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Inhibition of protein kinase C but not protein kinase A attenuates morphine withdrawal excitation of rat hypothalamus–pituitary–adrenal axis

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Abstract

Our previous studies have shown an enhanced activity of the hypothalamus–pituitary–adrenocortical axis response in rats withdrawn from morphine, which results from an increase in the hypothalamic paraventricular nucleus noradrenergic activity that is dependent on α-adrenoceptor activation. The first objective of this work was to examine the effect of protein kinase A (PKA) and protein kinase C (PKC) inhibitors on morphine withdrawal-induced changes in corticosterone release (an index of the hypothalamus–pituitary–adrenocortical axis activity) and in catecholaminergic turnover in the paraventricular nucleus. Plasma corticosterone levels as well as the concentration of noradrenaline, 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) in the paraventricular nucleus were determined. The second purpose of the study was to assess whether kinase inhibitors, administered continuously through s.c. osmotic minipumps, get into the brain. Chronic pretreatment for 7 days with the selective PKA inhibitor *N*-(2′guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004) concomitantly with morphine did not affect the increase in corticosterone release observed after naloxone-precipitated morphine withdrawal. However, pretreatment with the selective PKC inhibitor, calphostin-C significantly antagonized the corticosterone hypersecretion in morphine-withdrawn rats. Neither HA-1004 nor calphostin-C co-administered with morphine for 7 days did modify the morphine withdrawal-induced increase in noradrenaline turnover. Pretreatment with HA-1004 inhibits the increase in dopamine turnover during morphine withdrawal, whereas calphostin-C did not affect the DOPAC/dopamine ratio. Our results might indicate that expression of morphine dependence for hypothalamus–pituitary–adrenocortical axis hyperactivity involves PKC but not PKA signaling mechanisms. It is suggested that in rats PKC may be up-regulated during morphine dependence. High-performance liquid chromatography (HPLC) analysis of hypothalamic tissue from rats perfused with kinase inhibitors demonstrates that both calphostin-C and HA-1004 can cross the blood–brain barrier when administered peripherally.

Keywords: Hypothalamus–pituitary–adrenocortical axis; Morphine withdrawal; PKA (protein kinase A); PKC (protein kinase C); Corticosterone; Noradrenaline turnover; Dopamine turnover

1. Introduction

Chronic use of opioids produces tolerance/dependence, but the molecular mechanisms underlying these phenomena are not well defined. Morphine withdrawal induces a state of neuronal hyperexcitability in the brain which has been linked to alteration in a number of second messenger systems and neurotransmitters (Maldonado, 1997; Nestler and Aghajanian, 1997). Morphine withdrawal also produces a complex endocrine alteration in rats, including the activation of the hypothalamus–pituitary–adrenocortical axis, which constitutes the major axis responsible for maintenance of homeostasis. This alteration of the axis is characterized by Fos protein expression in the hypothalamic paraventricular nucleus (Laorden et al., 2002) as well as by an increased adrenocorticotropic hormone (ACTH) and corticosterone secretion possibly due to the overproduction and release of corticotropin-releasing factor (CRF) in the paraventricular nucleus (Gonzálvez et al., 1994; Ignar and Kuhn, 1990; Milanés et al., 1998; Vargas et al., 1997).

It is known that the catecholaminergic systems play an important role in the development and maintenance of opioid dependence and in the effects upon drug withdrawal (Maldonado, 1997; Nestler, 1992; Self and Nestler, 1995). Noradrenergic neurons in the nucleus of the solitary tract-A_2 have been shown to project to the parvocellular paraven-

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tricular nucleus, the primary location of CRF (Cunningham and Sawchenko, 1988). The pattern of innervation of neurosecretory CRF neurons by noradrenergic pathways clearly indicates an important role for noradrenaline neurotransmission in the regulation of CRF secretion and in the hypothalamus–pituitary–adrenocortical axis activity. On the other hand, the paraventricular nucleus also receives dopaminergic innervation from incertohypothalamic neurons, which have been suggested to be implicated in the regulation of neurosecretory neurons located in the paraventricular nucleus (Eaton et al., 1996). Recent findings from our laboratory have demonstrated that the increased activity of the hypothalamus–pituitary–adrenocortical axis seen during morphine withdrawal occurs concurrently with a pronounced enhance in noradrenaline and dopamine turnover in the paraventricular nucleus (Fuertes et al., 2000a; Milanés et al., 1998). In addition, the secretory activity of the axis after morphine withdrawal results from an increase in noradrenergic activity that is dependent on α_1- and α_2-adrenoceptor activation (Laorden et al., 2000). However, the signal transduction mechanisms responsible for chronic opioid effects at the hypothalamic level are incompletely understood.

Opioid receptors are coupled by G_i/G_0 proteins to intracellular signaling responses by acting on effector molecules such as adenylate cyclase or phospholipase or regulating ion channel function. The protein kinases pathway is now well known as a major pathway for signal transduction from cell surface opioid receptors to nuclear transcriptional activation. Although the μ-opioid receptor is negatively coupled to the adenylate cyclase/cAMP-dependent protein kinase (PKA) pathway upon acute stimulation (Childers, 1991), the PKA pathway has been shown to be up-regulated in several brain areas with chronic morphine treatment (Nestler, 1992). Therefore, up-regulation of the adenylate cyclase/cAMP transduction system is currently the best characterized potential mechanism for opioid tolerance and dependence. Recently, a number of intracellular pathways have been suggested to play a role in opioid tolerance/dependence. The chronic adaptive molecular mechanisms in these phenomena involve protein kinases (PKA, protein kinase C [PKC]- and mitogen-activated protein [MAP]-kinase) and intracellular calcium, which are relevant for a wide variety of cellular regulatory and signaling processes involving protein phosphorylation and gene expression (Mao et al., 1995; Nestler and Aghajanian, 1997; Nestler, 1992; Schulz and Höllt, 1998; van Haasteren et al., 1999).

The present experiments tested if alteration in the PKA and/or PKC underlies the changes in hypothalamus–pituitary–adrenocortical axis activity associated with chronic morphine treatment and withdrawal. This was assessed by chronically infusing specific inhibitors of PKA (*N*-(2′guanidinoethyl)-5-isoquinolinesulfonamide, HA-1004) and PKC (calphostin-C). We reported the results of a series of experiments in an attempt to elucidate the signaling pathways of morphine withdrawal leading to corticosterone secretion and paraventricular nucleus catecholaminergic activation. To ensure that the kinase inhibitors used in the present study can act at the central nervous system when administered peripherally, in a parallel set of experiments, we investigated whether calphostin-C and/or HA-1004 can cross the blood–brain barrier. This was assessed by high-performance liquid chromatography (HPLC) analysis of the kinase inhibitors in the hypothalamic tissue from rats chronically perfused with calphostin-C or HA-1004 via s.c. osmotic minipumps.

2. Materials and methods

2.1. Animals and experimental procedure

Male Sprague–Dawley rats (220–230 g at the beginning of the experiments) were housed four to five per cage under a 12-h light/dark cycle (L: 8:00–20:00 h) in a room with controlled temperature (22 °C), humidity (50 ± 10%) and food and water available ad libitum. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Local Committee. As the stress can affect the activity of the hypothalamus–pituitary–adrenocortical axis, the experimental design included efforts to reduce the potential effect of stress. For that, animals were handled daily (between 9:00 and 10:00 h) along 7 days before the experimental day in the experimental room to adapt them to manipulation and minimise nonspecific stress responses.

Groups of 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 anesthesia. Control animals were implanted with placebo pellets containing lactose instead of morphine on the same time schedule. This morphine treatment paradigm has been shown to produce profound states of tolerance and dependence as well as to result in characteristic biochemical adaptations within the paraventricular nucleus and behavioral alterations (Couceyro and Douglass, 1995; Fuertes et al., 2000a,b; Milanés et al., 1998; Vargas et al., 1997). Animals were co-administered for 7 days with Milli-Q water (vehicle), via s.c. implantation of osmotic minipumps (Alzet mod. 2001, 1 μl/h). On day 8, the animals pretreated with morphine or placebo pellets were injected with saline s.c. or naloxone (5 mg/kg s.c.) and were killed by decapitation 30 min later. Withdrawal signs were observed before and for 30 min after administration of the opioid receptor antagonist or saline, at which time many of the acute behavioral effects are manifest (Guitart and Nestler, 1989).

In order to determine the effect of inhibiting protein phosphorylation on the morphine withdrawal-induced hypothalamus–pituitary–adrenocortical axis activation, corticosterone secretion (as a marker of hypothalamus–pituitary–adrenocortical axis activity) and noradrenaline

and dopamine turnover were determined in tolerant/dependent and naive rats pretreated with inhibitors of PKA and PKC, and were compared with those observed in tolerant/dependent and naive animals that had not been so treated. Briefly, animals were continuously infused for 7 days, via s.c. osmotic minipumps (Alzet mod. 2001, 1 ~ μl/h), with HA-1004 (a PKA-selective inhibitor, (Hidaka et al., 1984); 40 nmol/day) or calphostin-C (a PKC-selective inhibitor, (Kobayashi et al., 1989); 40 pmol/day). Minipumps were implanted simultaneously with the chronic morphine or placebo pellets. Pumps were primed for 5 h before implantation at 37 °C in sterile saline in order to obtain an optimal flow rate (1 μl/h). On day 8, a withdrawal syndrome was induced by s.c. naloxone (5 mg/kg s.c.) or saline s.c. (control) injection and rats were decapitated 30 min later.

In order to determine whether kinase inhibitors get into the brain when administered peripherally, naïve animals were infused for 7 days, via s.c. osmotic minipumps, with HA-1004, calphostin-C or vehicle and, on day 8, were killed by decapitation.

2.2. Corticosterone assays

At the end of the treatment, rats were killed by decapitation between 10:00 and 11:00 a.m. to avoid circadian variations in plasma levels of corticosterone or in the hypothalamic content and turnover of noradrenaline and dopamine. Trunk blood was collected into ice-cooled tubes containing 5% EDTA and then was centrifuged (2500 rpm; 4 °C; 15 min). Plasma was separated, divided into two aliquots and stored at −30 °C until assayed for corticosterone. Plasma levels of corticosterone were estimated, as a sensitive marker of the hypothalamus–pituitary–adrenocortical axis activity, with a commercially available kit for rats (^{125}I-corticosterone radioimmunoassay, ICN, Biomedicals, USA). The sensitivity of the assay was 0.40 ng/ml. The inter- and intra-assay coefficients of variation were 6.5% and 4.4%, respectively. The antibody cross-reacted 100% with corticosterone and <0.5% with other steroids.

2.3. Estimation of catecholamines and their metabolites in the paraventricular nucleus

After decapitation, the brains were removed rapidly, fresh-frozen and stored immediately at −80 °C until use. The hypothalamic tissue containing the paraventricular nucleus was dissected from a coronal brain slice according the technique of Palkovits (1973) and the paraventricular nucleus corresponds to those in plates 25–26, 1800–2100 μm caudally to the bregma (Palkovits and Brownstein, 1988). Noradrenaline, its metabolite in the central nervous system (CNS) MHPG, dopamine, and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were determined by HPLC with electrochemical detection (HPLC/ED), as reported previously (Fuertes et al., 2000a). Briefly, bilateral tissue samples were weighed, were placed in cold perchloric acid (0.1 M) and were homogenised. The homogenates were then centrifuged and the supernatants taken for analysis. Two aliquots of the supernatant from the same tissue sample were used, the first for analysis of noradrenaline, dopamine and DOPAC, and the second for analysis of MHPG. Ten microliters of the first aliquot of each sample was injected into a 5-μm C_{18} reverse-phase column. The mobile phase consisted of a 95:5 (v/v) mixture of water and methanol with sodium acetate (50 mM), citric acid (20 mM), 1-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 and chromatographic data were analysed with a Millennium 2010 Chromatography Manager (Millipore) equipment. DOPAC, noradrenaline and dopamine were simultaneously detected by the described HPLC method. The method for the determination of total MHPG in the paraventricular nucleus is based on the acid-catalysed hydrolysis of MHPG-sulphate (Artigas et al., 1986; Lookingland et al., 1991). The eluent for MHPG determination was as described above, but without 1-octyl-sodium sulphonate. Noradrenaline, dopamine and their respective metabolites 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 peak heights measured. The content of noradrenaline, MHPG, dopamine and DOPAC in the paraventricular nucleus was expressed as ng/g wet weight of tissue.

2.4. Estimation of HA-1004 and calphostin-C in hypothalamic tissues

After decapitation, the brains were removed and the hypothalamic tissue was dissected according to the atlas of Palkovits and Brownstein (1988). Tissue samples were homogenised in acetonitrile and centrifuged; the supernatant was taken for the analysis and was filtered through 0.22 μm GV. Calphostin-C and HA-1004 were determined by HPLC (Shimadzu, Japan) with a diode array detector, using recent HPLC conditions (Chen et al., 1999a). One hundred microliters of calphostin-C and 20 μl of HA-1004 of each sample were injected into a 250 × 4.6 mm Kromasil 100 C_{18} (5 μm) column. Acetonitrile/water (70:30 v/v) containing 0.1% trifluoracetic acid and 0.1% triethylamine was used as the mobile phase for determination of calphostin-C. The column was eluted under isocratic conditions, and the detection wavelength was set at 479 nm. For determination of HA-1004, reverse-phase HPLC was used. Acetonitrile/water containing 0.5% trifluoracetic acid was used as mobile phase and the eluate was monitored at the wavelength of 220 nm. Calibration curves were run at the beginning and at the end of each series assays.

2.5. Drugs and chemicals

Pellets of morphine base (Alcaliber Labs., Madrid, Spain) and lactose were prepared by the Department of Pharmacy and Pharmaceutic Technology (School of Pharmacy, Granada, Spain); noradrenaline bitartrate, MHPG hemipiperazinium salt, dopamine HCl, DOPAC (used as HPLC standards for catecholamines), and naloxone HCl were purchased from Sigma (St. Louis, MO). Naloxone HCl was dissolved in sterile 0.9% NaCl (saline) and given in a volume of 0.1 ml/100 g. Triethylamine and trifluoracetic acid were obtained from Merck (Germany). HA-1004 HCl (*N*-(2′guanidinoethyl)-5-isoquinolinesulfonamide), was purchased from Sigma and was dissolved in Milli-Q (Millipore, Bedford, MA) 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)-1-ethylethyl carbonic acid 4-hydroxyphenyl ester

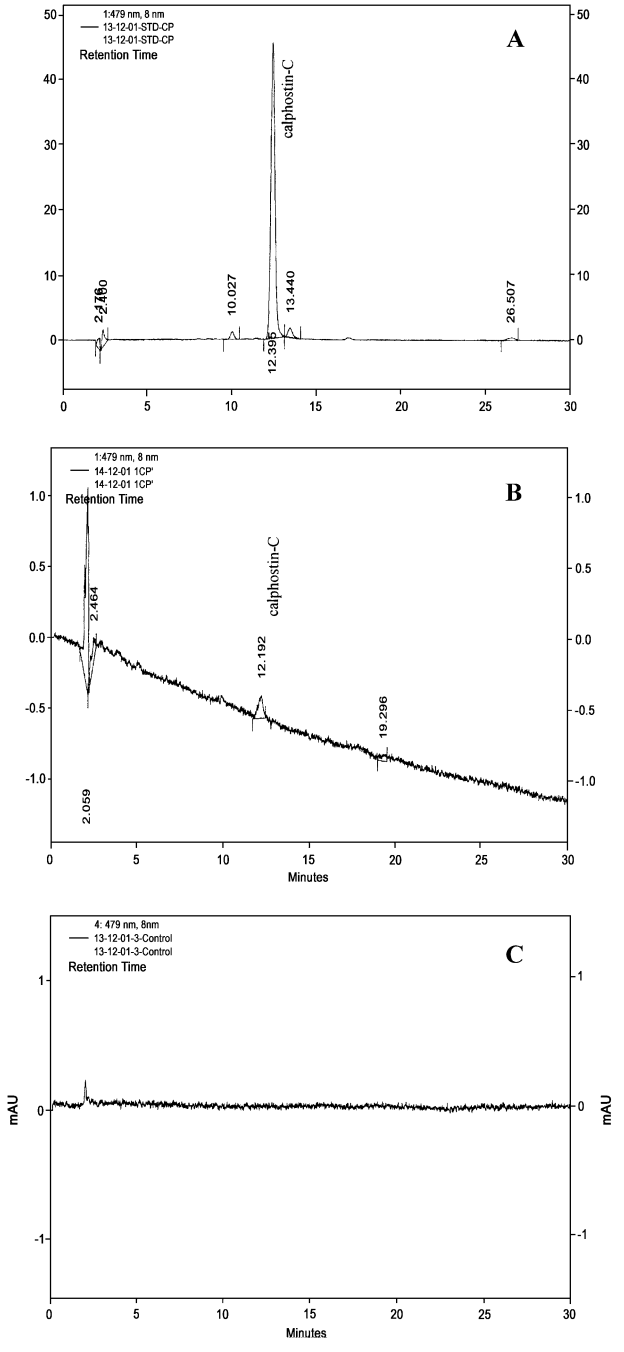

Fig. 1. Representative chromatograms of (A) standard of calphostin-C, (B) hypothalamic sample from animals chronically treated with calphostin-C, and (C) blank hypothalamic sample from control rats treated with vehicle.

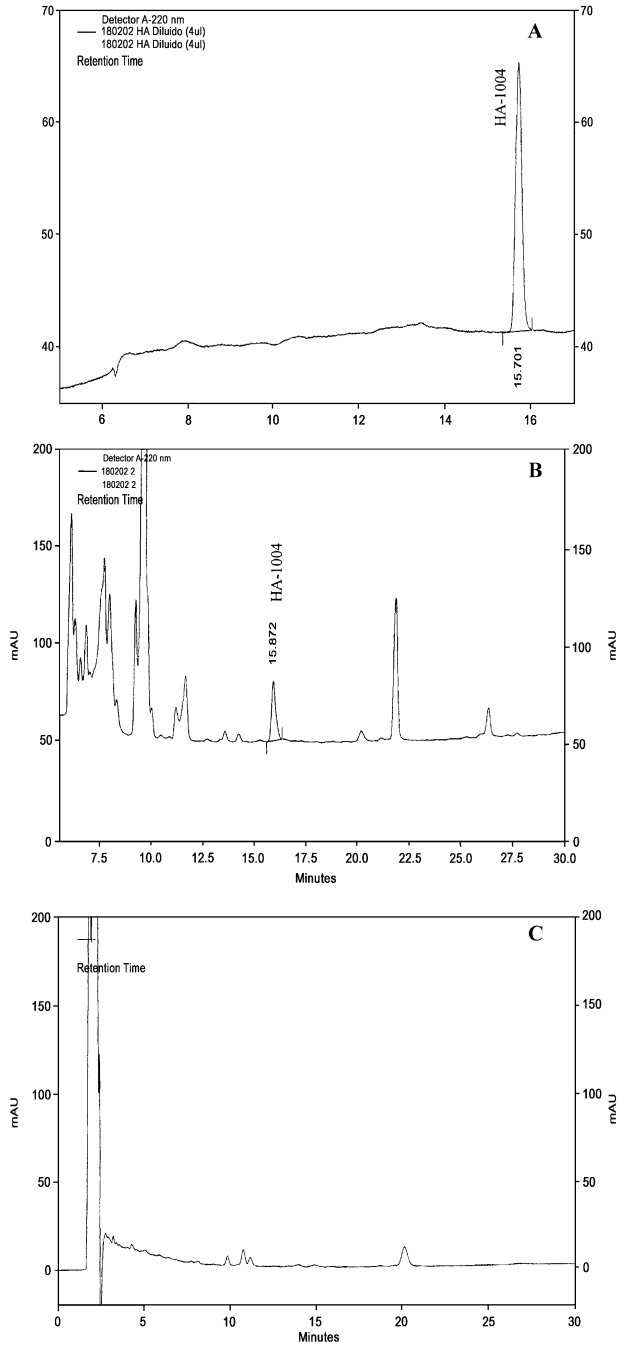

Fig. 2. Representative chromatograms of (A) standard of HA-1004, (B) hypothalamic sample from animals chronically treated with HA-1004, and (C) blank hypothalamic sample from control rats treated with vehicle.

was purchased from RBI (Natick, MA), was dissolved in dimethyl sulfoxide (DMSO) and was serially diluted in Milli-Q water (final concentration of DMSO was 0.06%); aliquots of the stock solutions were stored at −30 °C until used for experimentation. The chronic delivery of HA-1004 and calphostin-C was by means of Alzet 2001 osmotic minipumps (Alza, Palo Alto, CA), which deliver at 1 μl/h. Drugs were prepared fresh everyday. Other reagents were of analytical grade.

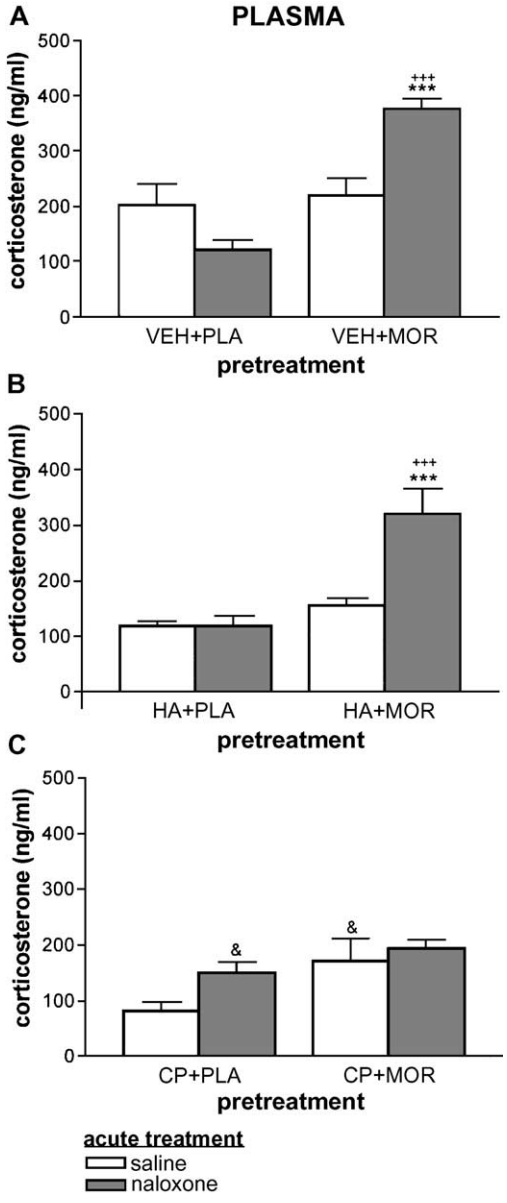

Fig. 3. Plasma corticosterone concentration in naïve and in morphine-dependent rats after injection of saline or naloxone. Animals received s.c. implantation of placebo or morphine (75 mg) pellets for 7 days and concomitantly were infused with vehicle (A), HA-1004 (40 nmol/day; B) or calphostin-C (40 pmol/day; C). On day 8, rats were injected with saline s.c. or naloxone (5 mg/kg) and were decapitated 30 min later. Data are the means ± S.E.M. ($n = 5–8$). $^{***}P < 0.001$ significantly different versus their respective morphine-dependent group receiving saline instead of naloxone; $^{+++}P < 0.001$ versus groups pretreated with placebo receiving naloxone. $^{\&}P < 0.05$ versus control group receiving saline.

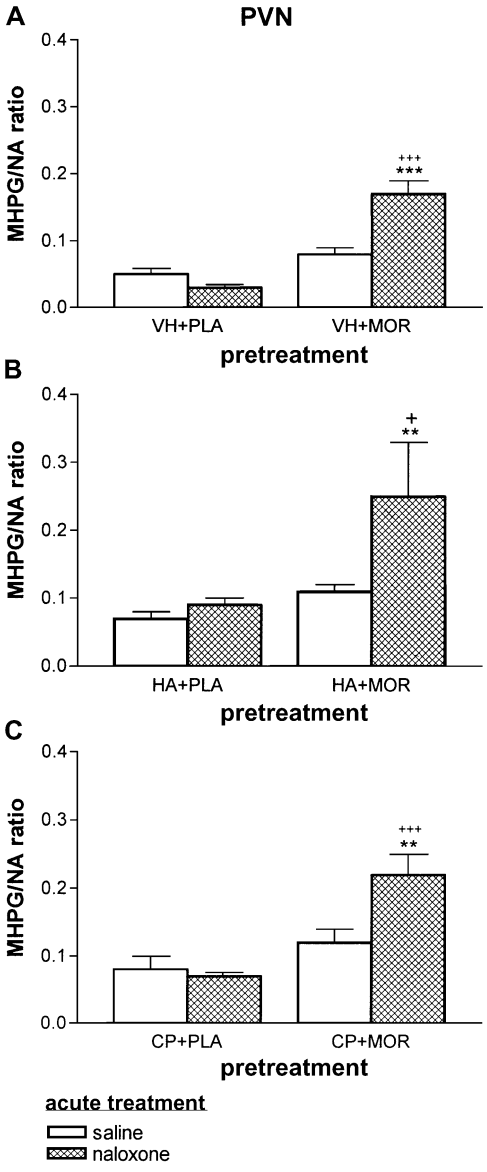

Fig. 4. Turnover of noradrenaline (as estimated by the MHPG/noradrenaline ratio) in naïve and in morphine-dependent rats 30 min after administration of saline or naloxone. The treatment was carried out as described in the legend of Fig. 1. Each column represents the mean ± S.E.M. ($n = 5–8$). $^{**}P < 0.01$; $^{***}P < 0.001$ significantly different versus dependent group treated with saline; $^{+}P < 0.05$, $^{+++}P < 0.001$ versus placebo group receiving naloxone.

2.6. Data analysis

The data are expressed as means ± S.E.M. Data from plasma corticosterone and catecholamines were analysed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Unpaired Student's *t*-test was used when comparing the mean of body weight change. One-way ANOVA, followed by the Dunnett's multiple

comparison test, was used when required. Significance level was taken as $P<0.05$.

3. Results

The weight of each animal was recorded on the day of implantation and on the day of decapitation, before receiving any injection. In all experimental groups, rats treated with morphine showed significantly ($P<0.001$) lower (22.37 ± 2.6 g) body weight gain than animals receiving placebo pellets (52.08 ± 3.1 g). Administration of naloxone (5 mg/kg s.c.) to chronically morphine-treated rats resulted in an important weight loss (14.67 ± 1.0 g) 30 min after naloxone injection ($P<0.001$) when compared with the control group receiving naloxone (5.00 ± 0.9 g). In addition, all animals undergoing morphine withdrawal displayed characteristic abstinence symptoms: wet dog-shakes, teeth chattering, tremor, piloerection, lacrimation, ptosis and spontaneous jumping. Signs of withdrawal were not observed in the placebo groups receiving saline or naloxone. Rats implanted with pellets of morphine receiving saline did not show any signs of abstinence.

Representative HPLC chromatograms of hypothalamic samples from rats treated for 7 days with calphostin-C or HA-1004 via s.c. minipumps are shown in Figs. 1 and 2, respectively. Under the described specific chromatographic conditions for calphostin-C analysis, one peak was found, which was identified as calphostin-C. At the retention time of this drug, no interfering peaks were detected in the blank hypothalamic tissue control. Under the chromatographic conditions for HA-1004, one peak was detected in hypothalamic samples from rats perfused for 7 days with the PKA inhibitor, which was identified as HA-1004. At its retention time, no interfering peaks were detected in the blank hypothalamic samples from control rats (Figs. 1 and 2).

3.1. Effects of protein kinase inhibitors on morphine withdrawal-induced corticosterone secretion

Plasma corticosterone levels were not modified 30 min after naloxone injection to control rats, but increased significantly ($P<0.001$) during morphine withdrawal (Fig. 3A). Chronic pretreatment with the selective PKA inhibitor, HA-1004 concomitantly with morphine did not affect the increase in corticosterone release observed after naloxone-precipitated morphine withdrawal (Fig. 3B). However, pretreatment with calphostin-C (a selective PKC inhibitor) significantly antagonized the corticosterone secretion during morphine withdrawal (Fig. 3C). On the other hand, in animals pretreated with calphostin-C, the placebo group treated with naloxone and the dependent group receiving saline showed concentrations of plasma corticosterone higher ($P<0.05$) than the control group injected with saline.

When HA-1004 or calphostin-C was administered to placebo-pelleted rats, both of them produced a decrease in plasma corticosterone levels ($P<0.05$; $P<0.01$, respectively) compared with control group receiving vehicle instead HA-1004 or calphostin-C (Dunnett's test).

3.2. Effects of PKA and PKC inhibitors on noradrenaline content, MHPG production and noradrenaline turnover in the paraventricular nucleus

As shown in Fig. 4A, in morphine-withdrawn rats, the noradrenaline turnover increased significantly ($P<0.001$). HA-1004 co-administered with morphine for 7 days did not alter the morphine withdrawal-induced noradrenaline turnover increase. Fig. 4B depicts that there was an enhanced noradrenaline turnover during morphine withdrawal in rats pretreated with HA-1004 compared with respective control groups receiving placebo plus naloxone ($P<0.05$) or morphine plus saline ($P<0.01$). Chronic co-administration of calphostin-C with morphine did not

Table 1
Effects of pretreatment with HA-1004 (HA) or calphostin-C (CP) on catecholamine content in the paraventricular nucleus

Treatment	Noradrenaline	MHPG	Dopamine	DOPAC
PLA + VEH + SAL	1688 ± 159.3	85 ± 12.7	3067 ± 79.0	536.4 ± 47.9
PLA + VEH + NX	1868 ± 182.9	65.8 ± 14.8	1780 ± 197.0	348.0 ± 44.6
MOR + VEH + SAL	1751 ± 45.4	153.0 ± 22.4	2255 ± 518.2	449.5 ± 91.4
MOR + VEH + NX	1233 ± 111.3[a,b]	213.0 ± 40.8[a]	2332 ± 423.3	880.0 ± 116.0[a,b]
PLA + HA + SAL	2440 ± 179.3	168.8 ± 21.9	1340 ± 159.7	501.0 ± 79.4
PLA + HA + NX	1924 ± 364.3	179.3 ± 27.0	821.5 ± 137.6	400.3 ± 92.6
MOR + HA + SAL	1939 ± 103.7	206.6 ± 20.2	1003 ± 251.1	358.9 ± 63.5
MOR + HA + NX	1365 ± 281.7	277.8 ± 55.3	1565 ± 353.8	590.5 ± 98.1
PLA + CP + SAL	2401 ± 216.0	197.7 ± 34.4	1376 ± 272.6	365.5 ± 57.0
PLA + CP + NX	2262 ± 158.1	147.3 ± 10.6	1285 ± 204.8	375.3 ± 55.1
MOR + CP + SAL	1883 ± 112.1	217.8 ± 37.4	1829 ± 177.9	664.0 ± 77.0
MOR + CP + NX	1477 ± 167.2[a]	307.8 ± 25.4[a,b]	1714 ± 385.9	747.5 ± 104.4[a]

HA-1004 or calphostin-C were administered concomitantly with morphine or placebo. Testing occurred 30 min after saline or naloxone (5 mg/kg s.c.) injection. Data are the mean ± S.E.M. of five to eight experiments.

[a] Significantly different versus its respective placebo-pretreated control.

[b] Significantly different versus respective group treated with saline.

affect the increase in noradrenaline turnover observed after naloxone-precipitated morphine withdrawal. As shown in Fig. 4C, there was a significant increase in noradrenaline turnover during morphine withdrawal in rats pretreated with calphostin-C compared with those receiving placebo plus naloxone ($P<0.001$) or morphine plus saline ($P<0.01$). Chronic infusion of HA-1004 or calphostin-C did not modify the noradrenaline turnover in placebo groups (Dunnett's test).

Table 1 depicts noradrenaline content and MHPG production in the paraventricular nucleus for control rats and for rats rendered dependent on morphine and pretreated with vehicle, HA-1004 or calphostin-C. The morphine-pelleted group pretreated with vehicle had lower levels of noradrenaline ($P<0.05$) after naloxone injection than the control placebo-pelleted rats and than dependent rats receiving saline instead naloxone, whereas the content of MHPG was significantly ($P<0.001$) higher in morphine-withdrawn rats compared with placebo-pelleted rats receiving naloxone. Neither placebo- nor morphine-pelleted groups showed any significant modifications in the noradrenaline or MHPG levels when HA-1004 was administered concomitantly. In rats dependent on morphine and pretreated with calphostin-C, there was a decrease ($P<0.01$) in the noradrenaline content and an increase ($P<0.01$) in MHPG production after naloxone injection compared with the placebo-pelleted group receiving naloxone.

3.3. Effects of PKA and PKC inhibitors on dopamine content, DOPAC production and dopamine turnover in the paraventricular nucleus

Fig. 5A shows that administration of naloxone to morphine-dependent rats increased ($P<0.01$) the turnover of dopamine in the paraventricular nucleus compared with dependent rats receiving saline instead of naloxone and with placebo-pelleted rats injected with the opioid-receptor antagonist. Chronic pretreatment with HA-1004 concomitantly with morphine antagonized the increase in the dopamine turnover during morphine withdrawal (Fig. 5B). However, pretreatment with calphostin-C did not affect the increase in DOPAC/dopamine ratio observed after naloxone-precipitated morphine withdrawal (Fig. 5C).

When HA-1004 or calphostin-C was administered to placebo-pelleted rats, both of them produced an increase ($P<0.01$ and $P<0.05$, respectively) in the DOPAC/dopamine ratio compared with control group receiving vehicle instead of the protein kinase antagonist (Dunnett's test).

Table 1 shows dopamine and DOPAC levels in the paraventricular nucleus in placebo and dependent rats that were coadministered with vehicle, HA-1004 or calphostin-C. When naloxone was given to morphine-pelleted rats pretreated with vehicle, there were no modifications in the dopamine levels, but the DOPAC production increased significantly ($P<0.01$). In rats pretreated with HA-1004, there were no changes in dopamine level or DOPAC production during morphine withdrawal. On the other hand, the dependent group pretreated with calphostin-C showed an elevation ($P<0.01$) in the DOPAC production after naloxone injection.

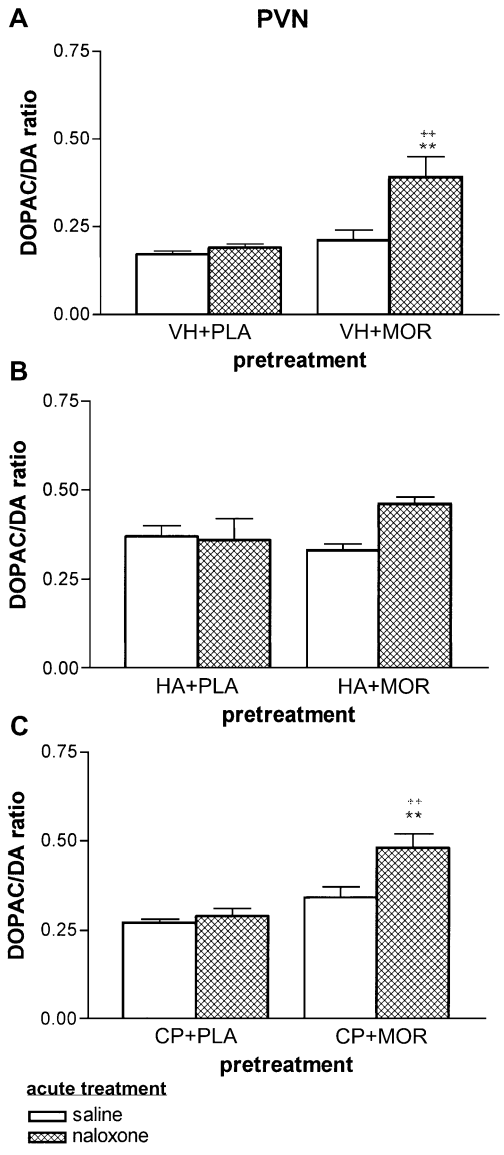

Fig. 5. Turnover of dopamine (as estimated by the DOPAC/dopamine ratio) in naïve and in morphine-dependent rats 30 min after administration of saline or naloxone. The treatment was carried out as described in the legend of Fig. 1. Each column represents the mean ± S.E.M. ($n=5-8$). $^{**}P<0.01$ significantly different versus dependent group treated with saline; $^{++}P<0.01$ versus placebo group receiving naloxone.

4. Discussion

This study was designed to investigate if alteration in the PKA and/or PKC underlies the changes in hypothalamus–pituitary–adrenocortical axis activity associated with morphine withdrawal. As expected, chronic morphine treatment produced physical dependence, as shown by naloxone-pre-

cipitated weight loss and behavioral signs of opioid withdrawal. Additionally, and consistent with previous results, the present data show that morphine withdrawal produced corticosterone hypersecretion (Fuertes et al., 2000a; Laorden et al., 2000; Vargas et al., 1997). This alteration in hypothalamus–pituitary–adrenocortical axis activity was accompanied by an overproduction of the brain metabolite, MHPG and an increase in noradrenaline turnover in the paraventricular nucleus. Furthermore, the DOPAC production and the dopamine turnover also were increased. It is well known that in rats dependent on morphine, the hypothalamus–pituitary–adrenocortical axis is characterized by a marked response after naloxone-induced withdrawal (Fuertes et al., 2000a; Laorden et al., 2000; Martínez-Piñero et al., 1994; Milanés et al., 1998; Pechnick, 1993). However, the chronic adaptive molecular mechanisms underlying these alterations are not well understood.

Protein phosphorylation represents one of the major intracellular regulatory mechanisms. Protein kinases are primary targets of intracellular second messengers in most signal cascades. These effectors are involved in the regulation of different cellular processes, including gene expression (Taylor et al., 1992). Alteration of both PKA and PKC pathways have been suggested as one of the molecular mechanisms of opioid tolerance and dependence (Nestler and Aghajanian, 1997; Tokuyama et al., 1995). Thus, up-regulation of PKA after chronic use of morphine has been suggested as one of the molecular mechanisms of opioid tolerance and addiction (Nestler and Aghajanian, 1997). In addition, a number of recent reports have demonstrated that chronic treatment with morphine enhances PKC activity (Mao et al., 1995; Narita et al., 1994; Tokuyama et al., 2000). Our research was a consequence of available data showing that the activity of the hypothalamus–pituitary–adrenocortical axis, such as the synthesis and release of CRF, might be regulated by PKA and/or PKC under physiological conditions (Itoi et al., 1996, 1998). It is unknown, however, whether increased protein kinase activity underlies the cellular neuroendocrine dependence on opioids observed in the hypothalamus–pituitary–adrenocortical axis with chronic morphine treatment.

The chromatographic data obtained with hypothalamic samples from control animals perfused for 7 days with calphostin-C or HA-1004 providing the first evidence that these kinase inhibitors get into the brain when administered peripherally. This finding agrees with recent data showing that the protein kinase inhibitor *N*-2-(*p*-bromo-cinnamylamino, ethyl-5-isoquinolinesulfonamide) (H-89) produced the same effects on the hypothalamus–pituitary–adrenocortical axis when administered subcutaneously than after its i.c.v. injection (Vargas et al., 2001). During the 7 days of treatment with calphostin-C or HA-1004, no toxic signs (e.g. weight loss, decrease in activity, diarrhea, etc.) were observed in any of the rats treated with these drugs, according to previous findings from Chen et al. (1999b). The results of the present study strongly suggest that the expression of morphine dependence for corticosterone hypersecretion (an index of hypothalamus–pituitary–adrenocortical axis hyperactivity) involves PKC but not PKA signaling mechanisms. Thus, the inhibition of PKA with HA-1004 did not modify the increased corticosterone secretion during morphine withdrawal. These data do not support a role for PKA-mediated phosphorylation in the morphine withdrawal-induced hypothalamus–pituitary–adrenocortical axis activation. However, present results do not preclude a role for this kinase in other sequelae of morphine dependence. Indeed, several studies have indicated that PKA is involved in opioid tolerance and dependence. Thus, withdrawal from morphine has been shown to be associated with marked increases in cAMP and PKA activity in the locus coeruleus (Nestler and Aghajanian, 1997; Nestler, 1992; van Haasteren et al., 1999). It is possible that the PKA pathways are differently affected by chronic morphine in different regions of the brain or that the paraventricular nucleus is not a target of PKA during morphine withdrawal.

Present data indicate that chronic inhibition of PKC with the selective inhibitor calphostin-C concurrently with morphine treatment significantly blocked the hypothalamus–pituitary–adrenocortical axis activity during morphine withdrawal. Our findings are consistent with the observation that PKC is up-regulated during chronic morphine treatment and suggest that PKC activity is necessary for the increased activity of the hypothalamus–pituitary–adrenocortical axis during morphine withdrawal. Present results and previous reports indicate that morphine withdrawal increases the turnover of catecholamines in the hypothalamic paraventricular nucleus concomitantly with an enhanced corticosterone secretion and a decreased CRF content in the paraventricular nucleus (Fuertes et al., 2000a; Milanés et al., 1998; Vargas et al., 1997). It is known that administration of noradrenaline or α-adrenergic agonists enhance the release of CRF, ACTH and corticosterone and also the expression of CRF mRNA in the parvocellular division of the paraventricular nucleus (Itoi et al., 1994; Lookingland et al., 1991). Recent results have shown that noradrenaline stimulates CRF gene expression at the transcriptional level by showing a rapid and marked increase in CRF hnRNA after noradrenaline microinjection into the paraventricular nucleus (Itoi et al., 1999). Additionally, the results of different findings have indicated that α_1-adrenoceptors may mediate the action of noradrenaline on CRF neurons (Itoi et al., 1994, 1998). A recent work from our laboratory has shown that the secretor activity in the hypothalamus–pituitary–adrenocortical axis after morphine withdrawal results from an increase in the activity of noradrenergic pathways innervating the paraventricular nucleus that is dependent of α-adrenergic receptor activation (Laorden et al., 2000), which agrees with many lines of evidence supporting the view that the α-adrenoceptor mediates noradrenergic transmission to hypothalamic CRF neurons (Itoi et al., 1994, 1998; Whitnall, 1993). PKC is activated in response to enhancement of intracellular diacylglycerol as

second messenger, activated, among the others by stimulation of receptors positively coupled to phospholipase C, like α_1-adrenoceptors. According to all these findings, the results of the present study strongly suggest the relevance of the PKC pathway in mediating the naloxone-induced morphine withdrawal activation of the hypothalamus–pituitary–adrenocortical axis activity.

Since the inhibition of PKC by calphostin did not affect the increase in noradrenaline or dopamine turnover that was seen in the paraventricular nucleus from morphine-withdrawn rats, present data suggest that the effect of calphostin inhibiting the hypothalamus–pituitary–adrenocortical axis hyperactivity might occur at the hypothalamic level. The PKA inhibitor, HA-1004, significantly inhibits the enhanced dopamine turnover during morphine withdrawal, whereas it was not able to antagonize the hypothalamus–pituitary–adrenocortical axis activity. This might indicate that dopaminergic pathways impinging the paraventricular nucleus are not implicated in the enhanced hypothalamus–pituitary–adrenocortical axis activity that results from naloxone-precipitated withdrawal.

In summary, present data together with previous findings suggest that the activity of the axis during morphine withdrawal might be under the control of PKC and indicate that changes in PKC activity are closely related to the expression of neuroendocrine dependence on morphine. Further studies are necessary to explore the molecular mechanisms underlying the alterations in the hypothalamus–pituitary–adrenocortical axis during chronic opioid treatment. Present results demonstrate for the first time that calphostin-C and HA-1004 get into the brain after their peripheral administration.

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References

Artigas, F., Sarrias, M.J., Adell, A., Gelpí, E., 1986. Quantitation of total MHPG in the rat brain using a non enzymatic hydrolysis procedure. Effects of drugs. Life Sci. 39, 1571–1578.

Chen, C.L., Chen, H., Zhu, D.M., Uckun, F.M., 1999a. Quantitative high-performance liquid chromatography-based detection method for calphostin C, a naturally occurring perylenequinone with potent antileukemic activity. J. Chromatogr. B 724, 157–162.

Chen, C.L., Tai, H.L., Zhu, D.M., Uckun, F.M., 1999b. Pharmacokinetic features and metabolism of calphostin C, a naturally occurring perylenequinone with antileukemic activity. Pharm. Res. 16, 1003–1009.

Childers, S.R., 1991. Opioid receptor-coupled second messengers systems. Life Sci. 48, 1991–2003.

Couceyro, P., Douglass, J., 1995. Precipitated morphine withdrawal stimulates multiple activator protein-1 signaling pathways in rat brain. Mol. Pharmacol. 47, 29–39.

Cunningham, E.T., Sawchenko, P.E., 1988. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. J. Comp. Neurol. 274, 60–76.

Eaton, M.J., Cheung, S., Moore, K.E., Lookingland, K.J., 1996. Dopamine receptor-mediated regulation of corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. Brain Res. 738, 60–66.

Fuertes, G., Laorden, M.L., Milanés, M.V., 2000a. Noradrenergic and dopaminergic activity in the hypothalamic paraventricular nucleus after naloxone-induced morphine withdrawal. Neuroendocrinology 71, 60–67.

Fuertes, G., Milanés, M.V., Rodríguez-Gago, M., Marín, M.T., Laorden, M.L., 2000b. Changes in hypothalamic paraventricular nucleus catecholaminergic activity after acute and chronic morphine administration. Eur. J. Pharmacol. 388, 49–56.

Gonzálvez, M.L., Milanés, M.V., Martínez-Piñero, M.G., Marín, M.T., Vargas, M.L., 1994. Effects of intracerebroventricular clonidine on the hypothalamic noradrenaline and plasma corticosterone levels of opiate naïve rats and after naloxone-induced withdrawal. Brain Res. 647, 199–203.

Guitart, X., Nestler, E.J., 1989. Identification of morphine- and cyclic AMP-regulated phosphoprotein (MARPPs) in the locus coeruleus and other regions of rat brain: regulation by acute and chronic morphine. J. Neurosci. 9, 4371–4387.

Hidaka, H., Imagaki, M., Kawamoto, S., Sasaki, V., 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23, 5036–5041.

Ignar, D.M., Kuhn, C.M., 1990. Effects of specific Mu and Kappa opiate tolerance and abstinence on hypothalamo–pituitary–adrenal axis secretion in the rat. J. Pharmacol. Exp. Ther. 255, 1287–1295.

Itoi, K., Suda, T., Tozawa, F., Dobashi, I., Ohmori, N., Sakai, Y., Abe, K., Demura, H., 1994. Microinjection of norepinephrine into the paraventricular nucleus of the hypothalamus stimulates corticotropin-releasing factor gene expression in conscious rats. Endocrinology 135, 2177–2182.

Itoi, K., Horiba, N., Tozawa, F., Sakai, Y., Sakai, K., Demura, H., Suda, T., 1996. Major role of 3′,5′-cyclic adenosine monophosphate-dependent protein kinase A pathways in corticotropin-releasing factor gene expression in the rat hypothalamus in vivo. Endocrinology 137, 2389–2396.

Itoi, K., Seasholtz, A.F., Watson, S.J., 1998. Cellular and extracellular regulatory mechanisms of hypothalamic corticotropin-releasing hormone neurons. Endocr. J. 45, 13–33.

Itoi, K., Helmreich, D.L., Lopez-Figueroa, M., Watson, S.J., 1999. Differential regulation of corticotropin-releasing hormone and vasopressin gene transcription in the hypothalamus by norepinephrine. J. Neurosci. 19, 5464–5472.

Kobayashi, E., Nakano, H., Morimoto, M., Tamaoki, T., 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 159, 548–553.

Laorden, M.L., Fuertes, G., González-Cuello, A., Milanés, M.V., 2000. Changes in catecholaminergic pathways innervating paraventricular nucleus and pituitary–adrenal axis response during morphine dependence: implication of α_1- and α_2-adrenoceptors. J. Pharmacol. Exp. Ther. 293, 578–584.

Laorden, M.L., Castells, M.T., Milanés, M.V., 2002. Effects of morphine and morphine withdrawal on brainstem neurons innervating hypothalamic nuclei that control the pituitary–adrenocortical axis in rats. Br. J. Pharmacol. 136, 67–75.

Lookingland, K.J., Ireland, L.M., Gunnet, J.W., Manzanares, J., Tian, Y., Moore, K.E., 1991. 3-Methoxy-4-hydroxyphenylethyleneglycol concentrations in discrete hypothalamic nuclei reflect the activity of noradrenergic neurons. Brain Res. 559, 82–88.

Maldonado, R., 1997. Participation of noradrenergic pathways in the expression of opiate withdrawal: biochemical and pharmacological evidence. Neurosci. Biobehav. Rev. 1, 91–104.

Mao, J., Price, D.D., Mayer, D.J., 1995. Increases in protein kinase C gamma

immunoreactivity in the spinal cord of rats associated with tolerance to the analgesic effects of morphine. Brain Res. 677, 257–267.

Martínez-Piñero, M.G., Milanés, M.V., Alcaraz, C., Vargas, M.L., 1994. Catecholaminergic mediation of morphine-induced activation of pituitary–adrenocortical axis in the rat: implication of α- and β-adrenoceptors. Brain Res. 668, 122–128.

Milanés, M.V., Laorden, M.L., Chapleur-Chateau, M., Burlet, A., 1998. Alterations in corticotropin-releasing factor and vasopressin content in rat brain during morphine withdrawal. Correlation with hypothalamic noradrenergic activity and pituitary–adrenal response. J. Pharmacol. Exp. Ther. 285, 700–706.

Narita, M., Makimura, M., Feng, Y., Hoskins, B., Ho, I.K., 1994. Influence of chronic morphine treatment on protein kinase C activity: comparison with butorphanol and implication for opioid tolerance. Brain Res. 650, 175–179.

Nestler, E.J., 1992. Molecular mechanism of drug addiction. J. Neurosci. 12, 2439–2450.

Nestler, E.J., Aghajanian, G.K., 1997. Molecular and cellular basis of addiction. Science 278, 58–63.

Palkovits, M., 1973. Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res. 59, 449–450.

Palkovits, M., Brownstein, M.J., 1988. Maps and Guide to Microdissection of the Rat Brain. Elsevier, New York.

Pechnick, R.N., 1993. Effects of opioids on the hypothalamo–pituitary–adrenal axis. Annu. Rev. Pharmacol. Toxicol. 32, 353–382.

Schulz, S., Höllt, V., 1998. Opioid withdrawal activities MAP kinase in locus coeruleus neurons in morphine-dependent rats in vivo. Eur. J. Neurosci. 10, 1196–1201.

Self, D.W., Nestler, E.J., 1995. Molecular mechanisms of drug reinforcement and addiction. Annu. Rev. Neurosci. 18, 463–495.

Taylor, S.S., Knighton, D.R., Zheng, J., Ten Eyck, L.F., Sowadski, J.M., 1992. Structural framework for the protein kinase family. Annu. Rev. Cell Biol. 8, 429–462.

Tokuyama, S., Feng, Y., Wakabayashi, H., Ho, I.K., 1995. Possible involvement of protein kinases in physical dependence on opioids: studies using protein kinase inhibitors, H-7 and H-8. Eur. J. Pharmacol. 284, 101–107.

Tokuyama, S., Ho, I.K., Yamamoto, T., 2000. A protein kinase inhibitor, H-7, blocks naloxone-precipitated changes in dopamine and its metabolites in the brains of opioid-dependent rats. Brain Res. Bull. 52, 363–369.

Van Haasteren, G., Li, S., Muda, M., Susini, S., Schlegel, W., 1999. Calcium signalling and gene expression. J. Recept. Signal Transduct. Res. 19, 481–492.

Vargas, M.L., Martínez-Piñero, M.G., Milanés, M.V., 1997. Neurochemical activity of noradrenergic neurons and pituitary–adrenal response after naloxone-induced withdrawal: the role of calcium channels. Naunyn-Schmiedeberg's Arch. Pharmacol. 355, 501–506.

Vargas, M.L., Abella, C., Hernández, J., 2001. Diazepam increases the hypothalamic–pituitary–adrenocortical (hypothalamus–pituitary–adrenocortical) axis activity by a cyclic AMP-dependent mechanism. Br. J. Pharmacol. 133, 1355–1361.

Whitnall, M.H., 1993. Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. Prog. Neurobiol. 40, 573–629.