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Characterization of the signal transduction pathways mediating morphine withdrawal-stimulated c-*fos* expression in hypothalamic nuclei

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Abstract

The transcription factor, Fos, is considered as a functional marker of activated neurons. We have shown previously that acute administration of morphine induces the expression of Fos in hypothalamic nuclei associated with control of the hypothalamus-pituitary-adrenocortex axis, such as the paraventricular nucleus and the supraoptic nucleus. In the current study, we examined the role of protein kinase A, protein kinase C and Ca^{2+} entry through L-type Ca^{2+} channels in naloxone-precipitated Fos expression in the paraventricular and supraoptic nuclei. After 7 days of morphine treatment, we did not observe any modification in Fos production. However, when opioid withdrawal was precipitated with naloxone a dramatic increase in Fos immunoreactivity was observed in the parvocellular division of the paraventricular nucleus and in the supraoptic nucleus. Chronic co-administration of chelerythrine (a selective protein kinase C inhibitor acting at its catalytic domain) with morphine did not affect the increase in Fos expression observed in nuclei from morphine withdrawn rats. In addition, infusion of calphostin C (another protein kinase C inhibitor, which interacts with its regulatory domain) did not modify the morphine withdrawal-induced expression of Fos. In contrast, when the selective protein kinase A inhibitor, *N*-(2′guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004), was infused it greatly diminished the increased Fos production observed in morphine-withdrawn rats. Furthermore, chronic infusion of the selective L-type Ca^{2+} channel antagonist, nimodipine, significantly inhibited the enhancement of Fos induction in the paraventricular and supraoptic nuclei from morphine-withdrawn animals. Taken together, these data might indicate that protein kinase A activity is necessary for the expression of Fos during morphine withdrawal and that an up-regulated Ca^{2+} system might contribute to the activation of Fos. The present findings suggest that protein kinase A and Ca^{2+} influx through L-type Ca^{2+} channels might contribute to the activation of neuroendocrine cells in the paraventricular and supraoptic nuclei.

Keywords: Morphine withdrawal; c-*fos*; Paraventricular nucleus; Supraoptic nucleus; Protein kinase A; Protein kinase C; Ca^{2+} channel

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 changes 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–adrenocortex axis, the major axis responsible for the maintenance of homeostasis. This alteration of the axis is characterized by an increase in adrenocorticotropic hormone (ACTH) and corticosterone secretion, possibly due to the overproduction of corticotropin-releasing factor (CRF) in the hypothalamic paraventricular nucleus (Gonzálvez et al., 1994; Ignar and Kuhn, 1990; Martínez et al., 1990; Vargas et al., 1997). These effects may be mediated by noradrenaline: morphine withdrawal is reported to increase noradrenaline turnover in the paraventricular nucleus, and this effect is blocked by adrenoceptor antagonists (Fuertes et al., 2000a; Laorden et al., 2000b; Milanés et al., 1998; Vargas et al., 1997). However, the signal transduction mechanisms responsible for chronic effects of opioids at a hypothalamic level are incompletely understood.

Opioid receptors are coupled by G_i/G_0 protein to intracellular signalling responses by acting on effector

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molecules such as adenylate cyclase or phospholipase or by regulating ion channel function. The protein kinase pathway is now 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 (protein kinase A) pathway upon acute stimulation (Childers, 1991), the protein kinase A pathway has been shown to be up-regulated in several brain areas during 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 (protein kinase A, protein kinase C and mitogen-activated protein kinase) and intracellular Ca^{2+}, which are involved in a wide variety of cellular regulatory and signalling 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).

Many drugs of abuse, including opioids, induce immediate-early gene expression (Hughes and Dragunow, 1995). The immediate-early gene, c-*fos*, and the expression of its protein product (the transcription factor Fos) have been proposed to reflect second messenger activation and to serve as sensitive indicators of neuronal activation, leading to longer term adaptive responses mediated by the regulation of other delayed response genes (Hughes and Dragunow, 1995; Morgan and Curran, 1991). Previous studies from this laboratory and others have demonstrated that acute activation of opioid receptors causes induction of the immediate-early gene c-*fos* in localized areas of the brain (Curran et al., 1996; Gutstein et al., 1998; Laorden et al., 2000a). Thus, administration of morphine induced the neuronal expression of Fos within neurons of the parvocellular division of the hypothalamic paraventricular nucleus, which regulates the hypothalamus–pituitary–adrenocortex axis (Laorden et al., 2000a). In addition, chronic morphine administration has been shown to alter immediate-early genes that are expressed in specific brain areas that are normally responsive to acute morphine treatment (Frankel et al., 1999; Nye and Nestler, 1996). We have previously demonstrated that during chronic morphine administration, tolerance develops towards the activation of Fos expression within hypothalamic nuclei. In addition, morphine withdrawal induces the expression of Fos protein in neurons that correspond to the parvocellular paraventricular nucleus, the primary location of the tuberoinfundibular CRF cells, which constitute the apex of the hypothalamus–pituitary–adrenocortex axis (unpublished data). The transcription factor Fos has also been shown to be altered in other brain areas following morphine withdrawal (Beckmann et al., 1995; Frankel et al., 1999).

Recent evidence suggests that long-term adaptations to chronic opioid exposure may involve changes in gene expression, which lead to changes in neurotransmission (Blendy and Maldonado, 1998; Nestler and Aghajanian, 1997). Transcription factors, such as c-AMP response element-binding protein (CREB) and the Fos/Jun family, have been proposed as a potential mechanism in opioid addiction. Several kinases are known to activate Fos expression, including protein kinases A and C (Hughes and Dragunow, 1995). In addition, Ca^{2+} influx has also been shown to trigger the expression of Fos (Cruzalegui and Bading, 2000; Van Haasteren et al., 1999). Therefore, protein kinases and intracellular Ca^{2+} may have an important role in the signal transduction to the nucleus.

The present experiments tested whether alterations of protein kinase A, protein kinase C and/or Ca^{2+} influx through L-type Ca^{2+} channels underlie the changes in c-*fos* expression in hypothalamic nuclei associated with chronic morphine treatment and withdrawal. This was assessed by chronically infusing specific inhibitors of protein kinase A and protein kinase C and a selective antagonist of L-type Ca^{2+} channels. We report the results of a series of experiments performed to elucidate the signalling pathways of morphine withdrawal which lead to Fos expression. Our data suggest that Ca^{2+} entry and protein kinase A activation are required for c-*fos* induction during withdrawal in hypothalamic nuclei that control hypothalamus–pituitary–adrenocortex axis activity.

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: 08:00-20:00 h) in a room with controlled temperature (22 ± 2 °C), humidity ($50 \pm 10\%$), 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.

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 and 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-treated 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.). 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 and to test the involvement of Ca^{2+} in the morphine-withdrawal induced expression of immediate-early genes, Fos expression was determined in tolerant/dependent and naive rats pretreated with inhibitors of protein kinase A, protein kinase C and L-type Ca^{2+} channels, and compared with that 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 protein kinase A-selective inhibitor; 40 nmol/day), chelerythrine (a protein kinase C-selective inhibitor; 10, 20 or 40 nmol/day), calphostin C (a protein kinase C selective inhibitor; 40 pmol/day) or nimodipine (a specific L-type Ca^{2+} channels antagonist; 48 μg/day). The selection of the drug doses used in this study was based on the IC_{50} value observed in rat brain for protein kinase A and protein kinase C inhibitors (Herbert et al., 1990; Kobayashi et al., 1989) and on the dose that has been demonstrated to be effective in inhibiting some hypothalamic biochemical changes induced during morphine withdrawal (Fundytus and Coderre, 1996; Vargas et al., 1997). 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. injection of naloxone (5 mg/kg s.c.) or saline (controls).

2.2. Tissue preparation and Fos-immunohistochemistry

Rats were killed with an overdose of pentobarbital (100 mg/kg i.p.) 90 min after s.c. administration of naloxone or saline. The delay of 90 min after the opioid antagonist or control injection was chosen since it was previously demonstrated that the peak of stimulated Fos in the brain occurs at 90 min (Morgan and Curran, 1991). After anesthesia, rats were perfused transcardially with 300 ml of phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of cold, 4% paraformaldehyde in PBS by (pH 7.4). Prepared on a Vibratome, a total of 18 hypothalamic sections were taken for each animal, corresponding to Plates 23–26 in the atlas of Palkovits and Brownstein (1988), and contained the hypothalamic supraoptic and paraventricular nuclei (plane of sections relative to bregma: −1.3 mm for supraoptic nuclei; −1.8 mm for paraventricular nuclei (Paxinos and Watson, 1998).

To optimize antibody penetration, the expression of Fos protein was examined in free floating sections, which were collected serially in adjacent sets, shaken in PBS for at least 30 min to remove the fixative and processed for immunohistochemistry, as described previously (Laorden et al., 2000a). Briefly, the sections were preincubated for 20 min in absolute methanol plus 30% H_2O_2 to block endogenous peroxidase activity. They were rinsed in PBS twice (15 min each) and treated with NSS-PBS (PBS containing 1% normal swine serum (Dako, Gostrup. Denmark) and 0.5% Triton X-100) for 30 min. All sections were then reacted with the primary polyclonal Fos antibody (dilution 1:3000 in NSS-PBS; sc253, Santa Cruz Biotechnology, Santa Cruz, CA) for 36 h at 4 °C. The bound primary antibody was then localized by biotynilated anti-rabbit IgG (dilution 1:200 in NSS-PBS, Vector, Burlingame, CA) and subsequently with the avidin–biotin complex (ABC kits; Vector) at room temperature for 1 h each. Visualization of the antigen-antibody reaction sites was achieved with 0.033% 3,3′-diaminobenzidine (Sigma) and 0.014% H_2O_2 in 0.05 M Tris–HCl buffer for 7 min. The reaction was stopped with PBS. The sections were mounted onto glass slides coated with gelatine, air dried, dehydrated through graded alcohol solutions, cleared in xylene and cover-slipped with DPX.

2.3. Quantification of Fos immunoreactivity

Fos-like immunoreactivity was detected under a light microscope. The density of Fos-like immunostaining of nuclei was evaluated with a computer-assisted image analysis system as described previously (Laorden et al., 2000a). This system consists of a Axioskop microscope (Carl Zeiss, Germany) connected to a videocamera and an Imco 10 computer (Kontron Instrument, Bildanalyse, Germany) with Microm Image Processing software (Microm, Spain). The 3–4 sections of each nuclei showing the highest level of Fos immunoreactivity were selected for quantitative image analysis. A square field (93.5 m side) was superimposed upon the captured image to be used as a reference area. The area of Fos-immunolabeling included in this square was used to estimate the immunoreactivity, and the percentage of Fos immunoreactivity was evaluated. Based on orientation criteria, the medial parvocellular neurosecretory portion of the paraventricular nucleus was defined after the adjacent boundaries of the posterior magnocellular and periventricular parts had been identified (Swanson and Simmons, 1989). For this nucleus, the density of Fos-like immunostaining at 3–4 rostrocaudal levels encompassing the parvocellular zone, the primary location of tuberoinfundibular CRF cells, was used to estimate the Fos immunoreactivity. The percentage of Fos immunoreactivity of both the right and left sides of 3–4 sections for each nucleus was averaged per animal. Measures were also averaged in each experimental group for the paraventricular and supraoptic nuclei.

2.4. Drugs

Pellets of morphine base (Ministerio de Sanidad y Consumo, Madrid, Spain) and lactose (placebo) were prepared by the Department of Pharmacy and Pharmaceutic Technology (University of Granada, Granada, Spain). Naloxone HCl was purchased from Sigma (St Louis, MO), dissolved in sterile 0.9% NaCl (saline) and given in a volume of 0.1 ml/100 g. Nimodipine (isopropyl-(2-methoxyethyl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate) was purchased from Sigma and diluted in polyethylene glycol 400 (PEG); all manipulations were carried out under sodium light. HA-1004 HCl (*N*-(2′guanidinoethyl)-5-isoquinolinesulfonamide, a protein kinase A-selective inhibitor (Hidaka et al., 1984), was purchased from Sigma and dissolved in Milli-Q (Millipore, Bedford, MA) sterile water. Chelerythrine HCl, a protein kinase C-selective inhibitor (Herbert et al., 1990; L.C. Labs., Woburn, MA), was prepared in Milli-Q 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-methylethyl carbonic acid 4-hydroxyphenyl ester, another specific protein kinase C inhibitor (Kobayashi et al., 1989), was purchased from RBI (Natick, MA), dissolved in dimethyl sulfoxide (DMSO) and 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, and those of nimodipine were protected from light at all times. The chronic delivery of nimodipine, HA-1004, chelerythrine and calphostin C was achieved by means of Alzet 2001 osmotic minipumps (Alza, Palo Alto, CA), which deliver at a rate of 1 μl/h. DMSO and other reagents for immunohistochemistry were purchased from Sigma.

2.5. Statistical analysis

All values are expressed as means ± S.E.M. Data were analyzed by analysis of variance (ANOVA), followed by the Newman–Keul's post hoc test. Differences with a P value less than 0.05 were considered significant.

3. Results

3.1. Chronic morphine regulation of Fos immunoreactivity in the paraventricular and supraoptic nuclei

To allow for any effects on Fos expression that 0.06% DMSO (the vehicle of calphostin C) or PEG (the vehicle of nimodipine) could have, in preliminary experiments Fos immunoreactivity was determined in the paraventricular and supraoptic nuclei in rats chronically infused with DMSO, PEG or Milli-Q water. Fos immunoreactivity was not different among the three treatments. So, in the remaining studies Fos immunoreactivity values obtained after pretreatment with kinase or L-type Ca^{2+} channel modifiers were compared with those for appropriate vehicle (Milli-Q water)-treated controls.

To assess the effects of chronic morphine administration on Fos protein production, animals received a 7-day pellet treatment regimen, which is known to produce tolerance and dependence (see Materials and methods), concomitantly with infusion of vehicle via osmotic minipumps. Our previous report has demonstrated that c-*fos* is induced in the hypothalamic paraventricular and supraoptic nuclei after acute morphine administration (Laorden et al., 2000a). As shown in Fig. 1A and C, no immunoreactivity was induced in either the paraventricular nucleus or the supraoptic nucleus 90 min after saline injection in rats treated chronically with morphine (tolerance) compared with the respective control group receiving placebo instead of morphine.

3.2. Induction of Fos immunoreactivity during naloxone-precipitated morphine withdrawal

After the 7-day chronic treatment regimen, physical opioid withdrawal was precipitated by administration of

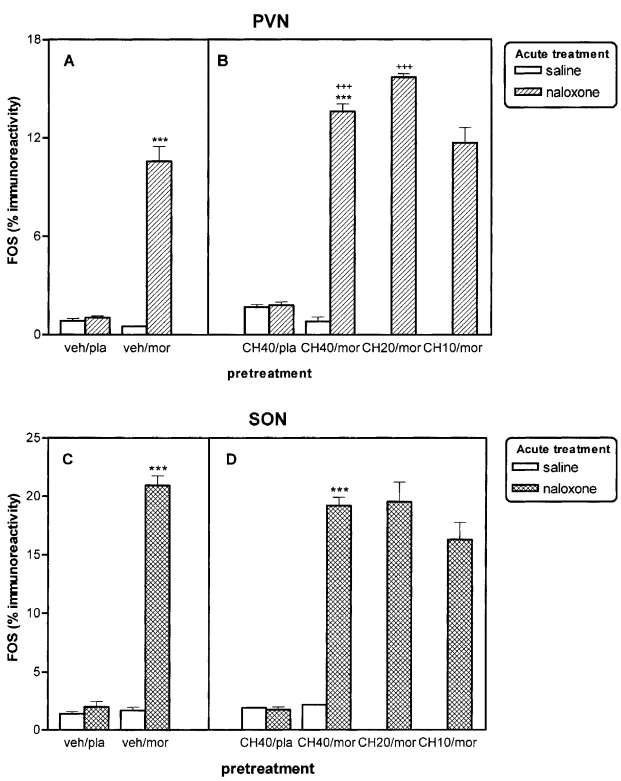

Fig. 1. Quantitative analysis of Fos immunoreactivity in the paraventricular and supraoptic nuclei after naloxone-precipitated withdrawal in vehicle-infused rats (A,C) and in animals chronically administered with chelerythrine (B,D). Animals received s.c. implantation of placebo or morphine (75 mg) pellets for 7 days and concomitantly were infused with vehicle or chelerythrine (10, 20 or 40 nmol/day). On day 8, rats were injected with naloxone (5 mg /kg s.c.) or s.c. saline and 90 min later the paraventricular and supraoptic nuclei were analyzed for Fos immunoreactivity by immunohistochemistry. Data are expressed as means ± S.E.M. for 4–6 animals in each treatment group. $^{***}P < 0.001$ compared with its corresponding control group; $^{+++}P < 0.001$ compared with animals treated with vehicle plus morphine plus naloxone.

naloxone (5 mg/kg s.c.) on day 8. All animals demonstrated behaviors and autonomic characteristics of morphine withdrawal, including teeth chattering, tremor, lacrimation, rhinorrhea, ptosis, wet-dog shakes, jumping and irritability. Consistent with our earlier observations, after 90 min of withdrawal there was a dramatic increase ($P < 0.001$) in Fos immunoreactivity in the paraventricular and supraoptic nuclei compared with that in the naive group injected with naloxone or that in the dependent group injected with saline (Figs. 1A,C, 2A and 3A). In rats chronically treated with placebo, the administration of naloxone (5 mg/kg) did not modify Fos expression in the paraventricular nucleus or in the supraoptic nucleus compared with that in control animals receiving s.c. saline instead of naloxone (Fig. 1A and C).

3.3. Expression of dependence on morphine as determined by Fos induction does not require protein kinase C signalling

Chronic co-administration of chelerythrine (10, 20 or 40 nmol/day) with morphine did not affect the increase in Fos expression observed after naloxone-precipitated morphine withdrawal in the paraventricular and supraoptic nuclei. Examples are shown in Figs. 2B and 3B: many Fos-positive nuclei were observed in the paraventricular and supraoptic nuclei in rats co-administered with chelerythrine and morphine pellets. Quantitative analysis showed that administration of various doses (10, 20 or 40 nmol/day for 7 days) of this selective protein kinase C inhibitor did not block the morphine withdrawal-induced expression of Fos in hypothalamic nuclei (Fig. 1B and D). In addition, the Fos immunoreactivity in the paraventricular nucleus was significantly ($P < 0.001$) higher when chelerythrine was infused at doses of 20 and 40 nmol than that in dependent animals receiving vehicle instead of chelerythrine (Fig. 1A,B). Chronic infusion of chelerythrine at doses of 10, 20 (data not shown) or 40 nmol/day/7 days did not modify the Fos immunoreactivity in placebo groups injected with saline or naloxone or in the tolerant group given saline when compared with the corresponding control groups infused with vehicle instead of chelerythrine (Fig. 1).

Chelerythrine selectively inhibits the activation of protein kinase C by interacting directly with its catalytic domain (Herbert et al., 1990). To test whether inhibition at the protein kinase C regulatory site would affect the morphine withdrawal-induced expression of Fos in the hypothalamic nuclei, another protein kinase C inhibitor, calphostin C, was tested. This agent interacts with the regulatory domain of protein kinase C (Kobayashi et al., 1989). Calphostin C (40 pmol/day) coadministered with morphine for 7 days did not alter the morphine withdrawal-induced expression of Fos either in the paraventricular or in the supraoptic nuclei. As shown in Figs. 2D and 3D, many Fos-positive nuclei were seen in the parvocellular part of the paraventricular nucleus and in the supraoptic nucleus. Compared with vehicle-infused morphine-dependent animals, rats administered calphostin C showed a similar expression of Fos protein in the paraventricular

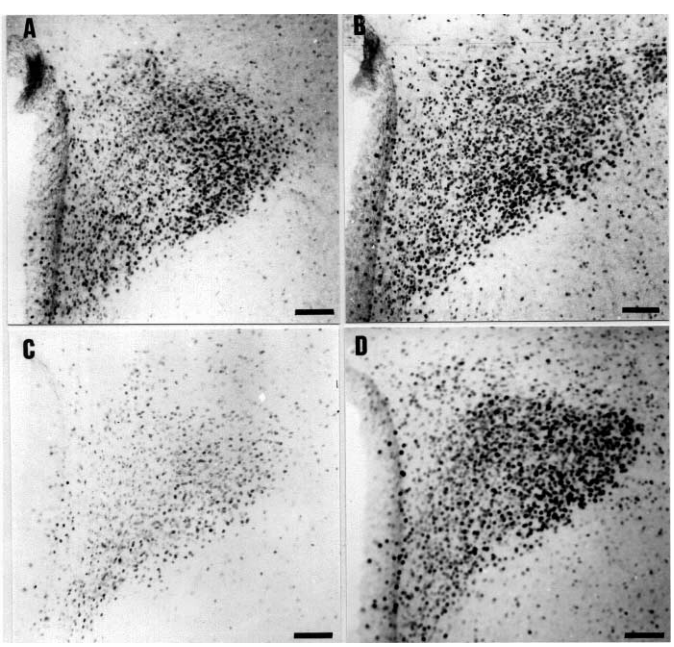

Fig. 2. Photomicrographs of Fos immunoreactivity in the paraventricular nucleus 90 min after naloxone (5 mg/kg s.c.) injection to morphine-dependent rats cotreated for 7 days with vehicle (A), chelerythrine 20 nmol/day (B), HA-1004 40 nmol/day (C) or calphostin C 40 pmol/day (D). Scale bar, 118 μm.

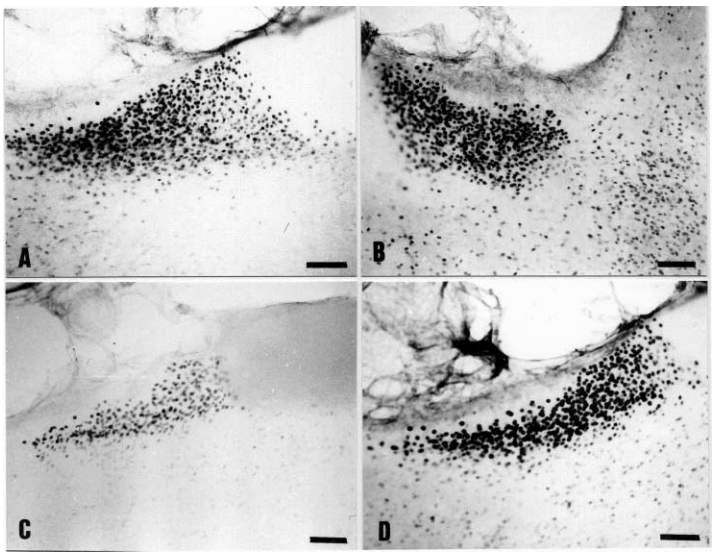

Fig. 3. Photomicrographs of Fos immunoreactivity in the supraoptic nucleus 90 min after naloxone (5 mg/kg s.c.) injection to morphine-dependent rats cotreated for 7 days with vehicle (A), chelerythrine 10 nmol/day (B), HA-1004 40 nmol/day (C) or calphostin C 40 pmol/day (D). Scale bar, 118 μm.

nucleus (Fig. 4B) and the supraoptic nucleus (Fig. 4D). As Fig. 4 depicts, neither the placebo group treated with saline or naloxone nor the tolerant group injected with saline showed any modifications in Fos immunoreactivity in the hypothalamic nuclei when compared with the corresponding control groups infused with vehicle instead of calphostin C.

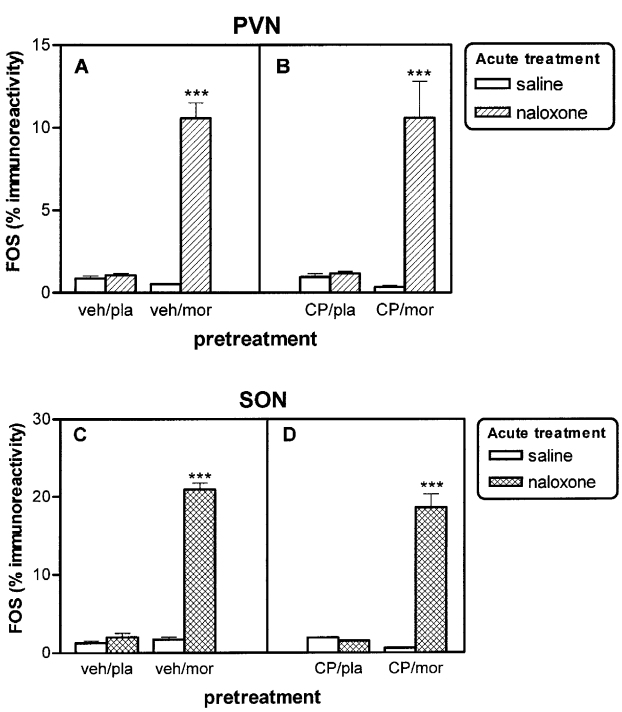

Fig. 4. Quantitative analysis of Fos immunoreactivity in the paraventricular and supraoptic nuclei after naloxone-precipitated withdrawal in vehicle-infused rats (A,C) and in animals chronically administered with calphostin C (B,D). Animals received s.c. implantation of placebo or morphine (75 mg) pellets for 7 days and concomitantly were infused with vehicle or calphostin C (40 pmol/day) for 7 days. On day 8, rats were injected with naloxone (5 mg /kg s.c.) or s.c. saline and 90 min later the paraventricular and supraoptic nuclei were analyzed for Fos immunoreactivity by immunohistochemistry. Data are expressed as means ± S.E.M. for 4–6 animals in each treatment group. $^{***}P < 0.001$ compared with its corresponding control group.

3.4. Involvement of protein kinase A in the morphine withdrawal-stimulated Fos induction

Having established that protein kinase C inhibitors did not affect Fos induction during morphine withdrawal, we then sought to determine whether increased protein kinase A activity is responsible for the naloxone-induced expression of c-*fos* in morphine-dependent rats. For this purpose, the selective protein kinase A inhibitor HA-1004 (40 nmol/day) was coadministered with morphine for 7 days and Fos immunoreactivity was examined on day 8, 90 min after naloxone injection. In morphine-dependent rats infused with the protein kinase A inhibitor, naloxone-induced Fos expression was prevented in the paraventricular nucleus (Fig. 2C). Similar results were obtained in the supraoptic nucleus (Fig. 3C). Quantitative analysis showed that the Fos immunoreactivity measured after naloxone injection to rats chronically treated with morphine plus HA-1004 was significantly ($P < 0.001$) lower in the paraventricular and supraoptic nuclei (Fig. 5B,D) than that in animals receiving vehicle instead of HA-1004. In addition, there were no significant differences in Fos immunoreactivity among dependent animals infused with HA-1004 and injected with naloxone, the dependent rats infused with HA-1004 and injected with saline, and the placebo group infused with the protein kinase A inhibitor and injected with naloxone. Chronic infusion of HA-1004 did not modify the basal Fos immunoreactivity in placebo control rats when compared with that obtained in the placebo group infused with vehicle instead of the protein

kinase A inhibitor (Fig. 5). These data suggest that protein kinase A-dependent mechanisms might play a role in the expression of morphine dependence, as estimated by c-*fos* expression in hypothalamic nuclei that regulate the hypothalamus-pituitary-adrenocortex axis.

3.5. Involvement of Ca^{2+} entry through L-type Ca^{2+} channels on the increased Fos expression during morphine withdrawal

We have shown previously that chronic perfusion of the L-type Ca^{2+} channel blocker, nimodipine, attenuates the hyperactivity of the hypothalamus-pituitary-adrenocortex axis (as estimated by increased noradrenaline turnover in the paraventricular nucleus and augmented corticosterone secretion) after naloxone precipitated-withdrawal in morphine-dependent rats (Vargas et al., 1997). To determine whether the blockade of Ca^{2+} entry can further attenuate Fos expression in paraventricular and supraoptic nuclei from morphine-dependent rats, we tested the effect of the selective L-type Ca^{2+} channel blocker, nimodipine on the induction of Fos protein in morphine-dependent rats. Nimodipine (48 μg/day) was coadministered with morphine for 7 days. Nimodipine significantly ($P < 0.001$) reduced the enhancement of Fos immunoreactivity in the paraventricular nucleus 90 min after naloxone injection in morphine-dependent rats when compared to that in the dependent group infused with vehicle instead of nimodipine (Fig. 6). Moreover, nimodipine dramatically suppressed the expression of Fos in the supraoptic nucleus in placebo groups and in the morphine-dependent animals. These results indicate that L-type Ca^{2+} channels play a prominent role in the enhancement of Fos protein in hypothalamic nuclei during morphine withdrawal.

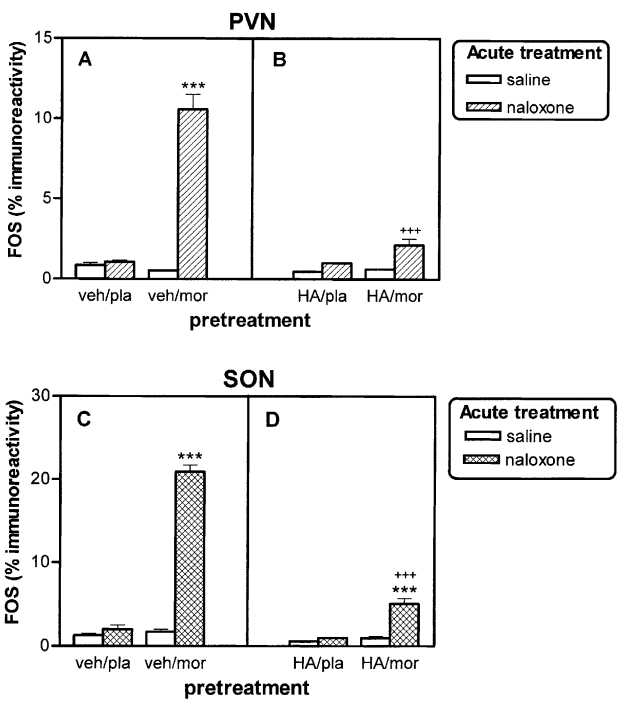

Fig. 5. Quantitative analysis of Fos immunoreactivity in the paraventricular and supraoptic nuclei after naloxone-precipitated withdrawal in vehicle-infused rats (A,C) and in animals chronically administered with HA-1004 (B,D). Animals received s.c. implantation of placebo or morphine (75 mg) for 7 days and concomitantly were infused with vehicle or HA-1004 (40 nmol/day). On day 8, rats were injected with naloxone (5 mg/kg s.c.) or s.c. saline and 90 min later the paraventricular and supraoptic nuclei were analyzed for Fos immunoreactivity by immunohistochemistry. Data are expressed as means ± S.E.M. for 4–6 animals in each treatment group. $^{***}P < 0.001$ compared with its corresponding control group; $^{+++}P < 0.001$ compared with animals treated with vehicle plus morphine plus naloxone.

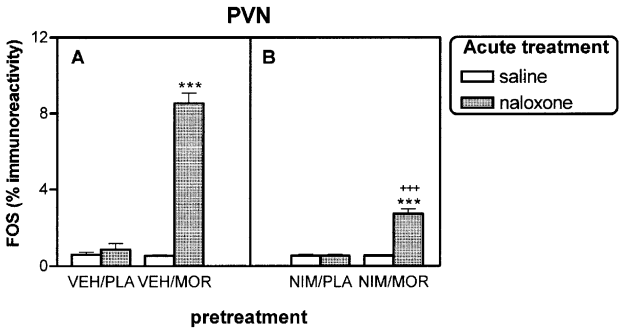

Fig. 6. Quantitative analysis of Fos immunoreactivity in the paraventricular nucleus after naloxone-precipitated withdrawal in vehicle-infused rats (A) and in animals chronically administered with nimodipine (B). Animals received s.c. implantation of placebo or morphine (75 mg) pellets for 7 days and concomitantly were infused with vehicle or nimodipine (48 μg/day). On day 8, rats were injected with naloxone (5 mg /kg s.c.) or s.c. saline and 90 min later the paraventricular nucleus was analyzed for Fos immunoreactivity by immunohistochemistry. Data are expressed as means ± S.E.M. for 4–6 animals in each treatment group. $^{***}P < 0.001$ compared with its corresponding control group; $^{+++}P < 0.001$ compared with animals treated with vehicle plus morphine plus naloxone.

4. Discussion

The present study shows that naloxone-precipitated morphine withdrawal is manifested by the neuronal expression of the transcription factor, Fos, within neurons that anatomically correspond to the parvocellular division of the paraventricular nucleus. This nucleus is the main site of tuberoinfundibular CRF and vasopressin (AVP) cells, which constitute the apex of the hypothalamus–pituitary–adrenocortex axis. This transcription factor may regulate a subsequent pattern of gene expression, thereby mediating the long-term consequences of trans-synaptic stimulation at a hypothalamic level (Kovács, 1998). The present investigation also shows that morphine withdrawal promotes the expression of Fos in the supraoptic nucleus, which is implicated in the neuroendocrine control of the paraventricular nucleus (Blake et al., 1996; Klein et al., 1991). In rats dependent on morphine, the hypothalamus-pituitary-adrenocortex axis is characterized by a marked response after naloxone-induced withdrawal (Martínez et al., 1990;

Pechnick, 1993). In particular, there is an increase in the release of ACTH and corticosterone (Fuertes et al., 2000a; Pechnick, 1993). This secretory activity of the axis results from an increase in noradrenergic pathways impinging on the paraventricular nucleus (Laorden et al., 2000b; Milanés et al., 1998). Furthermore, increased activity of the hypothalamus–pituitary–adrenocortex axis can be seen at a hypothalamic level, where there is an increase of CRF messenger RNA translation and changes in CRF and AVP content in the paraventricular nucleus and median eminence during morphine withdrawal (Lightman and Young, 1988; Milanés et al., 1998). Increased c-*fos* mRNA or Fos immunoreactivity within the central nervous system has been used as a marker of neuronal activation (Hughes and Dragunow, 1995; Morgan and Curran, 1991). As Fos protein functions as a transcription factor, its induction may extend to the level of gene regulation of critical hypothalamic hormone precursors, such as CRF and/or AVP, which trigger hypothalamus–pituitary–adrenocortex activation. Thus, the activation of parvocellular neurons of the paraventricular nucleus from morphine withdrawn rats might be related to the activation of Fos protein.

Protein phosphorylation represents a major intracellular regulatory mechanism. Protein kinases are primary targets of intracellular second messengers in most signalling cascades. These effectors are involved in the regulation of different cellular processes, including gene expression (Taylor et al., 1992). Changes in protein kinase A and protein kinase C pathways have been suggested as one of the molecular mechanisms of opioid tolerance and dependence (Nestler and Aghajanian, 1997; Tokuyama et al., 1995). Based on their respective sites of action (Hofmann, 1997), the protein kinase C inhibitors used in the present study would be predicted to inhibit most, if not all, of the isoforms of protein kinase C that have been cloned from mammalian species. The results of the present study strongly suggest that the expression of morphine dependence in terms of Fos induction in hypothalamic nuclei involves protein kinase A but not protein kinase C signalling mechanisms. Thus, the inhibition of protein kinase C activity at either the catalytic or the regulatory site (with chelerythrine or calphostin C, respectively) did not modify the increased Fos expression during morphine withdrawal. Moreover, chelerythrine at doses of 20 and 40 nmol/day potentiated the morphine withdrawal-induced expression of Fos in the paraventricular nucleus. Thus, these data do not support a role for protein kinase C-mediated phosphorylation in the morphine withdrawal-induced expression of c-*fos* in hypothalamic nuclei. However, the present results do not preclude a role for this kinase in other sequelae of morphine dependence. Indeed, several studies have indicated that protein kinase C is involved in opioid tolerance and dependence (Narita et al., 1994a; Smart and Lambert, 1996). It is possible that the protein kinase C pathway is differentially affected by morphine withdrawal in different regions of the brain or that Fos protein is not a target of protein kinase C during morphine withdrawal at a hypothalamic level.

The present data indicate that chronic inhibition of protein kinase A (with the selective protein kinase A inhibitor, HA-1004) concurrently with morphine treatment significantly blocks the Fos production during morphine withdrawal in both the paraventricular and supraoptic nuclei. Withdrawal from morphine has been shown to be associated with marked increases in cAMP and protein kinase A activity in the locus ceruleus (Nestler and Aghajanian, 1997; Nestler, 1992). Our findings are consistent with the observation that protein kinase A is up-regulated during chronic morphine administration and suggest that protein kinase A activity is necessary for the expression of Fos during morphine withdrawal. It has been established that protein kinase A can phosphorylate Fos (Hughes and Dragunow, 1995). In addition, both protein kinase A and protein kinase C have been shown to induce c-*fos* (Hagiwara et al., 1996). From the present results, we can affirm that one of the components of the morphine withdrawal signal transduction pathway that is a target for protein kinase A in hypothalamic nuclei controlling the hypothalamus–pituitary–adrenocortex axis is the c-*fos* gene. Transactivation of c-*fos* by protein kinase A might lead to increased expression of genes with activator protein-1 (AP-1) sites in their promoters, such as AVP or tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines (Kovács, 1998). Previous studies have also revealed AP-1 sites on the CRF gene, although it has not been clarified whether these sites are functionally linked to transcriptional activation of the CRF gene (Vamvakopoulos and Chrousos, 1993). The protein kinase A inhibitor HA-1004 was not able to modify the basal Fos immunoreactivity in the nuclei from either the naive animals or the tolerant rats receiving saline s.c. instead of naloxone. This indicates that the ability of the protein kinase A inhibitor to abolish the increase in Fos immunoreactivity is due to its ability to antagonize a biochemical event that results from naloxone-precipitated withdrawal and which does not occur under physiological conditions or during the development of morphine tolerance/dependence at a hypothalamic level. Whereas the present study does not address the role of protein kinase A in the increased Fos expression seen in the paraventricular and supraoptic nuclei during the development of morphine tolerance, such a role has been described for protein kinase A in the development of antinociceptive tolerance (Narita et al., 1994b).

Recently, we have shown that the blockade of Ca^{2+} channels attenuates the increase in the hypothalamus–pituitary–adrenocortex axis activity observed after naloxone-induced morphine withdrawal (Vargas et al., 1997). Thus, hypothalamic noradrenaline turnover and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG; the metabolite of noradrenaline in the central nervous system) production, which are both elevated during withdrawal,

returned to control levels in rats perfused chronically with nimodipine, concomitantly with a reduction in corticosterone secretion. In the present study, we further examined the possible requirement for Ca^{2+} entry in the activation of Fos expression in morphine-withdrawn rats. Ca^{2+} not only regulates neurotransmitter release but also acts as a key mediator of adaptive responses that involve changes in gene transcription. For example, Ca^{2+} influx through L-type Ca^{2+} channels potently stimulates transcription mediated by the cAMP response element (CRE). The predominant mechanism by which Ca^{2+} signals are further processed within the cytoplasm and the nucleus involves protein kinase cascades (Cruzalegui and Bading, 2000). In addition, it is accepted that genes coding for transcription factors (fos, jun) are the immediate targets of Ca^{2+} signalling (Van Haasteren et al., 1999).

The present results indicate that the increase in Fos immunoreactivity in the parvocellular division of the paraventricular nucleus seen after naloxone-induced withdrawal was significantly attenuated by chronic pretreatment with the L-type Ca^{2+} channel blocker nimodipine (48 μg/day), consistent with a role for Ca^{2+} influx in the expression of morphine dependence, as estimated by Fos induction. In addition, nimodipine dramatically abolished the Fos production in the supraoptic nucleus in both the placebo groups and the tolerant/dependent rats. Taken together, these data indicate that an up-regulated Ca^{2+} system might contribute to the activation of neuroendocrine cells in the parvocellular division of the paraventricular nucleus and in the supraoptic nucleus. Our data agree with the finding that chronic administration of Ca^{2+} channel blockers can attenuate the expression of dependence (Kochuvelikakam and Levine, 1997; Tokuyama et al., 1995). Recent reports have shown that activation of opioid receptors can augment several components of neuronal Ca^{2+} signalling pathways and, as a consequence, enhance the intracellular Ca^{2+} signal, which can contribute to the long-term effects of opioids (Przewlocki et al., 1999).

In summary, we demonstrated that the increased expression of Fos in the parvocellular part of the paraventricular nucleus and in the supraoptic nucleus from morphine-withdrawn rats occurs via a protein kinase A-dependent pathway. We further showed that Fos expression after naloxone-precipitated morphine withdrawal also appears to require Ca^{2+} influx through L-type Ca^{2+} channels. Clearly, a better understanding of the functional role played by c-*fos* during morphine withdrawal requires the identification of the genes influenced by it in the neurosecretory cells that regulate the activity of the hypothalamus–pituitary–adrenocortex axis.

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References

Beckmann, A.M., Matsumoto, I., Wilce, P.A., 1995. Immediate early gene expression during morphine withdrawal. Neuropharmacology 34, 1183–1189.

Blake, A.D., Bot, G., Reisine, T., 1996. Structure-function of the cloned opiate receptors: peptide and small molecule interactions. Chem. Biol. 3, 967–972.

Blendy, J.A., Maldonado, R., 1998. Genetic analysis of drug addiction: the role of cAMP response element binding protein. J. Mol. Med. 76, 104–110.

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.

Cruzalegui, F.H., Bading, H., 2000. Calcium-regulated protein kinase cascades and their transcription factor targets. Cell. Mol. Life Sci. 57, 402–410.

Curran, E., Akil, H., Watson, S., 1996. Psychomotor stimulant- and opiate-induced c-fos mRNA expression patterns in the rat forebrain: comparisons between acute drug treatment and a drug challenge in sensitized animals. Neurochem. Res. 21, 1425–1435.

Frankel, P.S., Harlan, R.E., Garcia, M.M., 1999. Chronic administration of morphine alters immediate-early gene expression in the forebrain of post-dependent rats. Brain Res. 835, 204–212.

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.

Fundytus, M.E., Coderre, T.J., 1996. Chronic inhibition of intracellular Ca^{++} release or protein kinase C activation significantly reduces the development of morphine dependence. Eur. J. Pharmacol. 300, 173–181.

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.

Gutstein, H.B., Thome, J.L., Fine, J.L., Watson, S.J., Akil, H., 1998. Pattern of c-fos mRNA induction in rat brain by acute morphine. Can. J. Physiol. Pharmacol. 76, 294–303.

Hagiwara, M., Shimoruma, A., Yoshida, K., Imaki, J., 1996. Gene expression and CREB phosphorylation induced by cAMP and Ca^{2+} in neuronal cells. Adv. Pharmacol. 36, 277–285.

Herbert, J.M., Augereau, J.M., Gleye, J., Maffrand, J.P., 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 172, 993–999.

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.

Hofmann, J., 1997. The potential for isoenzyme-selective modulation of protein kinase C. FASEB J. 11, 649–669.

Hughes, P., Dragunow, M., 1995. Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. Pharmacol. Rev. 47, 133–178.

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.

Klein, D.C., Moore, R.Y., Reppert, S.M., 1991. Suprachiasmatic Nucleus: The Mind's Clock. Oxford Univ. Press, New York.

Kobayashi, E., Nakano, H., Morimoto, M., 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.

Kochuvelikakam, O.A., Levine, J.D., 1997. Different mechanisms mediate development and expression of tolerance and dependence for peripheral μ-opioid antiniciception in rat. J. Neurosci. 17, 8018–8023.

Kovács, K., 1998. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem. Int. 33, 287–297.

Laorden, M.L., Castells, M.T., Martínez, M.D., Martínez, P.J., Milanés, M.V., 2000a. Activation of c-fos expression in hypothalamic nuclei by mu- and kappa-receptor agonists. Correlation with catecholaminergic activity in the hypothalamic paraventricular nucleus. Endocrinology 141, 1366–1376.

Laorden, M.L., Fuertes, G., González-Cuello, A., Milanés, M.V., 2000b. 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.

Lightman, S.L., Young, W.S., 1988. Corticotrophin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. J. Physiol. 403, 511–523.

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, J.A., Vargas, M.L., Fuente, T., del Río-García, J., Milanés, M.V., 1990. Plasma β-endorphin and cortisol levels in morphine-tolerant rats and in naloxone-induced withdrawal. Eur. J. Pharmacol. 182, 117–123.

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.

Morgan, J.I., Curran, T., 1991. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. Ann. Rev. Neurosci. 14, 421–451.

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

Narita, M., Feng, Y., Makimura, M., Hoskins, B., Ho, I.K., 1994b. A protein kinase inhibitor, H-7, inhibits the development of tolerance to opioid antiniciception. Eur. J. Pharmacol. 271, 543–545.

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.

Nye, H.E., Nestler, E.J., 1996. Induction of chronic fos-related antigens in rat brain by chronic morphine administration. Mol. Pharmacol. 49, 636–645.

Palkovits, M., Brownstein, M.J., 1988. Maps and guide to microdissection of the rat brain. Elsevier, New York.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates. 4th edn. Academic Press, San Diego.

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

Przewlocki, R., Parsons, K.L., Sweeney, D.D., Trotter, C., Netzeband, J.G., Siggins, G.R., Gruol, D.L., 1999. Opioid enhancement of calcium oscillations and burst events involving NMDA receptors and L-type calcium channels in cultured hippocampal neurons. J. Neurosci. 19, 9705–9715.

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

Smart, D., Lambert, D.G., 1996. The stimulatory effects of opioids and their possible role in the development of tolerance. Trends Pharmacol. Sci. 17, 264–269.

Swanson, L.W., Simmons, D.M., 1989. Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. J. Comp. Neurol. 285, 413–435.

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

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

Vamvakopoulos, N.C., Chrousos, G.P., 1993. Structural organization of the 5′ flanking region of the human corticotropin releasing hormone gene. DNA Seq. 4, 197–206.

Van Haasteren, G., Li, S., Muda, M., Susini, S., Schlegel, W., 1999. Calcium signalling and gene expression. J. Recept. Signal Transduction 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.