulated by intracellular PKA (Hussain *et al*., 1999; Kamp and Hell, 2000). Previous studies in our laboratory have demonstrated that naloxone administration to morphine-dependent rats leads to an increase in the force and rate of contraction in different cardiac tissues (Rabadán *et al*., 1997, 1998). In addition, it has been demonstrated that withdrawal from morphine is associated with a marked increase in the ventricular levels of cAMP in parallel with an enhancement of noradrenaline (NA) turnover (Milanés *et al*., 2000).

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Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated extracellular kinase (MEK; mitogen-activated PK) family, transduces a broad range of extracellular stimuli into diverse intracellular responses. It is now well established that the ERK signalling pathway is a critical player in synaptic and neuronal plasticity (Adams and Sweatt, 2002) and its pharmacological blockade prevents rewarding effects of cocaine (Lu *et al.*, 2005). Recently, several studies have shown that this pathway contributes to naloxone-precipitated withdrawal in morphine-dependent rats (Ren *et al.*, 2004; Cao *et al.*, 2005; Almela *et al.*, 2007).

On the other hand, TH, the rate-limiting enzyme in catecholamine synthesis, plays important roles in the regulation of sympathetic nervous system and its impact on cardiac function. TH expression is subjected to intricate regulation by a number of mechanisms, including transcriptional and post-transcriptional processes (Kumer and Vrana, 1996; Mallet, 1999). Short-term regulation of catecholamine biosynthesis occurs through the modulation of the state of phosphorylation of TH. TH phosphorylation and activation is the primary mechanism responsible for the maintenance of catecholamine levels in tissues after catecholamine secretion. TH can be phosphorylated at serine (Ser) residues 8, 19, 31 and 40 by a variety of PKs (Campbell *et al.*, 1986). PKA and PKC phosphorylate TH only at Ser40 (Roskoski *et al.*, 1987; Funakoshi *et al.*, 1991). ERK1 and ERK2 were shown to phosphorylate Ser31 *in situ* (Haycock *et al.*, 1992). The phosphorylation of Ser40 increases the enzyme's activity *in vitro*, *in situ* and *in vivo*. Phosphorylation at Ser31 also increases the activity but to a much lesser extent than Ser40 phosphorylation. The phosphorylation of TH at Ser19 or Ser8 has no direct effect on TH activity (Dunkley *et al.*, 2004). The intrinsic effects of most of these phosphorylation events have not been established.

One of the most consistent biochemical changes in response to morphine exposure is an upregulation of TH, which is evident after naloxone administration in several brain areas (Nestler and Aghajanian, 1997; Boundy *et al.*, 1998). Recent results from this laboratory have shown an increase in the non-phosphorylated TH levels in the heart from rats withdrawn from morphine (González-Cuello *et al.*, 2004). However, the mechanism(s) involved in TH phosphorylation and its involvement in the adaptive changes that occur during morphine withdrawal remain unclear. Therefore, this study was designed to assess the changes in TH phosphorylation after chronic morphine administration and following morphine withdrawal in the heart. To this end, we first evaluated the changes in TH phosphorylation at Ser40 and Ser31 in addition to total levels of TH, TH enzymatic activity and NA turnover. It is known that phosphorylation of TH at each site is associated with distinct signalling pathways. As PKA phosphorylates Ser40 (Dunkley *et al.*, 2004) and ERK phosphorylates Ser31 (Haycock, 1993; Dunkley *et al.*, 2004), we then tested whether the activation of PKA and ERK1/2 pathways contributed to morphine withdrawal-induced TH phosphorylation at Ser40 and Ser31, respectively. This was achieved by measuring the effects of HA-1004, a selective inhibitor of PKA (Hidaka *et al.*, 1984) on the levels of TH phosphorylated at Ser40 and the effects of SL327, a selective inhibitor of ERK activation (Atkins *et al.*, 1998) on the levels of TH phosphorylated at Ser31.

Materials and methods

Animals and treatments

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 Animal Ethics Committee. Male Sprague–Dawley rats (220–240 g at the beginning of the experiments) were housed four to five per cage under a 12-h light/dark cycle (light: 8:00–20:00 h) in a room with controlled temperature (22 ± 2 °C), humidity (50 ± 10%), food and water available *ad libitum* and handled for several days preceding the experiment to minimize stress, as described previously (Laorden *et al.*, 2000). The drug and molecular target nomenclature used in this study conforms to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

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.*, 1997; Milanés and Laorden, 2000; Milanés *et al.*, 2000). On day 8, the animals pretreated with morphine or placebo pellets were injected with saline (s.c.) or naloxone ($2\,mg\,kg^{-1}$, s.c.). The weight gain of the rats 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). In addition, body weight loss was determined as the difference between the weight determined immediately before saline or naloxone injection and a second determination made 60 and 90 min later.

To determine the effects of PKA and PKC on the morphine withdrawal-induced changes in TH phosphorylation, animals were continuously treated for 7 days through s.c. osmotic minipumps (Alzet mod. 2001, which deliver at $1\,\mu L\,h^{-1}$; Alza, Palo Alto, CA, USA), with HA-1004, a PKA selective inhibitor (Hidaka *et al.*, 1984; $40\,nmol\,day^{-1}$), calphostin C, a PKC selective inhibitor (Kobayashi *et al.*, 1989) ($40\,pmol\,day^{-1}$) or vehicle. The PKC inhibitor was dissolved in dimethyl sulphoxide (DMSO) and serially diluted in MilliQ-water (final concentration of DMSO was 0.06%). 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 to obtain an optimal flow rate ($1\,\mu L\,h^{-1}$). On day 8, a withdrawal syndrome was induced by s.c. naloxone ($2\,mg\,kg^{-1}$) injection. Previously, PKA and PKCδ levels were determined in morphine-dependent and control rats treated with HA-1004

or calphostin C. To determine the effect of inhibiting ERK phosphorylation on the morphine withdrawal-induced changes in TH phosphorylation at the heart, levels of TH phosphorylated at Ser31 were determined in morphine-dependent and control rats treated with SL327, a selective inhibitor of MEK (Atkins *et al*., 1998), administered 1 h before the injection of naloxone or saline. This inhibitor was dissolved in DMSO (100%) and injected intraperitoneally at an injection volume of 1 mL kg^{-1} and at doses of 50 and 100 mg kg^{-1}. On the basis of our previous experiments (Almela *et al*., 2007) of SL327-induced inhibition of ERK phosphorylation in the heart, the 100 mg kg^{-1} dose was chosen for our experiments.

Estimation of NA and its metabolite normetanephrine in the right ventricle

Rats were decapitated 60 or 90 min after saline s.c. or naloxone (2 mg kg^{-1} s.c.), the chest was opened with a midsternal incision and the right ventricle was dissected and stored immediately at −80°C. NA and its metabolite normetanephrine were determined by high-performance liquid chromatography with electrochemical detection. Each tissue was weighed, placed in a dry cooled propylene vial and homogenized with a Polytron-type homogenizer in 1.5 mL perchloric acid (0.1 M). The homogenates were then centrifuged (8000 *g* 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, USA) and centrifuged (6000 *g* 4°C, 20 min) again through Ultra free MC 0.2 (Millipore). From each sample, 10 μL was injected into a 5 μm C_{18} reverse phase column (Waters, Milford, MA, USA) through a Rheodyne 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% (v/v) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), L-octyl-sodium sulphonate (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 Equipment (Millipore). NA and normetanephrine were simultaneously detected by the described high-performance liquid chromatography method at an elution time of 4.25 and 7.32 min, respectively. NA and normetanephrine were quantified by reference to calibration curves run at the beginning and at 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 normetanephrine in the right ventricle was expressed as ng/gram tissue.

Measurement of TH activity

After 90 min of saline or naloxone injection to placebo- or morphine-pelleted animals, rats were killed by decapitation, the heart removed and the right ventricle was dissected, fresh-frozen and stored immediately at −80 °C until use. TH catalyses the hydroxylation of tyrosine to generate L-DOPA and water using tetrahydropterine as a cofactor. TH activity is measured by quantifying tritiated water production from 3,5-[^{3}H]L-tyrosine. Right ventricle samples were placed in homogenization buffer (phosphate-buffered saline, protease inhibitors plus a phosphatase inhibitor Cocktail Set) and homogenized before centrifugation at 4000 *g* for 10 min at 4 °C. Then, 25 μL of the supernatants were incubated at 37 °C in a final volume of 50 μL of a reaction mixture containing the following components: 0.2 M Tris HCl (pH, 7), 1 mM tetrahydrobiopterin, 10 mM β-mercapto-ethanol, 0.02% catalase, 50 μM isotopically diluted L-[3,5-^{3}H] tyrosine (radioactive concentration 10 μCi mL^{-1}, specific activity 0.2 mCi μmol^{-1}; Amersham-Pharmacia-Biotechnology, Madrid, Spain). For the blank reaction, samples were replaced by sodium orthovanadate. After 4 h of incubation, the reaction was stopped by the addition of 1% trichloroacetic acid and the radioactive organic compounds were separated from tritiated water by absorption into activated charcoal. After centrifugation (4800 *g*, 4 °C, 5 min), tritiated water was quantified in the supernatant by scintillation counting in a Wallac 1409 liquid scintillation counter. The assays were performed in duplicate.

Western blot analysis

Animals were killed by decapitation 60 or 90 min after administration of naloxone or saline for PKA, for PKCδ, for total TH determination, for TH phosphorylation analysis and for phosphorylated ERKs evaluation. The hearts were rapidly removed and the right ventricle was dissected, fresh-frozen and stored immediately at −80 °C until use. Samples were placed in homogenization buffer (phosphate-buffered saline, 2% SDS plus protease and phosphatase inhibitors) and homogenized for 50 s before centrifugation at 15 000 r.p.m. for 20 min at 4 °C. Total protein concentrations were determined spectrophotometrically using the bicinchoninic acid method (Wiechelman *et al*., 1988).The optimal amount of protein to be loaded was determined in the preliminary experiments by loading gels with increasing protein contents (25–100 μg) from samples of each experimental group. Equal amounts of protein (50 μg/lane) from each sample were loaded on a 10% SDS-polyacrylamide gel, separated by electrophoresis, and transferred onto poly vinylidene difluoride membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratory, CA, USA). Non-specific binding of antibodies was prevented by incubating membranes in 1% BSA in Tris buffer saline tween (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20). The blots were incubated overnight at room temperature (for total TH and pTH) or at 4 °C (for pERK, PKA, PKC), with the following primary antibodies: specific PKA catalytic subunit antibody (1:2000 polyclonal antibody; sc-903, Santa Cruz Biotecnologia, Santa Cruz, CA, USA); polyclonal anti-PKCδ (1:1000 dilution; p8333, Sigma Chemical Co., St Louis, MO, USA); polyclonal antitotal TH (against phosphorylated and non-phosphorylated TH; 1:1000 dilution; AB152, Chemicon International, Temecula, CA, USA), polyclonal anti-pSer40 TH (1:500 dilution; AB5935, Chemicon International), polyclonal anti-pSer31 TH (1:250 dilution; AB5423, Chemicon International), or monoclonal anti-pERK1/2 (1:1000; sc-7383, Santa Cruz Biotechnology, Santa Cruz) in Tris buffer

saline tween with BSA. After extensive washings with Tris buffer saline tween, the membranes were incubated for 1 h, at room temperature, with peroxidase-labelled secondary antibodies (antirabbit sc-2004 for PKA, PKCδ and total TH, pTH, Santa Cruz; antimouse sc-2005 for phospho-ERK1/2, Santa Cruz) 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. Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25 mM and SDS 1%) pH2, for 1 h at 37 °C. Blots were subsequently reblocked and probed with 1:1000 anti-β actin (Cell signalling, 43 kDa). Quantification of immunoreactivity corresponding to PKA (42 kDa), PKCδ (78 kDa), total TH, TH phosphorylated at Ser40 or Ser31 (60 kDa) and phospho-ERK1/2 (42 and 44 kDa) bands was carried out by densitometry (AlphaImager, Nucliber, Madrid, Spain). The integrated optical density of bands was normalized to the background values. Relative variations between bands of experimental samples and control samples were calculated in the same image.

Statistical analysis

The results are expressed as the mean ± s.e.mean Data were analysed by ANOVA followed by the Newman–Keuls *post hoc* test. Body weight gain and loss in naive and morphine-dependent rats were analysed by unpaired Student's *t*-test. One-way ANOVA followed by Dunnett's multiple comparison test was used when required. Differences with a *P*-value <0.05 were considered significant.

Drugs and chemicals

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); SDS, polyacrylamide gel and poly vinylidene difluoride membrane were obtained from Bio-Rad Laboratory (Teknovas, Bilbao, Spain). NA bitartrate, normetanephrine (used as an high-performance liquid chromatography standard), naloxone HCl and western blot reagents were purchased from Sigma Chemical Co. Naloxone HCl was dissolved in sterile 0.9% NaCl (saline) and administered in volumes of 0.1 mL $100\,g^{-1}$ body weight. HA-1004 (N-2′ guanidinoethyl-5-isoquinolinesulphonamide) was purchased from Sigma Chemical Co. and dissolved in Milli-Q (Millipore, Bedford, MA, USA) sterile water. Calphostin C (2-(12-(2-(benzoyloxy) propyl)-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl)-1menthylethyl carbonic acid 4-hydroxyphenyl ester) was purchased from RBI (Natick, MA, USA). The chronic delivery of HA-1004 or calphostin C was achieved by means of Alzet 2001 osmotic minipumps (Alza, Palo Alto, CA, USA), which deliver at $1\,\mu L\,h^{-1}$. SL327 (Ascent, Scientific, Bristol, UK). DMSO was purchased from Sigma. Drugs were prepared freshly every day. Reagents: protease inhibitors (Boehringer Mannhein, Germany); phosphatase inhibitor Cocktail Set (Calbiochem, Germany). TH activity reagents were purchased from Sigma.

Results

Before performing the immunodetection assays, we assessed the efficacy of chronic treatment with morphine by implantation of pellets, which has been previously shown to induce tolerance and dependence to the effects of morphine (Laorden *et al*., 2002; González-Cuello *et al*., 2003; Martínez *et al*., 2003). For this purpose, the weight of the animals was recorded on the days of pellet implantation and on the day of killing (day 8), before receiving any injections. Rats treated with morphine showed a significantly lower ($P<0.01$; *t*-test) body weight gain (22.01 ± 1.40 g, $n=20$) than animals receiving placebo pellets (40.20 ± 2.30 g, $n=20$). Administration of naloxone to control rats resulted in no significant changes in body weight when measured 60 (3.38 ± 0.50 g, $n=10$) or 90 min (3.25 ± 0.1 g, $n=10$) after drug injection, as compared to control rats receiving saline (3.50 ± 0.61 g, $n=10$, 3.39 ± 0.2 g, $n=12$, respectively; *t*-test). However, chronic morphine-treated animals showed a significant weight loss ($P<0.001$; *t*-test) 60 (19.36 ± 0.32 g, $n=10$) or 90 min (18.48 ± 0.40 g, $n=20$) after naloxone injection when compared with placebo-pelleted group also receiving naloxone. This reduction was due to an increase in the locomotor activity. The injection of naloxone in rats chronically treated with HA-1004 or calphostin C concomitantly with morphine or in rats acutely treated with SL327 induced a weight loss, similar to that described in the group chronically pretreated with vehicle plus morphine (data not shown). All the animals undergoing morphine withdrawal displayed characteristic abstinence symptoms: wet-dog shakes, teeth chattering, ptosis, tremor, piloerection, lacrimation, rhinorrhea, chromodiacryorrhea and spontaneous jumping.

Effects of HA-1004 and calphostin C on PKA and PKCδ expression in the right ventricle

In this study, we have evaluated PKA or PKCδ levels in the right ventricle after inhibition of PKA or PKCδ by HA-1004 or calphostin C, respectively. As shown in Figure 1a, chronic pretreatment with HA-1004 concomitantly with morphine antagonized the expression of PKA in both controls and morphine-withdrawn animals. Regarding calphostin C, this inhibitor blocked the expression of PKCδ after naloxone-induced withdrawal in the morphine-dependent animals (Figure 1b). These experiments demonstrated that the doses of HA-1004 and calphostin C used in this study are useful to inhibit the expression of PKA or PKCδ, one of the main PKC isoforms involved in the adaptive changes in hearts, observed during morphine withdrawal (Cerezo *et al*., 2005).

Effects of PKA and PKC inhibitors on NA turnover in the heart

In agreement with our previous investigations (González-Cuello *et al*., 2004; Almela *et al*., 2006), this study shows that 60 or 90 min after naloxone administration to morphine-dependent rats, there was an increase ($P<0.001$) of NA turnover in the right ventricle when compared with naïve rats injected with naloxone (Figures 2a and b). Chronic pretreatment with HA-1004 concomitantly with morphine

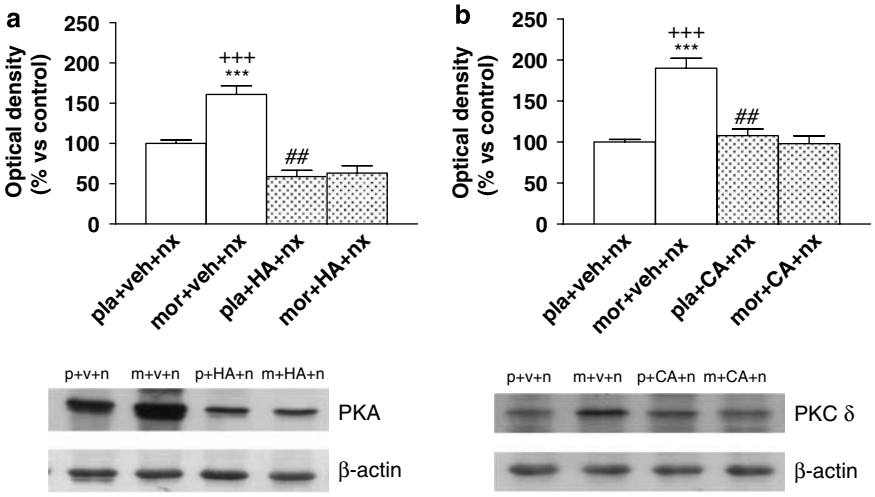

Figure 1 Immunoblots of PKA (**a**) and PKCδ (**b**) in right ventricle from placebo (pla, p)- or morphine (mor, m)-dependent rats after naloxone (nx, n)-precipitated withdrawal in vehicle (veh, v) infused rats and in animals chronically administered with HA-1004 (HA) or calphostin C (CA). Animals received s.c. implantation of pla or mor (75 mg) pellets for 7 days and concomitantly were infused with veh, HA (40 nmol day^{-1}) or CA (40 pmol day^{-1}). On day 8, rats were injected with saline (s) s.c. or nx; 2 mg kg^{-1}) and were decapitated 90 min later. The immunoreactivity corresponding to PKA or PKCδ is expressed as a percentage of that in the control group (pla + veh + nx; defined as 100%). Data are the mean ± s.e.mean ($n=4$). ***$P<0.001$ versus the (pla + veh + nx;); $^{+++}P<0.001$ versus the group treated with (mor + HA + nx) or (mor + CA + nx); $^{\#\#}P<0.01$ versus (pla + veh + nx). Bottom panels: representative bands from autoradiograms at the known apparent molecular weight for PKA and PKCδ. β-actin was used as an internal loading control.

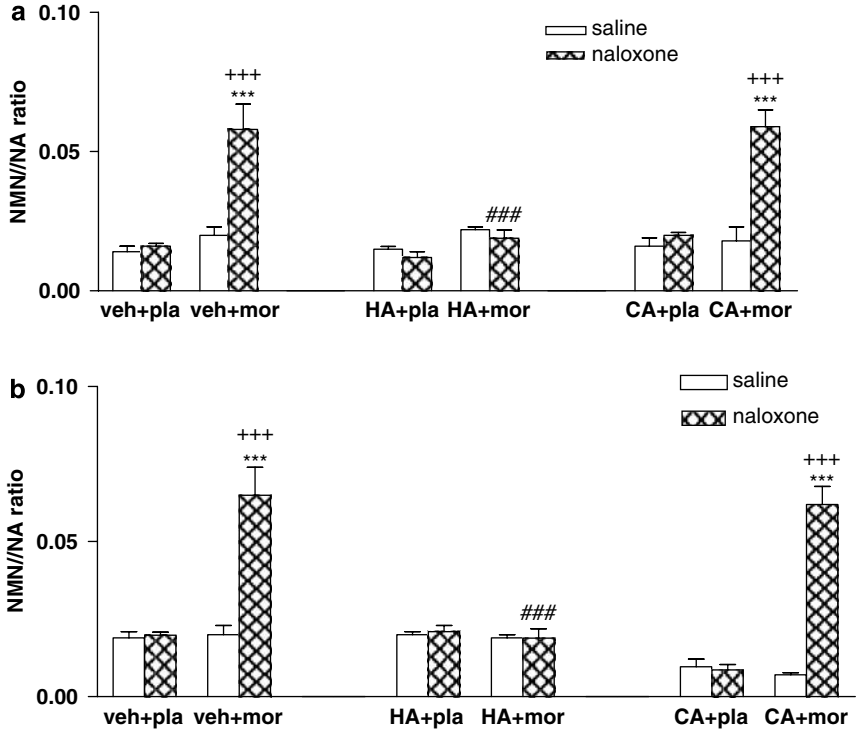

Figure 2 Normetanephrine (NMN)/noradrenaline (NA) ratio in the right ventricle 60 (**a**) or 90 min (**b**) after saline or naloxone (nx, n) administration to placebo-(pla, p) or morphine (mor, m)-treated rats receiving vehicle (veh, v), HA1004 or calphostin. Data are the mean ± s.e.mean ($n=6$–7). ***$P<0.001$ versus the group receiving saline instead of nx; $^{+++}P<0.001$ versus the group treated with pla instead of mor; $^{\#\#\#}P<0.001$ versus the group treated with veh + mor + nx.

antagonized the increase in the NA turnover observed during morphine withdrawal (Figures 2a and b). Having established that PKA inhibitor antagonized the increased NA turnover observed during morphine withdrawal in the heart, we then sought to determine whether increased PKC activity was also responsible for the naloxone-induced increases of NA turnover in morphine-withdrawn rats. For this purpose, the selective PKC inhibitor calphostin C was coadministered

with morphine. The administration of calphostin C did not prevent the increase in NA turnover observed in the right ventricle 60 or 90 min after the injection of naloxone to morphine-dependent rats (Figures 2a and b).

Effects of morphine withdrawal on total TH

As altered TH is one of the most robust findings in different areas with chronic morphine exposure, we studied TH protein levels by western blot analysis in the right ventricle and we next investigated the effect of PKA or PKC inhibition on the response of TH to morphine withdrawal. To assess the relationships between PKA or PKC and morphine-induced biochemical adaptations in the heart, we examined the ability of HA-1004 or calphostin C, infused chronically through osmotic minipumps, to modify total TH levels in the right ventricle. Control animals received infusion of vehicle. As shown in Figure 3, total TH immunoreactivity was elevated in the right ventricle 60 (Figure 3a) or 90 min (Figure 3b) after naloxone injection to morphine-dependent rats compared with the control group receiving naloxone or the dependent group receiving saline instead of naloxone. Chronic infusion of HA-1004 completely blocked the ability of naloxone-precipitated morphine withdrawal to induce total TH immunoreactivity in the right ventricle (Figure 3). In contrast, chronic infusion of calphostin C did not modify the increase in total TH expression observed in the right ventricle 60 or 90 min after naloxone injection to morphine-dependent rats when compared with the corresponding group infused with vehicle instead of calphostin C (Figure 3). These results demonstrated that the PKC inhibitor, calphostin C, fails to prevent the enhancement of TH expression observed during morphine withdrawal.

Effects of HA-1004 and SL 327 on TH phosphorylation at Ser40 or Ser31

Additional experiments were performed in the right ventricle to determine whether naloxone-induced morphine withdrawal would activate phosphorylation of TH at Ser40

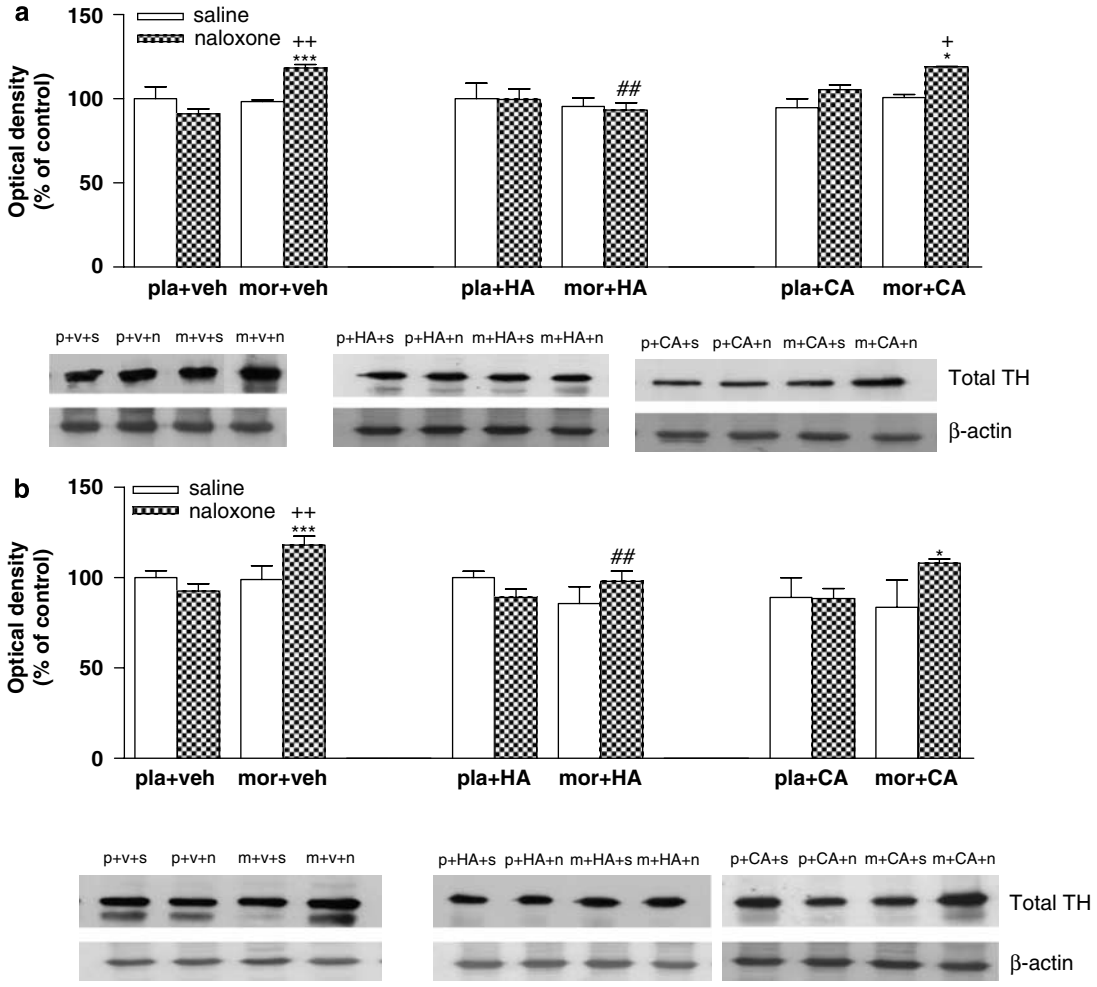

Figure 3 Western blotting analysis of TH immunoreactivity levels in the right ventricle 60 (**a**) or 90 min (**b**) after saline or naloxone (nx, n) administration to placebo–(pla, p) or morphine (mor, m)-treated rats receiving vehicle (veh, v), HA1004 or calphostin. The immunoreactivity corresponding to total TH is expressed as a percentage of that in the control group (pla + veh + saline; defined as 100%). Data are the mean ± s.e.mean ($n = 4$–6). *$P<0.05$, ***$P<0.001$ versus the group receiving saline instead of nx; $^{+}P<0.05$, $^{++}P<0.01$ versus the group pretreated with pla instead of mor; $^{\#\#}P<0.01$ versus the group receiving veh instead of HA. Bottom panels: representative bands from autoradiograms at the known apparent molecular weight for TH. β-actin was used as an internal loading control.

or Ser31. These experiments demonstrated that, in the right ventricle, acute naloxone treatment had no effect on animals chronically treated with placebo and that phosphorylation at Ser40 or Ser31 was also unchanged after saline administration to morphine-dependent rats. However, rats chronically treated with morphine and given naloxone showed significant increases in phosphoSer40-TH levels in the right ventricle 60 (Figure 4a) or 90 min (Figure 4b) after the opioid antagonist injection compared with the corresponding control group receiving naloxone and with the morphine-dependent animals receiving saline.

In accordance with previous studies (Cerezo *et al*., 2005), the present results demonstrated that morphine withdrawal increased PKA levels in right ventricle. To asses the contribution of PKA to the regulation of TH, we have examined TH phosphorylation at Ser40 during morphine withdrawal in animals receiving the selective inhibitor of PKA HA-1004. Chronic infusion of HA-1004 completely prevents the ability of naloxone-precipitated morphine withdrawal to increase the levels of phospho-Ser40 TH in the right ventricle (Figures 4a and b). Thus, these results suggest that TH phosphorylation at Ser40 following morphine withdrawal occurs downstream of PKA.

We also studied the phosphorylation of TH at Ser31 at different time points. As shown in Figure 5a, 60 min after naloxone-precipitated morphine withdrawal, there were no changes in the levels of phospho-Ser31 TH in the right ventricle. However, rats chronically treated with morphine and given naloxone showed significant increases in phospho-Ser31 TH in the right ventricle 90 min after the opioid antagonist injection compared with the corresponding control group receiving naloxone and with the morphine-dependent animals receiving saline (Figure 5b).

As TH phosphorylation at Ser31 is dependent on extracellular signal-regulated PKs 1 and 2 (Haycock *et al*., 1992), we analysed phospho-Ser31-TH levels in the right ventricle after inhibition of ERK by SL327, a drug that prevents the activation of ERK by inhibiting ERK kinase (MEK), the upstream kinase of ERK (Atkins *et al*., 1998).

First, we determined the basal levels of phosphorylated (activated) ERK1/2 in the right ventricle from control and from morphine-withdrawn rats pretreated with SL327. As

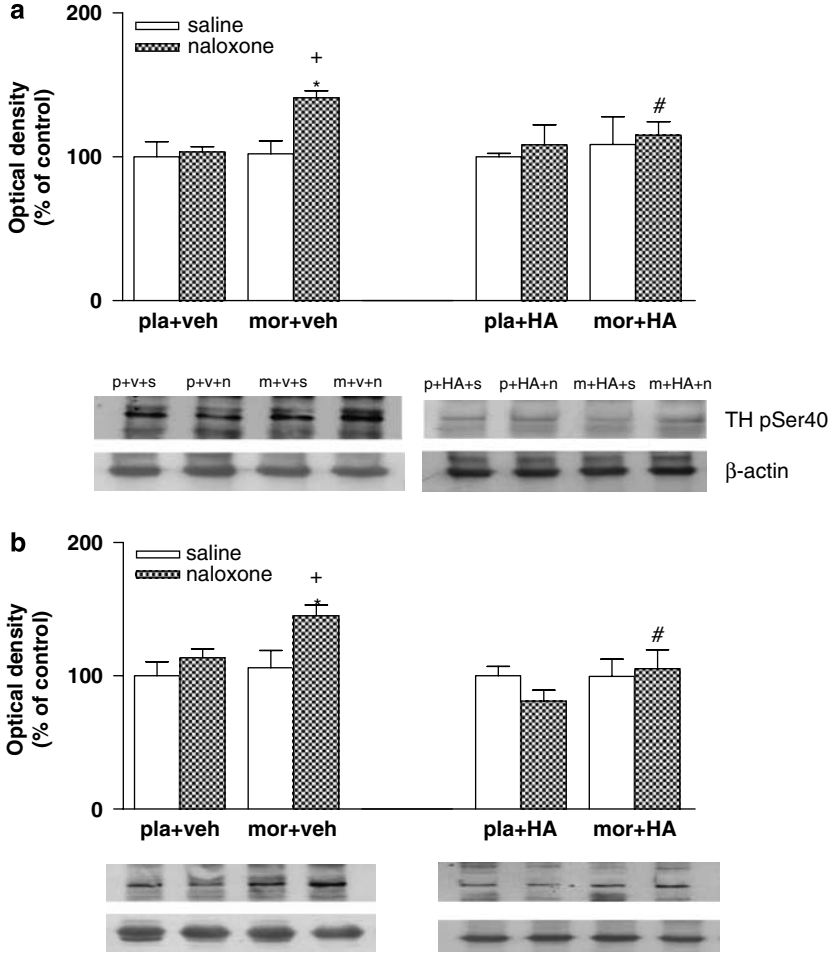

Figure 4 Western blotting analysis of TH phospho (p) Ser40 in the right ventricle 60 (**a**) or 90 min (**b**) after saline or naloxone (nx, n) administration to placebo- (pla, p) or morphine (mor, m)-treated rats receiving vehicle (veh, v), or HA1004. The immunoreactivity corresponding to TH phospho-Ser40 is expressed as a percentage of that in the control group (pla + veh + saline; defined as 100%). Data are the mean ± s.e.mean ($n = 4$–6). *$P < 0.05$ versus the group receiving saline instead of nx; $^{+}P < 0.05$ versus the group pretreated with pla instead of mor; $^{\#}P < 0.05$ versus the group receiving veh instead of HA. Bottom panels: representative bands from autoradiograms at the known apparent molecular weight for TH. β-actin was used as an internal loading control.

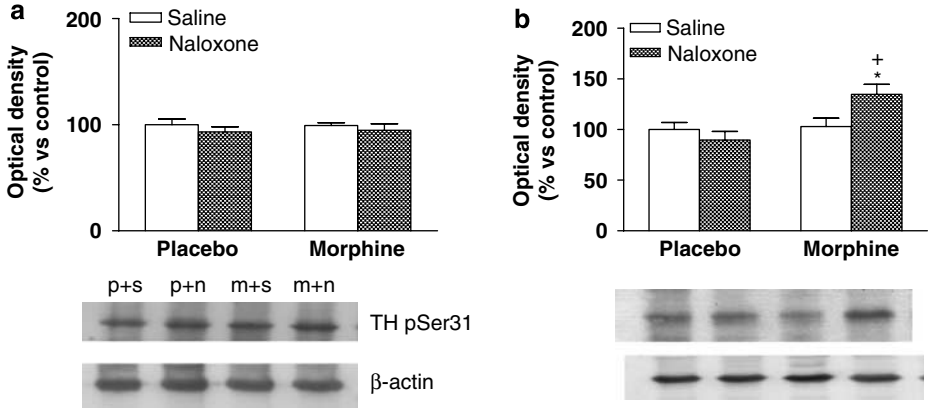

Figure 5 Western blotting analysis of TH phospho (p) Ser31 in the right ventricle 60 (**a**) or 90 min (**b**) after saline or naloxone (nx, n) administration to placebo- (pla, p) or morphine (mor, m)-treated rats. The immunoreactivity corresponding to TH phospho-Ser31 is expressed as a percentage of that in the control group (pla + saline; defined as 100%). Data are the mean ± s.e.mean ($n = 4$–6). *$P < 0.05$ versus the group receiving saline instead of nx; $^{+}P < 0.05$ versus the group pretreated with pla instead of mor. Bottom panels: representative bands from autoradiograms at the known apparent molecular weight for TH. β-actin was used as an internal loading control.

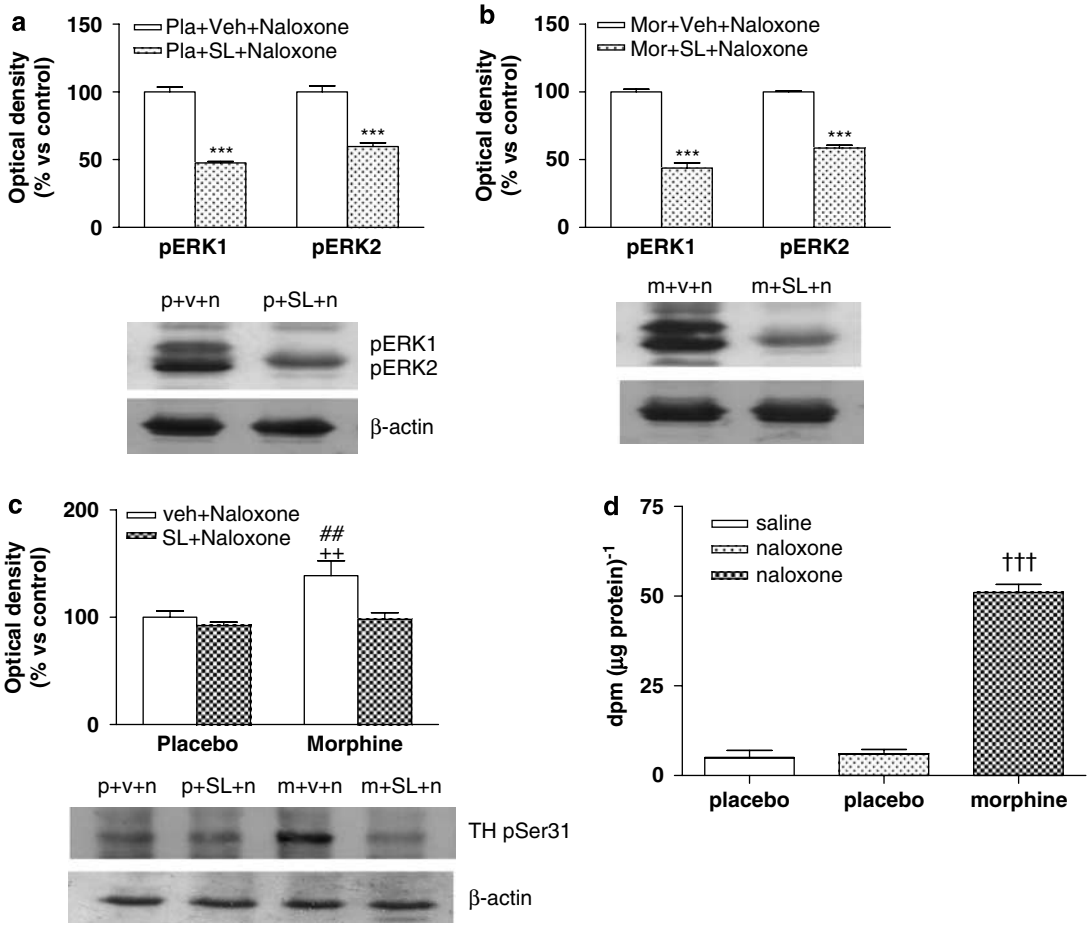

Figure 6 ERK1/2 (**a**, **b**) and TH phospho (p) Ser31 (**c**) immunoblots and TH activity (**d**) in right ventricle from placebo-(pla, p) or morphine(mor, m)-dependent rats 90 min after s.c. administration of naloxone (nx, n) in the absence or presence of SL327 (SL, 100 mg kg^{-1}, i.p.), 1 h before nx. Phospho(p)ERK1/2 or pSer31 TH immunoreactivity bands were measured, normalized to the background values and expressed as percentage of controls. Data are the mean ± s.e.mean ($n = 4$–6). ***$P < 0.001$ versus the group receiving vehicle (veh, v) instead of SL; $^{++}P < 0.01$ versus the group pretreated with pla instead of mor; $^{\#\#}P < 0.01$ versus mor + SL + nx; $^{\dagger\dagger\dagger}P < 0.001$ versus pla + nx.

shown in Figures 6a and b, phosphorylation of ERK1/2 was significantly decreased in the presence of SL327 (100 mg kg^{-1}) in both controls and morphine-withdrawn animals. As SL327 effectively reduced basal levels of phospho-ERK 1/2 immunoreactivity, we injected SL327 in control rats and in animals made dependent on morphine,

1 h before saline or naloxone and we have determined phospho-Ser31 TH in the right ventricle 90 min after the administration of the opioid antagonist. As shown in Figure 6c, phospho-Ser31 TH levels decreased in the right ventricle of morphine-dependent rats injected with SL327 before naloxone, when compared to morphine-dependent rats treated with vehicle instead of SL327. As mentioned above, SL327 effectively reduces basal levels of phospho-ERK 1/2 immunoreactivity, thereby suggesting that the decrease in phosphoSer-31 TH levels after treatment with SL327 is not caused by a non-specific action of the compound on MEK. Thus, these results suggest that TH phosphorylation at Ser31 following morphine withdrawal occurs downstream of ERK.

Effects of morphine withdrawal of TH activity

Phosphorylation of TH has been reported to increase TH activity (Dunkley *et al*., 2004). To examine whether TH phosphorylation was accompanied by the changes in enzymatic activity, we determined TH activity, using the ^{3}H-water assay, in extracts of the right ventricle. Consistent with the effect of morphine withdrawal on TH phosphorylation, present results show that TH activity was significantly augmented during morphine withdrawal compared with the placebo group receiving naloxone (Figure 6d).

When all the parameters investigated in this study were measured in the left ventricle, similar results were obtained (data not shown).

Discussion and conclusions

It is known that chronic morphine alters the level and/or activity of various μ-opioid receptor-signalling elements. These chronic adaptive molecular mechanisms involve some PKs, which are relevant for signalling processes involving protein phosphorylation (Liu and Anand, 2001). However, the intracellular signal transduction pathways that regulate TH in the heart during morphine withdrawal have not been defined. In this report, we have investigated the possible phosphorylation of TH by the PKA or ERK pathways, using HA-1004 (a selective PKA inhibitor) or SL327 (a selective inhibitor of MEK).

Chronic opiate exposure induces numerous neurochemical adaptations in the noradrenergic system, including increased expression of TH (Nestler, 1992; Maldonado, 1997; Nestler and Aghajanian, 1997; Boundy *et al*., 1998). Recent findings from our group have also shown the involvement of an upregulated PKA in activating TH levels during morphine withdrawal (González-Cuello *et al*., 2004; Almela *et al*., 2006). The present results show that naloxone-induced morphine withdrawal resulted in an increase in the NA turnover and in the expression of total TH in the right ventricle. This upregulation of TH would be expected to increase the capacity of noradrenergic neurons to synthesize NA, which could contribute to the increase in NA turnover seen in the heart during morphine dependence. As already reported (Martínez *et al*., 2003), the present data demonstrated that HA1004 but not calphostin C significantly blocks the enhancement of NA turnover and the total TH expression during morphine withdrawal in the right ventricle. Our finding suggests that PKA activity is necessary for the enhancement of NA turnover observed during morphine withdrawal in the heart.

On the other hand, changes in the state of phosphorylation of TH, the rate-limiting enzyme in the synthesis of catecholamines, are critically involved in the regulation of catecholamine synthesis and function. In particular, increases in the phosphorylation of Ser40 and Ser31 accelerate TH activity, thereby stimulating the production of neurotransmitter in catecholamine-containing terminals (Kumer and Vrana, 1996; Dunkley *et al*., 2004). In this study, we also assessed the changes in TH phosphorylation following morphine withdrawal in the heart. The results of this work provided evidence for TH phosphorylation during naloxone-induced morphine withdrawal in the noradrenergic nerve terminals innervating the right ventricle. Using phosphorylation state-specific antibodies directed towards Ser40 or Ser31 in this study, we have shown that naloxone-induced morphine withdrawal increased the level of TH phosphorylation at Ser40 and Ser31 in the right ventricle, concomitantly with enhanced TH activity. Altogether, these data suggest that Ser40 and Ser31 phosphorylation of TH may be important modulators of TH activity during naloxone induced-morphine withdrawal and might be directly involved in regulating NA turnover and in the increase in the force and rate of contraction observed during morphine withdrawal in morphine-withdrawn rats (Rabadán *et al*., 1997, 1998).

It is well known that the phosphorylation of Ser40 results in a considerable increase of TH activity (Dunkley *et al*., 2004) and is associated with TH activity and catecholamine synthesis *in vivo*. Striatal Ser40 phosphorylation is increased concomitantly with the enhanced TH activity and DOPA synthesis (Haycock and Haycock, 1991). Our results show that the levels of TH phosphorylation at Ser40 in the right ventricle were increased 60 or 90 min after naloxone administration to morphine-dependent rats. However, 60 min after naloxone administration to morphine-dependent rats, there was no change in the levels of TH phosphorylation at Ser31. In contrast, there was an enhancement of TH phosphorylation at Ser31 90 min after naloxone injection to morphine-dependent rats. As phosphorylation of TH is closely associated with the activation of the enzyme, present data are in agreement with the finding showing an increase in TH activity 90 min after naloxone induced-morphine withdrawal.

Agents that increase the cAMP pathway increase phosphorylation of TH at Ser40 (Haycock, 1990). Present results show that the administration of naloxone- to morphine-treated rats induced an increase of TH phosphorylation at Ser40 in the right ventricle. These data suggest that naloxone-induced withdrawal increases the phosphorylated form of the enzyme in noradrenergic terminals innervating the heart. The phosphorylated enzyme has a higher affinity for the pteridine cofactor, accelerating the synthesis of NA (Kumer and Vrana, 1996), and thus the phosphorylation of Ser40 could be involved in the enhancement of NA turnover. Present results are in agreement with previous studies demonstrating that morphine withdrawal increases PKA

levels in the right ventricle (Cerezo *et al*., 2005). To assess the contribution of PKA to the regulation of TH, we examined TH phosphorylation at Ser40 during morphine withdrawal in animals receiving the selective inhibitor of PKA HA-1004. This selective inhibitor has been previously used in different studies from our laboratory (Martínez *et al*., 2003; Benavides *et al*., 2005).

There is a strong evidence that PKA phosphorylates TH at Ser40 and leads to TH activation and catecholamines synthesis in complex systems and *in vivo* (Dunkley *et al*., 2004). Thus, it has been observed an upregulation of the cAMP pathway including activation of PKA and phosphorylation of cAMP response element binding protein during morphine withdrawal in specific brain areas, (Nestler and Aghajanian, 1997; Blendy and Maldonado, 1998; Williams *et al*., 2001). In addition, previous studies from our laboratory have demonstrated that withdrawal from morphine is associated with a marked increase in the ventricular cAMP and PKA levels in parallel with an enhancement of NA turnover (Milanés *et al*., 2000; Cerezo *et al*., 2005). Present results show that the enhancement of NA turnover, the increase of total TH levels and the phosphorylation of TH at Ser40 during morphine withdrawal are dependent on PKA. We have found that concurrent infusion of HA 1004 with morphine inhibited the enhancement of NA turnover, the increase in total TH and the phosphorylation (activation) of TH at Ser40 in the noradrenergic terminals innervating the heart. These results suggest that PKA pathway is directly involved in the adaptive changes that occur after naloxone-precipitated morphine withdrawal.

On the other hand, Peart and Gross (2006) have demonstrated that chronic morphine preconditioning is mediated by a PKC-independent pathway involving PKA. The results obtained in this study demonstrated that PKA but not PKC is involved in the cardiac adaptive changes observed during morphine withdrawal. Taken together, these results suggest that alterations in PKA pathway may be important in the regulation of cardiac function.

Recently, some studies have demonstrated that the modulation of ERK pathway in multiple brain regions was relevant to tolerance, dependence or reward after acute or chronic administration of addictive drugs (Valjent *et al*., 2004). Thus, acute or chronic morphine treatment resulted in the increases of pERK expression on the supraspinal level and spinal cord neurons (Schulz and Höllt, 1995; Cao *et al*., 2005). The only PK reported to phosphorylate TH at Ser31 *in vitro* was ERK (Haycock *et al*., 1992; Lindgren *et al*., 2002). *In situ* phosphorylation of TH at Ser31 increases TH activity and catecholamine synthesis (Dunkley *et al*., 2004). Given that TH is phosphorylated on a specific serine residue (Ser31) by the ERK, it is possible that activation of ERK1/2 in the heart provides a way in which TH is regulated under morphine dependence.

Previous results from our laboratory using western blot and inmunohistochemistry showed that naloxone induced-morphine withdrawal increases phosphorylated ERK1/2 in the heart, indicating that this treatment increases ERKs activity (Almela *et al*., 2007). In this study, we, therefore, utilized the MEK inhibitor SL327 to check the involvement of ERK1/2 in TH phosphorylation at Ser31 during morphine withdrawal. We found that the treatment with 100 mg kg^{-1}, a dose that selectively blocks MEK, (Atkins *et al*., 1998; Pozzi *et al*., 2003) decreased the morphine withdrawal stimulation of Ser31 phosphorylation in the right ventricle. These data are in agreement with a previous study in the CNS (Nuñez *et al*., 2007) and suggest that morphine withdrawal induced an activation of ERKs, which results in enhanced Ser31 phosphorylation.

In conclusion, this study suggests that morphine withdrawal might stimulate TH activity and accelerate NA turnover in the heart involving the activation of PKA and ERKs and phosphorylation of TH at Ser40 and Ser31. The novel signalling events reported here provide additional mechanisms by which naloxone-induced morphine withdrawal may modulate TH phosphorylation in the heart and may be an important contribution to understand the cardiac disorders that occur in morphine addicts.

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Conflict of interest

The authors state no conflict of interest.

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