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Cross-Talk between Protein Kinase A and Mitogen-Activated Protein Kinases Signalling in the Adaptive Changes Observed during Morphine Withdrawal in the Heart

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ABSTRACT

Our previous studies have shown that morphine withdrawal induced an increase in the expression of protein kinase (PK) A and mitogen-activated extracellular kinase (MAPK) pathways in the heart during morphine withdrawal. The purpose of the present study was to evaluate the interaction between PKA and extracellular signal-regulated kinase (ERK) signaling pathways mediating the cardiac adaptive changes observed after naloxone administration to morphine-dependent rats. Dependence on morphine was induced by a 7-day subcutaneous implantation of morphine pellets. Morphine withdrawal was precipitated on day 8 by an injection of naloxone (2 mg/kg). ERK1/2 and tyrosine hydroxylase (TH) phosphorylation was determined by quantitative blot immunolabeling using phosphorylation state-specific antibodies. Naloxone-induced morphine withdrawal activates ERK1/2 and phosphorylates TH at Ser31 in the right and left ventricle, with an increase in the mean arterial blood pressure and heart rate. When *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004), a PKA inhibitor, was infused, concomitantly with morphine, it diminished the expression of ERK1/2. In contrast, the infusion of calphostin C (a PKC inhibitor) did not modify the morphine withdrawal-induced activation of ERK1/2. The ability of morphine withdrawal to activate ERK that phosphorylates TH at Ser31 was reduced by HA-1004. The present findings demonstrate that the enhancement of ERK1/2 expression and the phosphorylation state of TH at Ser31 during morphine withdrawal are dependent on PKA and suggest cross-talk between PKA and ERK1/2 transduction pathway mediating morphine withdrawal-induced activation (phosphorylation) of TH.

The development of opioid addiction involves complex adaptive changes in opioid receptors and associated signaling systems, leading to neuronal plasticity in specific brain regions (Nestler and Aghajanian, 1997; Ueda, 2004). However, adaptive changes also occur in other tissues and cells expressing opioid receptors, such as in the heart (Pugsley, 2002). Although the μ-opioid receptor is negatively coupled to the adenylate cyclase/cAMP-dependent protein kinase (PK) A pathway upon acute stimulation (Childers, 1991), the pathway is up-regulated in cardiac tissues after chronic morphine treatment (Milanés et al., 1999). Furthermore, it has been demonstrated that PKA plays an important role in regulating protein phosphorylation and contraction in cardiac muscle (for review, see Sugden and Bogoyevitch, 1995). 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).

Extracellular signal-regulated kinase (ERK), one member of mitogen-activated extracellular kinase (MAPK) family, transduces a broad range of extracellular stimuli into diverse intracellular responses. The ERK signaling pathway could be important as a regulator of cardiac function (for review, see Michel et al., 2001). Recently, several studies have shown

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ABBREVIATIONS: PK, protein kinase; NA, noradrenaline; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated extracellular kinase; TH, tyrosine hydroxylase; HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide; DMSO, dimethyl sulfoxide; SL327, α-(amino((4-aminophenyl)thio)methylene)-2-(trifluoromethyl)benzeneacetonitrile; MEK, mitogen-activated protein kinase; TBST, Tris-buffered saline/Tween 20; p, phosphorylated; HR, heart rate; MAP, mean arterial blood pressure.

that this pathway contributes to naloxone-precipitated withdrawal in morphine-dependent rats (Ren et al., 2004; Almela et al., 2007b, 2008).

It is now appreciated that cross-talk among various signal pathways plays an important role in activation of intracellular and intranuclear signal transduction cascades. Different studies have shown a cross-talk between cAMP and MAPK (for review, see Stork and Schmitt, 2002). cAMP stimulates MAPK activity in cultured neurons (Villalba et al., 1997; Vossler et al., 1997) and is required for its nuclear translocation (Impey et al., 1998). Cross-talk between cAMP/PKA and MAPK pathways is necessary to regulate genetic expression (Sengupta et al., 2007). It has been demonstrated that PKA, MAPK, and mitogen- and stress-activated protein kinase are activated in the same subset of CA1 pyramidal neurons and that Ca^{2+}-stimulated adenyl cyclase activity is indispensable for the training-induced activation of MAPK, mitogen- and stress-activated protein kinase, and cAMP response element-binding protein (Sindreu et al., 2007). So, the increase in PKA activity may be necessary to support the activation of MAPK during morphine withdrawal in the heart. However, there is no evidence that PKA activation is required for stimulation of ERKs and subsequently phosphorylation of TH at Ser31 in morphine-dependent rats. Because the mechanism implicated in the ERK1/2 activation during morphine dependence and withdrawal remains unknown, the present study examined whether the interaction between PKA and ERK signaling pathways, in the heart, mediates the enhancement of TH phosphorylation observed after naloxone administration to morphine-dependent rats.

Materials and Methods

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 (lights on, 8:00 AM–8:00 PM) in a room with controlled temperature (22 ± 2°C), humidity (50 ± 10%), and food and water available ad libitum. Rats were prehandled for several days preceding the experiment to minimize stress, as described previously (Laorden et al., 2000). All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the local committee.

Experimental Procedure. Rats were rendered tolerant/dependent on morphine by subcutaneous implantation of morphine base pellets (75 mg), one on day 1, two on day 3, and three on day 5, under light ether anesthesia. 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 behaviorally and biochemically (Rabadán et al., 1997; Milanés et al., 2000). On day 8, the animals pretreated with morphine or placebo pellets were injected with saline subcutaneously or with naloxone (2 mg/kg s.c.). We used this model because the adaptive changes observed in the heart are more evident after naloxone-precipitated withdrawal than after deprivation from morphine.

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. 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 or 90 min later.

To determine the effects of PKA and PKC on the morphine withdrawal-induced changes in ERK1/2, animals were continuously infused for 7 days, via subcutaneous osmotic minipumps, which deliver at 1 μl/h (Alzet model 2001; Alza, Palo Alto, CA), with HA-1004, a PKA-selective inhibitor (40 nmol/day) (Hidaka et al., 1984); calphostin C, a PKC-selective inhibitor (40 pmol/day) (Kobayashi et al., 1989); or vehicle. PKA inhibitor was dissolved in sterile water and PKC inhibitor was dissolved in dimethyl sulfoxide (DMSO) and serially diluted in MiIli-Q water (final concentration of DMSO, 0.06%) (Millipore, Billerica, MA). 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 μl/h). On day 8, a withdrawal syndrome was induced by subcutaneous naloxone (2 mg/kg) injection. To determine the effect of inhibiting ERK phosphorylation on the morphine withdrawal-induced changes in TH phosphorylation in the heart, TH phosphorylated at Ser31 levels was determined in morphine-dependent and control rats treated with HA-1004 or SL327, a selective inhibitor of mitogen-activated protein kinase (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 at dose of 100 mg/kg (Almela et al., 2006).

Animals were killed by decapitation 60 or 90 min after administration of naloxone or saline for phosphorylated ERKs evaluation and for TH phosphorylation analysis. The hearts were rapidly removed, and the right and left ventricle were dissected, fresh-frozen, and stored immediately at −80°C until use. Samples were placed in homogenization buffer [phosphate-buffered saline, 2% SDS plus protease (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor Cocktail Set (Calbiochem, Schwalbach, Germany)], and homogenized for 50 s before centrifugation at 6000*g* 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 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, electrophoresed, and transferred onto polyvinylidene difluoride membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding of antibodies was prevented by incubating membranes in 1% bovine serum albumin in Tris-buffered saline/Tween 20 (TBST: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20). The blots were incubated overnight at room temperature [for phospho-(p)TH] or at 4°C (for pERK, PKA, PKCδ), with the following primary antibodies: specific polyclonal PKA catalytic subunit antibody (1:2000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); polyclonal anti PKCδ (1:1000 dilution; Sigma-Aldrich, St. Louis, MO); monoclonal anti-pERK1/2 (1:1000 dilution; Santa Cruz Biotechnology, Inc.), polyclonal anti-pSer31 TH (1:250 dilution; Millipore Bioscience Research Reagents, Temecula, CA), in TBST with bovine serum albumin. After extensive washings with TBST, the membranes were incubated for 1 h, at room temperature, with peroxidase-labeled secondary antibodies (anti-rabbit for PKA, PKCδ, and pTH, total-ERK; Santa Cruz Biotechnology, Inc.; anti-mouse for phospho-ERK1/2; Santa Cruz Biotechnology, Inc.) at 1:5000 dilution. After washing, immunoreactivity was detected with an enhanced chemiluminescence Western blot detection system (ECL; GE Healthcare, Madrid, Spain) and visualized by Hyperfilm-ECL (GE Healthcare). Quantification of PKA (42-kDa), PKCδ (78-kDa), phospho-ERK1/2 (42- and 44-kDa), and TH phosphorylated at Ser31 (60-kDa) bands was carried out by densitometry (AlphaImager; Nucliber, Madrid, Spain). The integrated optical density of bands was normalized to the background values. The optical density of the bands was normalized as a percentage of average of control. Relative variations between bands of experimental samples and control samples were calculated in the same image. We used β-actin or total ERK as our loading control for all the experiments. Before reprobing, blots were stripped by incubation with stripping buffer (25 mM

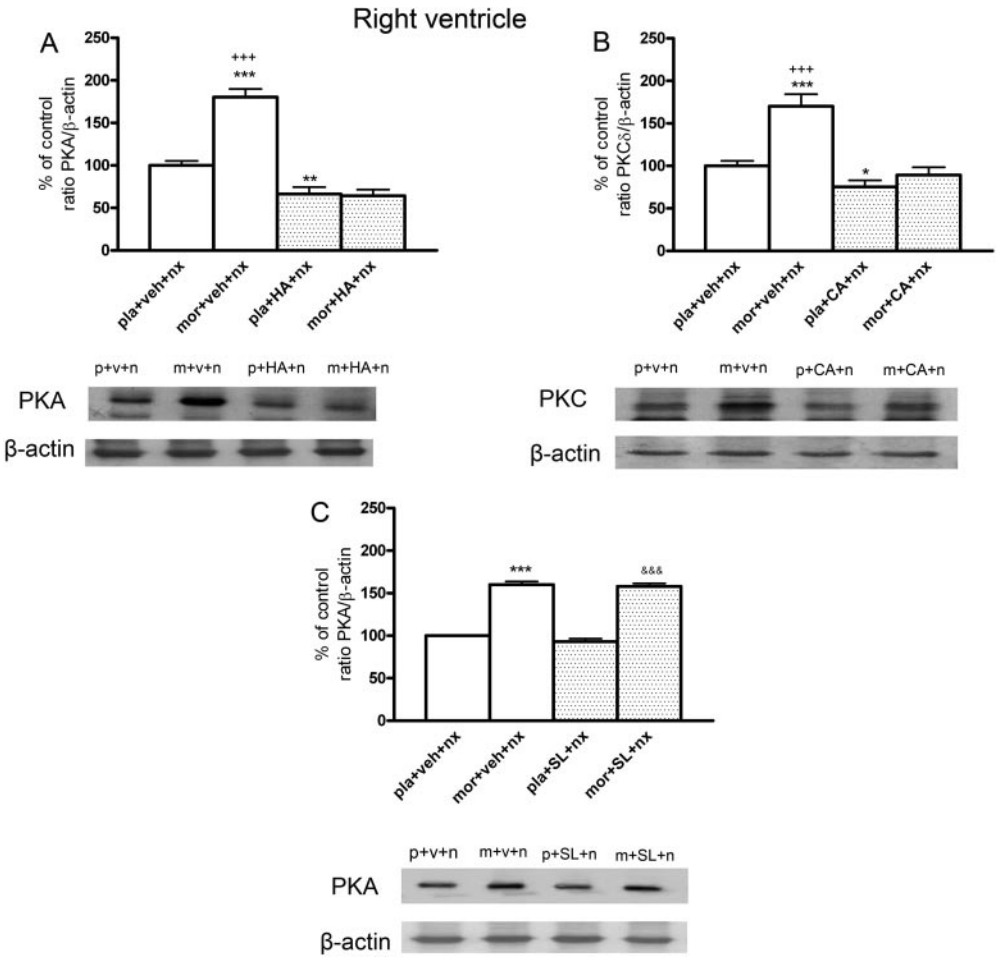

Fig. 1. Immunoblots of PKA (A) and PKCδ (B) in right ventricle from placebo (pla, p)- or morphine (mor, m)-dependent rats after naloxone-precipitated withdrawal in vehicle (veh, v)-infused rats and in animals chronically administered with HA-1004 (HA) or calphostin C (CA). Animals received subcutaneous implantation of placebo or morphine (75-mg) pellets for 7 days and concomitantly were infused with vehicle, HA (40 nmol/day), or CA (40 pmol/day). On day 8, rats were decapitated 90 min after naloxone (nx, n; 2 mg/kg s.c.) administration in presence of vehicle, HA, CA, or SL327 (SL; 100 mg/kg i.p.) (C). SL was administered 1 h before naloxone. The immunoreactivity corresponding to PKA or PKCδ is expressed as a percentage of that in the control group (pla + veh + nx; defined as 100% value). Data are the mean ± S.E.M. ($n = 4$–5). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus the pla + veh group receiving nx. +++, $p < 0.001$ versus the group treated with mor + HA + nx or mor + CA + nx. &&&, $p < 0.001$ versus pla + SL + nx. Bottom, representative bands from autoradiograms at the known apparent molecular weight for PKA and PKCδ.

glycine and 1% SDS), pH 2.0, for 1 h at 37°C. Blots were subsequently reblocked and probed with 1:1000 anti-β-actin (43 kDa; Cell Signaling Technology Inc., Danvers, MA) or anti-total ERK (Santa Cruz Biotechnology, Inc.). The ratio of PKA/β-actin, PKCδ/β-actin, phospho-ERK1/total ERK, phospho-ERK2/total ERK, and pTH/β-actin was plotted and analyzed.

Hemodynamic Parameters. The rats were anesthetized with thiopental sodium (40 mg/kg i.p.), intubated, and placed on a heated table to maintain body temperature at 37°C. A polyethylene-50 cannula was placed in the right femoral artery for measuring. Catheters were connected to pressure transducers (L969-A07; Abbott Ireland, Sligo, Republic of Ireland), and blood pressure and heart rate (HR) were monitored on a PowerLab8/30 (ADInstruments Pty Ltd., Oxford, UK) and analyzed with LabChart software (ADInstruments Pty Ltd.). Naloxone (2 mg/kg) was injected subcutaneously after a 30-min stabilization period, and its effect on mean arterial blood pressure (MAP) and HR was evaluated in rats implanted with placebo or morphine concomitantly treated with vehicle or HA-1004.

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 polyvinylidene difluoride membranes were obtained from Bio-Rad Laboratories (Teknovas, Bilbao, Spain). Naloxone HCl and Western blot reagents were purchased from Sigma-Aldrich. Naloxone HCl was dissolved in sterile 0.9% NaCl (saline) and administered in volumes of 0.1 ml/100 g body weight. HA-1004 was purchased from Sigma-Aldrich and dissolved in Milli-Q sterile water. Calphostin C [2-(12-(2-(benzoyloxy)propyl)-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetrametoxy-3,10-dioxo-1-perylenyl)-1menthylethyl carbonic acid 4-hydroxyphenyl ester] was purchased from Sigma/RBI (Natick, MA). The chronic delivery of HA-1004 or calphostin C was achieved by means of Alzet 2001 osmotic minipumps (Alza), which deliver at 1 μl/ h. SL327 was purchased from Ascent Scientific (Bristol, UK).

Statistical Analysis. The results are expressed as the mean ± S.E.M. Data were analyzed by one-way analysis of variance followed by the Newman-Keuls post hoc test. Body weight gain and loss in naive- and morphine-dependent rats was analyzed by unpaired Student's t test. One-way analysis of variance followed by Dunnett's multiple comparison test was used when required. Differences with a p value less than 0.05 were considered significant.

Results

Before performing the Western blot analysis, we assessed the efficacy of chronic treatment with morphine by pellets implantation, which has been shown previously to induce tolerance and dependence to the effects of morphine (González-Cuello et al., 2003). For this purpose, the weight of the animals was recorded on the days of pellets implantation and on the day of killing (day 8), before receiving any injection. Rats treated with morphine showed a significantly lower body weight gain than animals receiving placebo pellets (data not shown). Chronic morphine-treated animals showed a significant weight loss 60 or 90 min after naloxone injection compared with the placebo-pelleted group also receiving naloxone. The injection of naloxone in rats chronically treated with HA-1004, calphostin C, or SL327 concomitantly with morphine induced a weight loss, similar to the group chron-

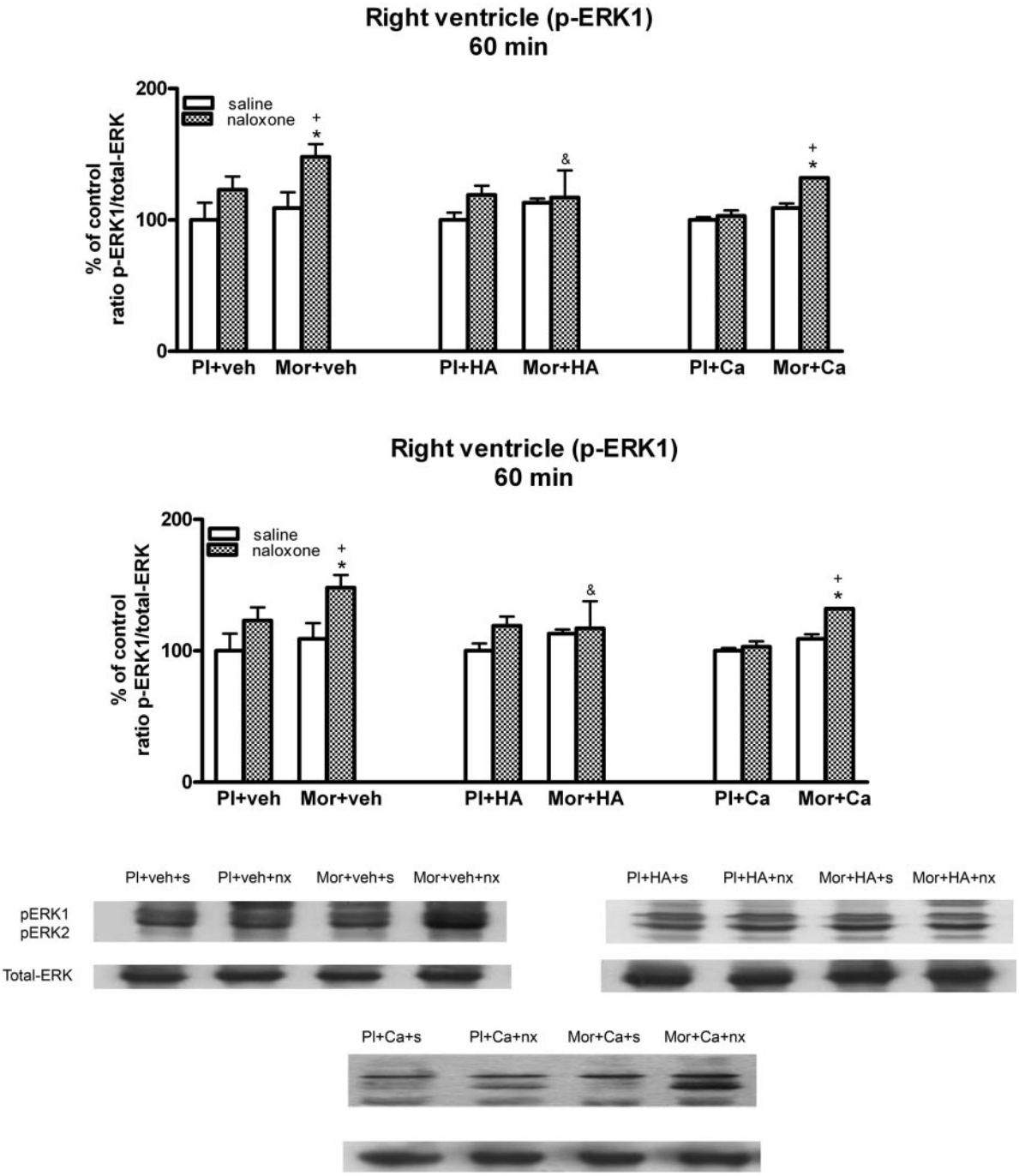

Fig. 2. Western blotting analysis of pERK1 and pERK2 immunoreactivity levels in the right ventricle 60 min after saline (s) or naloxone (nx) administration to placebo (Pl)- or morphine (Mor)-treated rats receiving vehicle (veh), HA-1004 (HA), or calphostin (Ca). The immunoreactivity corresponding to ERK1 or ERK2 is expressed as a percentage of that in the control group (Pl + veh + saline; defined as 100% value). Data are the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$ versus the dependent group receiving saline instead of naloxone. +, $p < 0.05$ versus the group pretreated with placebo instead of morphine injected with naloxone. &, $p < 0.05$ versus the group receiving vehicle instead of HA. Bottom, representative bands from autoradiograms at the known apparent molecular weight for pERK1 or pERK2.

ically pretreated with vehicle plus morphine (data not shown). All the animals treated with vehicle, HA-1004, calphostin C, or SL327 undergoing morphine withdrawal displayed characteristic abstinence symptoms: wet-dog shakes, teeth chattering, ptosis, tremor, piloerection, lacrimation, rhinorrhea, chromodacryorrhea, and spontaneous jumping.

PKA and PKC δ Expression in the Right Ventricle. In this study, we have evaluated PKA or PKCδ levels in the right ventricle after the inhibition of PKA by HA-1004 or SL327 and the inhibition of PKCδ by calphostin C. As shown in Fig. 1A, chronic pretreatment with HA-1004 concomitantly with morphine antagonized the expression of PKA in both controls and morphine-withdrawn animals. However, the administration of SL327 did not inhibit the increase in PKA expression observed after naloxone administration to morphine-dependent animals (Fig. 1C). Regarding calphostin C, this inhibitor blocked the expression of PKCδ observed after naloxone-induced withdrawal in

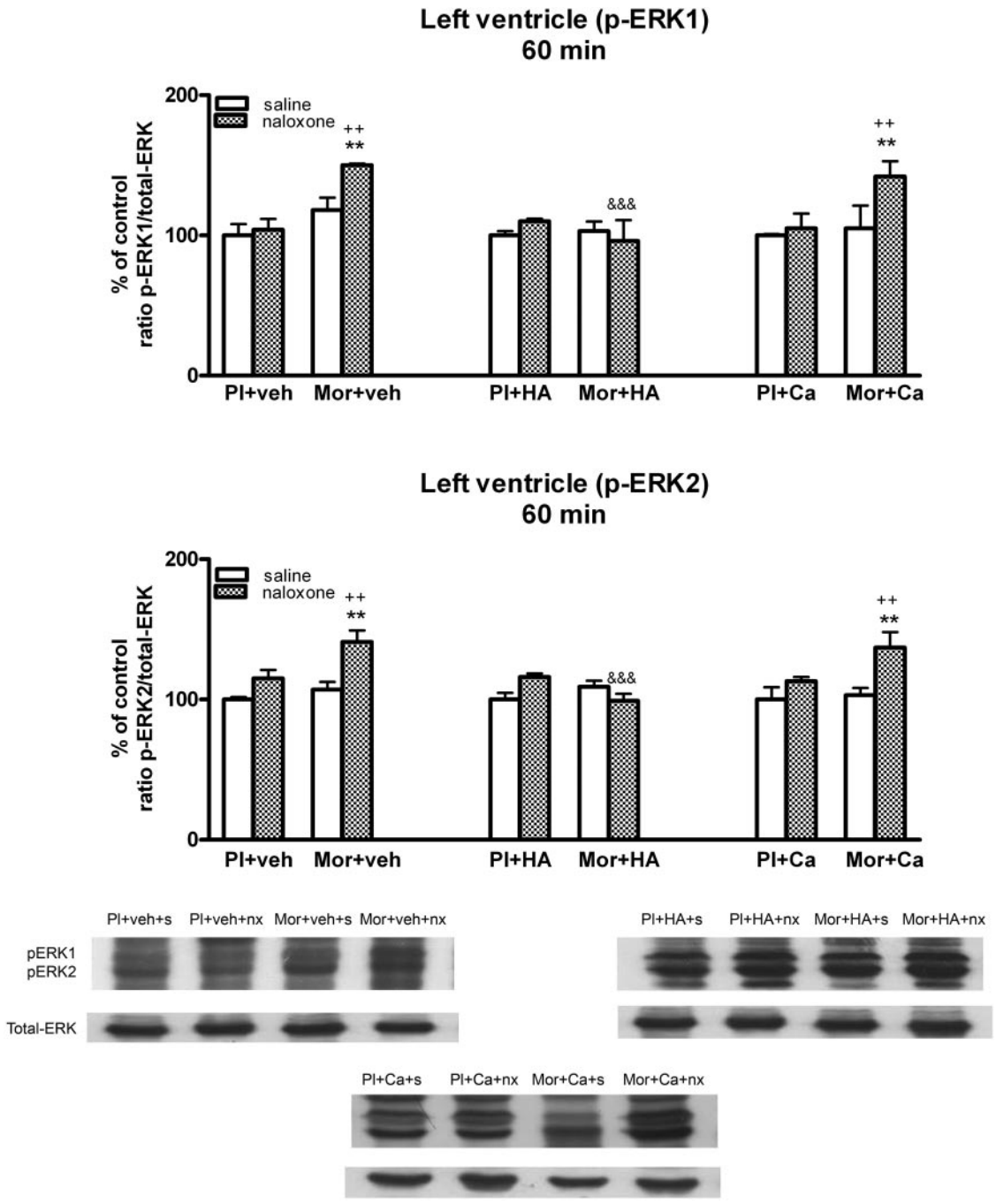

Fig. 3. Western blotting analysis of pERK1 and pERK2 immunoreactivity levels in the left ventricle 60 min after saline (s) or naloxone (nx) administration to placebo (Pl)- or morphine (Mor)-treated rats receiving vehicle (veh), HA-1004 (HA), or calphostin (Ca). The immunoreactivity corresponding to ERK1 or ERK2 is expressed as a percentage of that in the control group (Pl + veh + saline; defined as 100% value). Data are the mean ± S.E.M. ($n = 4–6$). **, $p < 0.01$ versus the dependent group receiving saline instead of naloxone. ++, $p < 0.01$ versus the group pretreated with placebo instead of morphine injected with naloxone. &&&, $p < 0.001$ versus the group receiving vehicle instead of HA. Bottom, representative bands from autoradiograms at the known apparent molecular weight for pERK1 or pERK2.

both control and morphine-dependent animals (Fig. 1B). Similar results were obtained in the left ventricle (data not shown).

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 observed in the heart during morphine withdrawal (Cerezo et al., 2005).

Effects of HA-1004 and Calphostin C on ERK Phosphorylation in the Heart. Having established that HA-1004 and calphostin C antagonized the expression of PKA and PKC observed during morphine withdrawal, we then sought to determine whether increased PKA or PKC activity was also responsible for the naloxone-induced increases of ERK phosphorylation in withdrawn rats. For this purpose, the selective PKA inhibitor HA-1004 or the PKC inhibitor calphostin C were coadministered with morphine. At different times, chronic pretreatment with HA-1004, concomitantly with morphine, antagonized the increase in the ERK1/2 phosphorylation observed during

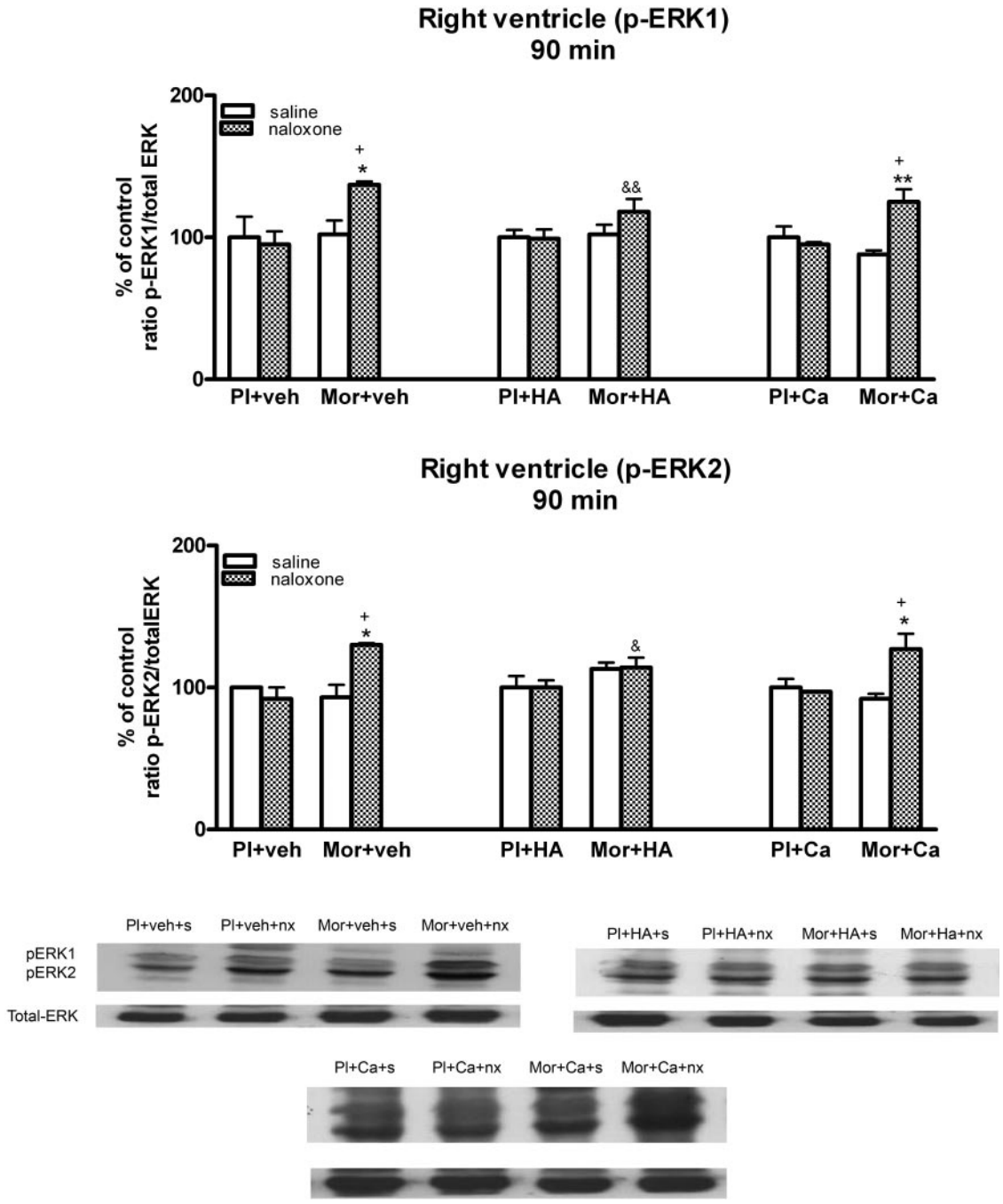

Fig. 4. Western blotting analysis of pERK1 and pERK2 immunoreactivity levels in the right ventricle 90 min after saline (s) or naloxone (nx) administration to placebo (Pl)- or morphine (Mor)-treated rats receiving vehicle (veh), HA-1004 (HA), or calphostin (Ca). The immunoreactivity corresponding to ERK1 or ERK2 is expressed as a percentage of that in the control group (Pl + veh + saline; defined as 100% value). Data are the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$ and **, $p < 0.01$ versus the dependent group receiving saline instead of naloxone. +, $p < 0.05$ versus the group pretreated with placebo instead of morphine injected with naloxone. &, $p < 0.05$ and &&, $p < 0.01$ versus the group receiving vehicle instead of HA. Bottom, representative bands from autoradiograms at the known apparent molecular weight for pERK1 or pERK2.

morphine withdrawal in the right (Figs. 2 and 4) and left (Figs. 3 and 5) ventricle. Calphostin C did not prevent the increase in ERK1/2 observed in the right ventricle or left ventricle 60 (Figs. 2 and 3) or 90 min (Figs. 4 and 5) after the injection of naloxone to morphine-dependent rats. These results suggest a cross-talk between PKA, but not PKC, and ERK pathways.

Effects of SL327 or HA-1004 on TH Phosphorylation at Ser31. Because TH phosphorylation at Ser31 is dependent on extracellular signal-regulated protein kinases 1 and 2 (Haycock et al., 1992), we analyzed phospho-Ser31 TH levels in the right and left ventricle after inhibition of ERK by SL327, a drug that prevents the activation of ERK by inhibiting MEK, the upstream kinase of ERK (Atkins et al., 1998). First, we determined the basal levels of phosphorylated (activated) ERK1/2 in the right and left ventricle from control and from morphine withdrawn rats pretreated with SL327. As shown in Fig. 6, phosphorylation of ERK1/2 was significantly decreased in the presence of SL327 in both controls and morphine-

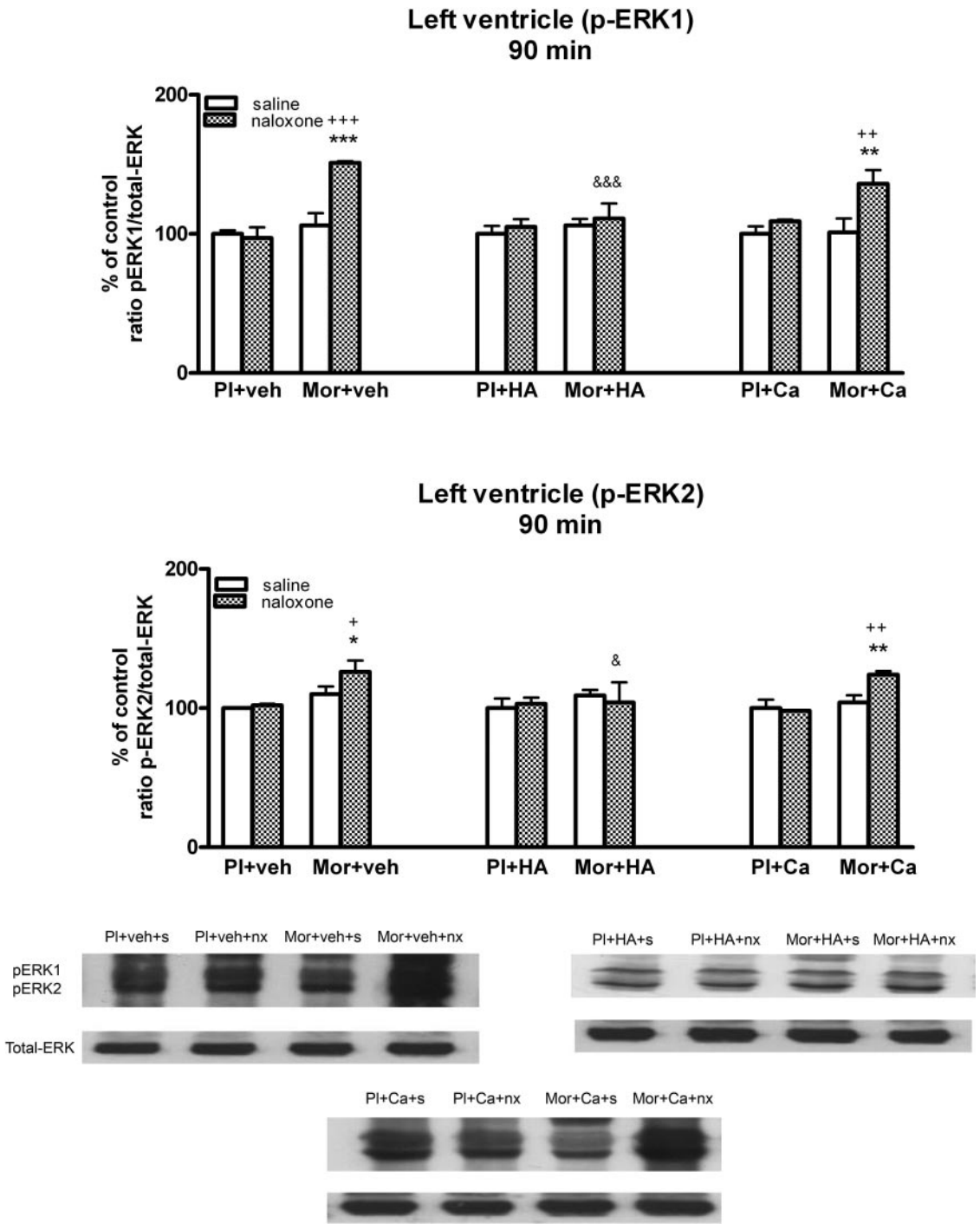

Fig. 5. Western blotting analysis of pERK1 and pERK2 immunoreactivity levels in the left ventricle 90 min after saline (s) or naloxone (nx) administration to placebo (Pl)- or morphine (Mor)-treated rats receiving vehicle (veh), HA-1004 (HA), or calphostin (Ca). The immunoreactivity corresponding to ERK1 or ERK2 is expressed as a percentage of that in the control group (Pl + veh + saline; defined as 100% value). Data are the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus the dependent group receiving saline instead of naloxone. +, $p < 0.05$; ++, $p < 0.01$; and +++, $p < 0.001$ versus the group pretreated with placebo instead of morphine injected with naloxone; &, $p < 0.05$ and &&&, $p < 0.001$ versus the group receiving vehicle instead of HA. Bottom, representative bands from autoradiograms at the known apparent molecular weight for pERK1 or pERK2.

withdrawn animals. Because 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 determined phospho-Ser31 TH in the right and left ventricle 90 min after the administration of the opioid antagonist.

As shown in Fig. 7, A and B, phospho-Ser31 TH levels decreased in the right and left ventricle of morphine-dependent rats injected with SL327 before naloxone, compared with morphine-dependent rats treated with vehicle instead of SL327. As mentioned above, SL327 effectively reduces basal levels of phospho-ERK1/2 immunoreactivity, thereby suggesting that the decrease in phospho-Ser31 TH levels after treatment with SL327 is not caused by a nonspecific action of

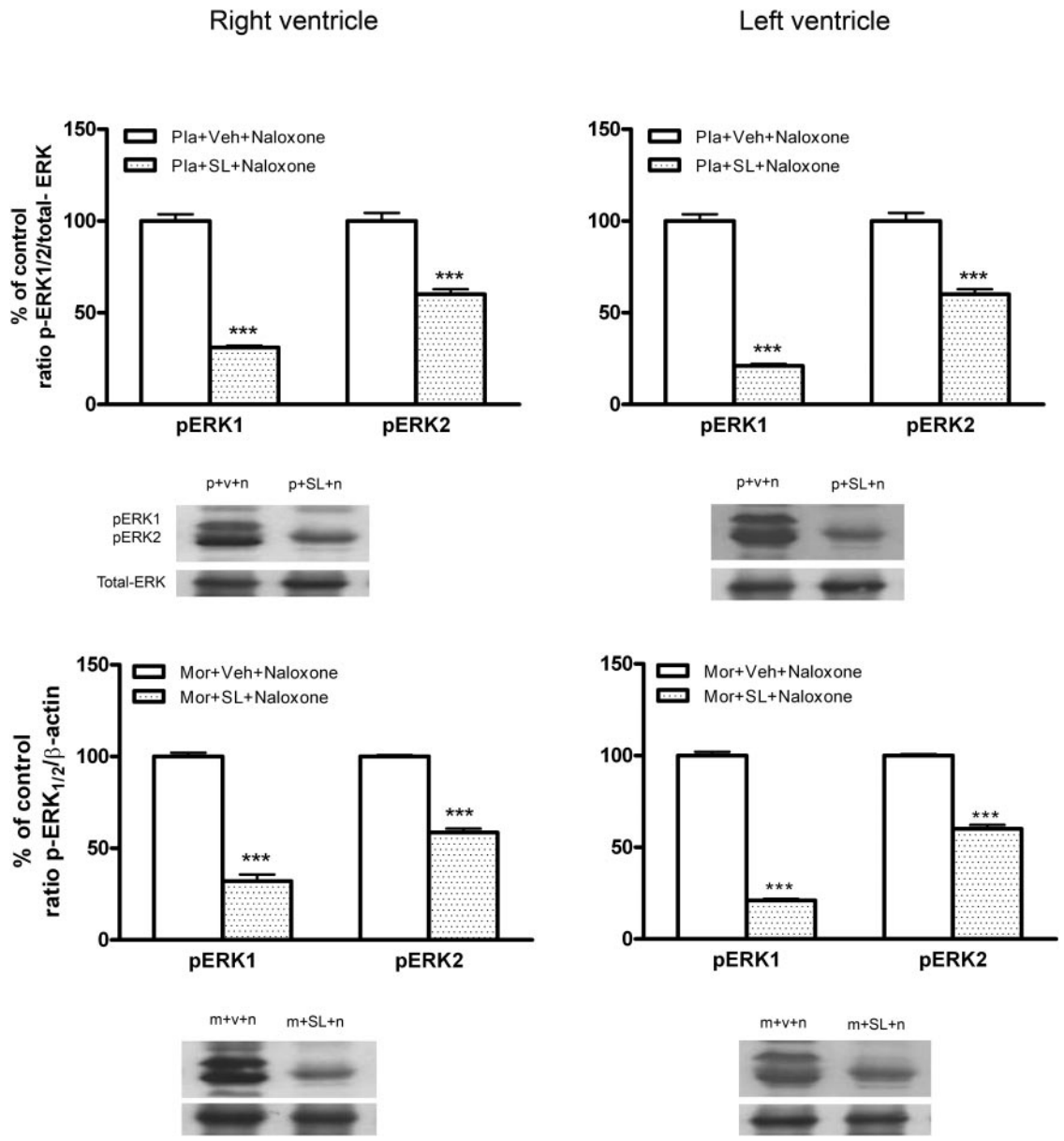

Fig. 6. Immunoblots of ERK1/2 in the right and left ventricles isolated from placebo (pla, p)- or morphine (mor, m)-dependent rats after subcutaneous administration of naloxone (n) in rats treated with SL327 (SL; 100 mg/kg i.p.) or vehicle (veh, v), 1 h before naloxone injection. pERK1 and pERK2 immunoreactive bands were measured, normalized to the background values and are expressed as percentages of controls. Data correspond to mean ± S.E.M. ($n = 4$). ***, $p < 0.001$ versus its control group. Bottom, representative bands from autoradiograms at the known apparent molecular weight for pERK1 or pERK2.

the compound on MEK. Thus, these results suggest that TH phosphorylation at Ser31 after morphine withdrawal occurs downstream of ERK.

According to a previous study (Almela et al., 2008), 60 min after naloxone-precipitated morphine withdrawal, there were no changes in the levels of phospho-Ser31 TH (data not shown). However, rats chronically treated with morphine and given naloxone showed significant increases in phospho-Ser31 TH in the right and left ventricle 90 min after the opioid antagonist injection compared with the corresponding control group receiving naloxone and with the morphine-dependent animals receiving saline (Fig. 7, C and D). To assess the contribution of PKA to the regulation of TH, we have examined TH phosphorylation at Ser31 during morphine withdrawal in animals receiving the selective inhibitor of PKA HA-1004. Chronic infusion of HA-1004 prevents the ability of naloxone-precipitated morphine withdrawal to increase the levels of phospho-Ser31 TH in the right and left ventricle (Fig. 7, C and D). Because SL327 failed to inhibit PKA expression in the right and left ventricle, these results demonstrated that PKA activity is required for ERK-mediated TH phosphorylation at Ser31 after morphine withdrawal.

Cardiovascular Effects of Naloxone Precipitated Morphine Withdrawal. As shown in Fig. 8, A and B, the pretreatment with HA instead of vehicle did not induce significant changes in MAP. However, the basal MAP was significantly decreased in rats treated chronically with morphine versus placebo (Fig. 8A). In addition, the injection of naloxone (2 mg/kg s.c.) in placebo- + vehicle- or placebo- +

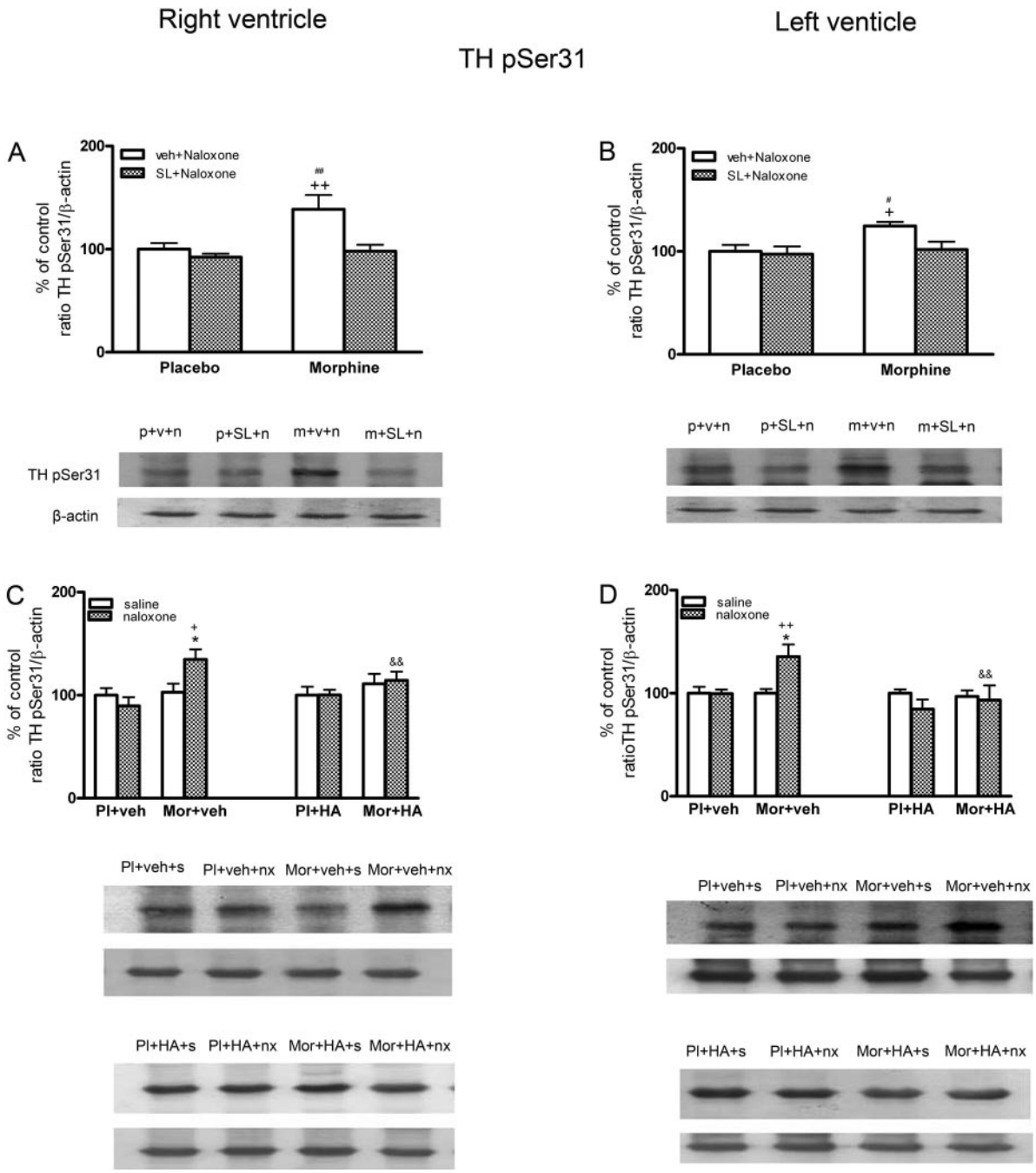

Fig. 7. Western blotting analysis of TH pSer31 in the right ventricle and left ventricle 90 min after saline (s) or naloxone (n, nx) administration to placebo (p, Pl)- or morphine (m, Mor)-treated rats receiving vehicle (v, veh) or SL327 (SL) (A and B), veh, or HA-1004 (HA) (C and D). The immunoreactivity corresponding to TH phospho-Ser31 is expressed as a percentage of that in the control group (p + v + n or Pl + veh + saline; defined as 100% value). Data are the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$ versus the dependent group receiving saline instead of naloxone. +, $p < 0.05$ and ++, $p < 0.01$ versus the group pretreated with placebo instead of morphine injected with naloxone. &&, $p < 0.001$ versus the group receiving vehicle instead of HA. #, $p < 0.05$ and ##, $p < 0.01$ versus the dependent group injected with SL327. Bottom, representative bands from autoradiograms at the known apparent molecular weight for TH phosphorylated at Ser31.

HA1004-treated rats evoked no significant changes in MAP. However, naloxone administration to morphine-dependent rats induced an immediate and significant increase in MAP (2–8 min) versus placebo + vehicle rats injected with naloxone (Fig. 8B). Similarly, the injection of naloxone in rats chronically treated with HA-1004 concomitantly with morphine induced significant changes in the MAP compared with the morphine-dependent groups treated with vehicle instead of HA-1004 (Fig. 8B).

The morphine chronic treatment also decreases the basal HR versus placebo treatment. The rats pretreated with HA-1004 instead of vehicle did not show changes in HR (Fig. 9A). However, 10 min after naloxone administration to rats treated with morphine + vehicle or morphine + HA-1004 there was a significant enhancement of HR. The time of peak effect was 20 min after naloxone injection (Fig. 9B), although HR remained elevated in the morphine-dependent groups for 1 h after naloxone (data not shown).

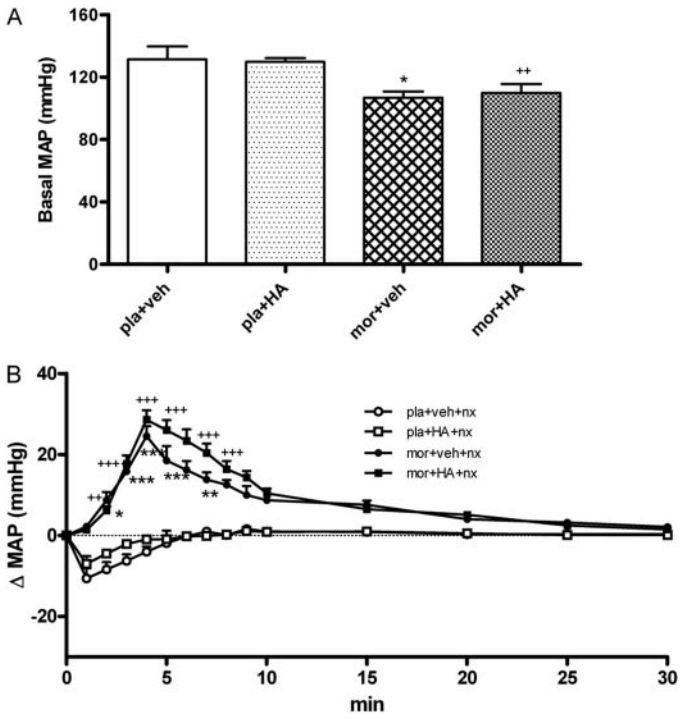

Fig. 8. Baseline MAP (mm Hg) in rats implanted with morphine (mor) or placebo (pla) pellets concomitantly with vehicle (veh) or HA-1004 (HA), just before naloxone injection (A). Effects of naloxone (2 mg/kg s.c.) on changes in MAP, in rats pretreated with mor or pla concomitantly with veh or HA. Naloxone (nx) was injected at time 0 (B). Data are the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus pla + veh. ++, $p < 0.01$ and +++, $p < 0.001$versus pla + HA.

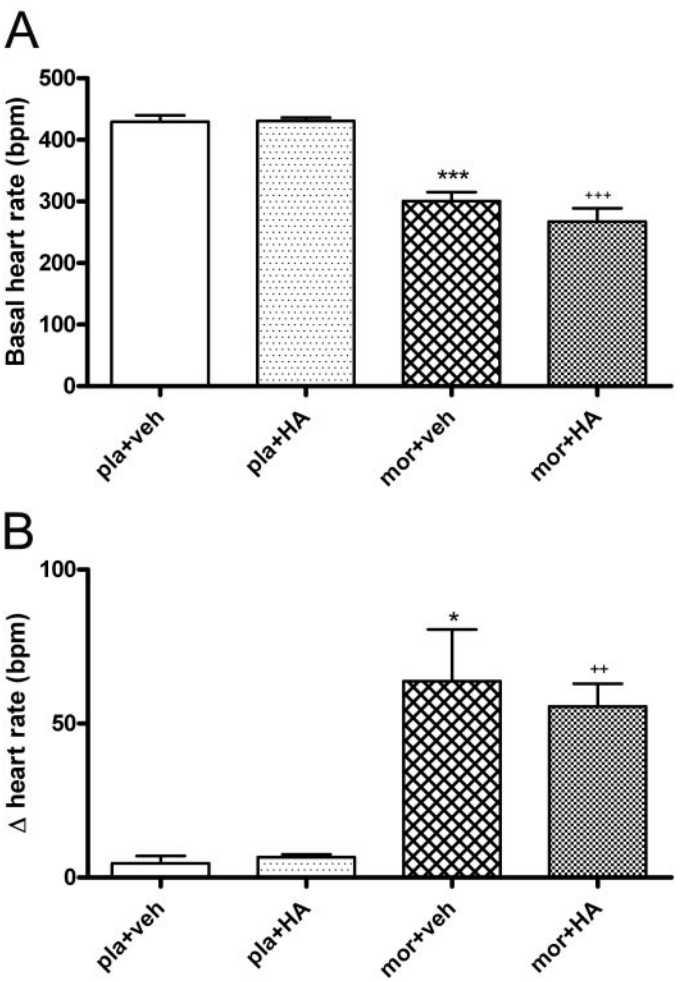

Fig. 9. Baseline HR (beats per minute, bpm) in rats with morphine (mor) or placebo (pla) pellets implantation concomitantly with vehicle (veh) or HA-1004 (HA), just before naloxone injection (A). Changes in HR 20 min after naloxone (2 mg/kg s.c.) injection, in rats pretreated with mor or pla concomitantly with veh or HA (B). Data are the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$ and ***, $p < 0.001$ versus pla + veh. ++, $p < 0.01$ and +++, $p < 0.001$ versus pla + HA.

Discussion

Although it is known that opioid receptors activation attenuates both contractile dysfunction and development of myocardial infarction (Gross et al., 2007; Peart et al., 2008), there is limited data about the potential cardiac effects of chronic opioid receptors stimulation. Chronic opiate exposure induces cardioprotective effects (Peart and Gross, 2006) and numerous neurochemical adaptations in the noradrenergic system at heart levels (González-Cuello et al., 2003).

Previous studies have demonstrated that chronic μ-opioid receptor stimulation decreases muscle sympathetic nerve activity (Kienbaum et al., 2001, 2002), NA plasma concentration (Kienbaum et al., 2001), and dopamine turnover in the heart (Rabadán et al., 1997). According with these data, present results demonstrated that chronic morphine treatment decreases baseline cardiovascular parameters (MAP and HR) compared with placebo-treated rats. However, μ-opioid receptor blockade by naloxone in patients with chronic opioid abuse or in morphine-dependent rats unmasks these effects, resulting in markedly increased muscle sympathetic nerve activity, NA plasma concentrations (Kienbaum et al., 2001), NA and dopamine turnover (Pugsley, 2002; Almela et al., 2008), and total TH expression (Almela et al., 2008). In addition, when naloxone was injected in morphine-treated rats, there was an increase in MAP and HR, two objective and accurate measurable signs of opioid withdrawal in humans. Altogether, these results suggest that an up-regulation of TH would be expected to increase the capacity of noradrenergic neurons to synthesize NA, which could contribute to the increase in NA turnover and in the hemodynamic changes seen in the heart during morphine dependence.

Previous results from our laboratory demonstrated that when the selective PKA inhibitor HA-1004 was infused concomitantly with morphine pellets implantation, it diminished the increase in NA turnover, total TH levels (Almela et al., 2007a), and phosphorylation of TH (Almela et al., 2008). However, present results show that HA-1004 failed to affect significantly MAP or HR during withdrawal. Because the cardiovascular response induced by morphine withdrawal was not altered by the PKA inhibitor, a dissociation could be made between the physiological and cellular/molecular responses to naloxone-precipitated morphine withdrawal. Hence, the data of the present study suggest that the cardiovascular response and the biochemical changes that occur during morphine withdrawal are mediated by different pathways and mechanisms.

Many pathways implicated in morphine dependence are subject to feedback mechanisms that can either amplify or suppress their own signaling, and there is considerable signaling from one pathway to another, a phenomenon known

as cross-talk. Consequently, the responses that cells mount to specific environmental conditions depend on the sum of the intensity and duration of signals from several pathways and how they interact with each other. Because there is not evidence that PKA activation is required for stimulation of MAPK induced by morphine withdrawal in the heart (Almela et al., 2007b), this study evaluates the cross-talk between cAMP/PKA and ERKs and subsequently phosphorylation of TH at Ser31 in morphine-dependent rats. These two ancient and conserved signaling pathways are involved in various central processes, including drug addiction (Ren et al., 2004)

Although several signaling pathways can mediate activity-dependent phosphorylation of TH, the only protein kinase 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 ERK, it is possible that activation of ERK1/2 in the heart provides a way in which TH is regulated under morphine dependence. In agreement with previous studies (Almela et al., 2008), we found that treatment with 100 mg/kg SL327, 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 and left ventricle. These data suggest that morphine withdrawal induced an activation of ERKs, which results in enhancement of TH phosphorylation at Ser31.

According to previous results from our laboratory using Western blot and immunohistochemistry (Almela et al., 2007b), present results showed that naloxone-induced morphine withdrawal increases phosphorylated ERK1/2 in the heart, indicating that this treatment enhanced ERKs activity. In the present study, we used the PKA or the PKC inhibitor to check the involvement of these pathways in the activation of ERKs during morphine withdrawal. Present results demonstrated that HA-1004 antagonized the increase in ERK1/2 observed during morphine withdrawal. These data indicate that PKA signaling pathway modulates the increased levels of ERK1/2 observed during morphine withdrawal and suggest a cross-talk between PKA and ERK in the heart that could mediate the adaptive changes observed after naloxone injection to morphine-dependent rats. However, the activation of ERK1/2 observed in the present study is independent of PKC pathway. According to this result, it has been indicated that there is no direct interaction between PKC and MAPK pathways mediating the effect of endothelin-1 on glucose transporter 1 transcription (Kao and Fong, 2008).

Although the mechanism of cross-talk between PKA and ERK pathways has not yet been clarified, it is possible that PKA pathway facilitates MEK1/2 that activates the ERK1/2 pathway (Stork and Schmitt, 2002; Obara et al., 2007). The activated ERK pathway increases the phosphorylation of proteins related to morphine dependence, including TH. Using phosphorylation state-specific antibodies directed toward TH at Ser31, in the present study we have shown that HA-1004 blocked the increase in the level of TH phosphorylation at Ser31 induced after naloxone injection to morphine-dependent rats in the right and left ventricle. These data suggest that cross-talk between PKA and ERK pathways is a key regulatory design necessary to regulate the Ser31 phosphorylation of TH.

In conclusion, our results indicate that TH phosphorylation at Ser31 is regulated by ERK and PKA pathways through cross-talk mechanisms. These findings provide a new explication to understand the complex mechanisms implicated in the adaptive changes observed during morphine withdrawal and could be useful for future treatment strategies focused on addictive processes.

References

Almela P, Cerezo M, González-Cuello A, Milanés MV, and Laorden ML (2007a) Differential involvement of 3′,5′-cyclic adenosine monophosphate-dependent protein kinase in regulation of Fos and tyrosine hydroxylase expression in the heart after naloxone induced morphine withdrawal. *Naunyn Schmiedebergs Arch Pharmacol* **374:**293–303.

Almela P, Milanés MV, and Laorden ML (2007b) Activation of ERK signalling pathway contributes to the adaptive changes in rat hearts during naloxone-induced morphine withdrawal. *Br J Pharmacol* **151:**787–797.

Almela P, Milanés M, and Laorden M (2008) The PKs PKA and ERK 1/2 are involved in phosphorylation of TH at serine 40 and 31 during morphine withdrawal in rat hearts. *Br J Pharmacol* **155:**73–83.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, and Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. *Nat Neurosci* **1:**602–609.

Cerezo M, Milanés MV, and Laorden ML (2005) Alterations in protein kinase A and different protein kinase C isoforms in the heart during morphine withdrawal. *Eur J Pharmacol* **522:**9–19.

Childers SR (1991) Opioid receptor-coupled second messengers systems. *Life Sci* **48:**1991–2003.

Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, and Dickson PW (2004) Tyrosine hydroxylase phosphorylation: regulation and consequences. *J Neurochem* **91:**1025–1043.

González-Cuello A, Milanés MV, Castells MT, and Laorden ML (2003) Activation c-Fos expression in the heart after morphine but not U-50,488H withdrawal. *Br J Pharmacol* **138:**626–633.

Gross ER, Hsu AK, and Gross GJ (2007) GSβ inhibition and K_{ATP} channel opening mediate acute opioid-induced cardioprotection at reperfusion. *Basic Res Cardiol* **102:**341–349.

Haycock JW, Ahn NG, Cobbe MH, and Krebs EG (1992) ERK 1 and ERK 2, Two Microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. *Proc Natl Acad Sci U S A* **89:**2365–2369.

Hidaka H, Inagaki M, Kawamoto S, and Sasaki Y (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:**5036–5041.

Hussain M, Drago GA, Bhogal M, Colyer J, and Orchard CH (1999) Effects of the protein kinase A inhibitor H-89 on Ca^{2+} regulation in isolated ferret ventricular myocytes. *Pflugers Arch* **437:**529–537.

Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, and Storm DR (1998) Crosstalk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* **21:**869–883.

Kamp TJ and Hell JW (2000) Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. *Circ Res* **87:**1095–1102.

Kao YS and Fong JC (2008) Endothelin-1 induction of Glut1 transcription in 3T-L1 adipocytes involves distinct PKCε- and p42/44 MAPK-dependent pathways. *Biochem Biophys Acta* **1780:**154–159.

Kienbaum P, Heuter T, Michel MC, Scherbaum N, Gastpar M, and Peters J (2001) Chronic μ-opioid receptor stimulation in humans decreases muscle sympathetic nerve activity. *Circulation* **103:**850–855.

Kienbaum P, Heuter T, Scherbaum N, Gastpar M, and Peters J (2002) Chronic μ-opioid receptor stimulation alters cardiovascular regulation in humans: differential effects on muscle sympathetic and heart rate responses to arterial hypotension. *J Cardiovasc Pharmacol* **40:**363–369.

Kobayashi E, Nakano H, Morimoto M, and Tamaoki T (1989) Calphostin C (UCN), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159:**548–553.

Laorden ML, Fuertes G, González-Cuello A, and Milanés MV (2000) Changes in catecholaminergic pathways innervating paraventricular nucleus and pituitary-adrenal axis response during morphine dependence: implication of A1 and A2 adrenoceptors. *J Pharmacol Exp Ther* **293:**578–584.

Lindgren N, Goiny M, Herrera-Marschitz M, Haycock JW, Hökfelt T, and Fisone G (2002) Activation of extracellular signal-regulated kinases 1 and 2 by depolarization stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat brain. *Eur J Neurosci* **15:**769–773.

Michel MC, Li Y, and Heusch G (2001) Mitogen-activated protein kinases in the heart. *Naunyn Schmiedebergs Arch Pharmacol* **363:**245–266.

Milanés MV, Fuente T, Marín MT, and Laorden ML (1999) Catecholaminergic activity and 3′,5′-cyclic adenosine monophosphate concentrations in the right ventricle after acute and chronic morphine administration in the rat. *Br J Anaesth* **83:**784–788.

Milanés MV, Fuente T, and Laorden ML (2000) Catecholaminergic activity and 3′,5′-cyclic adenosine monophosphate levels in heart right ventricle after naloxone induced withdrawal. *Naunyn Schmiedebergs Arch Pharmacol* **361:**61–66.

Nestler EJ and Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science* **278:**58–63.

Obara Y, Horgan AM, and Stork PJ (2007) The requirement of Ras and Rap1 for the

activation of ERKs by cAMP, PACAP, and KCL in cerebellar granule cells. *J Neurochem* **101:**470–482.
Peart JN and Gross GJ (2006) Cardioprotective effects of acute and chronic opioid treatment is mediated via different signalling pathways. *Am J Physiol Heart Circ Physiol* **291:**H1746–H1753.
Peart JN, Gross ER, Reichelt ME, Hsu A, Headrick JP, and Gross GJ (2008) Activation of kappa-opioid receptors at reperfusion affords cardioprotection in both rat and mouse hearts. *Basic Res Cardiol* **103:**454–463.
Pozzi L, Håkansson K, Usiello A, Borgkvist A, Lindskog M, Greengard P, and Fisone G (2003) Opposite regulation by typical and atypical anti-psychotics of ERK1/2, CREB and Elk-1 phosphorylation in mouse dorsal striatum. *J Neurochem* **86:**451–459.
Pugsley MK (2002) The diverse molecular mechanism responsible for the actions of opioids on the cardiovascular system. *Pharmacol Ther* **93:**51–75.
Rabadán JV, Milanés MV, and Laorden ML (1997) Effects of chronic morphine treatment on catecholamines content and mechanical response in the rat heart. *J Pharmacol Exp Ther* **280:**32–37.
Rabadán JV, Milanés MV, and Laorden ML (1998) Changes in right atrial catecholamine content in naïve rats and after naloxone-induced withdrawal. *Br J Anaesth* **80:**354–359.
Ren X, Noda Y, Mamiya T, Nagai T, and Nabeshima TA (2004) Neuroactive steroid, dehydroepiandrosterone sulfate, prevents the development of morphine dependence and tolerance via c-Fos expression linked to the extracellular signal-regulated protein kinase. *Behav Brain Res* **152:**243–250.
Sengupta N, Vinod PK, and Venkatesh KV (2007) Crosstalk between cAMP/PKA and MAP kinase pathways is a key regulatory design necessary to regulate FLO11 expression. *Biophys Chem* **125:**59–71.
Sindreu CB, Scheiner ZS, and Storm DR (2007) Ca^{2+}-stimulated adenylyl cyclases regulate ERK-dependent activation of MSK1 during fear conditioning. *Neuron* **53:**79–89.
Stork PJ and Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signalling in the regulation of cell proliferation. *Trends Cell Biol* **12:**258–266.
Sugden PH and Bogoyevitch MA (1995) Intracellular signalling through protein kinases in the heart. *Cardiovasc Res* **30:**478–492.
Ueda H (2004) Locus-specific involvements of anti-opioid systems in morphine tolerance and dependence. *Ann N Y Acad Sci* **1025:**376–382.
Villalba M, Bockaert J, and Journot L (1997) Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. *J Neurosci* **17:**83–90.
Vossler MR, Yao H, York RD, Pan MG, Rim CS, and Stork PJ (1997) cAMP activates MAP kinase and Elk-1 trough a B-Raf and Rap-1-dependent pathway. *Cell* **89:**73–82.
Wiechelman KJ, Braun RD, and Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for colour formation. *Anal Biochem* **175:**231–237.

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