Phorbol Ester Regulation of Opioid Peptide Gene Expression in Myocardial Cells

ROLE OF NUCLEAR PROTEIN KINASE C*

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Opioid peptide gene expression was characterized in adult rat ventricular cardiac myocytes that had been cultured in the absence or the presence of phorbol 12-myristate 13-acetate. The phorbol ester induced a concentration- and time-dependent increase of prodynorphin mRNA, the maximal effect being reached after 4 h of treatment. The increase in mRNA expression was suppressed by incubation of cardiomyocytes with staurosporine, a putative protein kinase C inhibitor, and was not observed when the cells were cultured in the presence of the inactive phorbol ester 4α-phorbol 12,13-didecanoate. Incubation of cardiac myocytes with phorbol 12-myristate 13-acetate also elicited a specific and staurosporine-sensitive increase in immunoreactive dynorphin B, a biologically active end product of the precursor, both in the myocardial cells and in the culture medium. In vitro run-off transcription assays indicated that transcription of the prodynorphin gene was increased both in nuclei isolated from phorbol ester-treated myocytes and in nuclei isolated from control cells and then exposed to phorbol 12-myristate 13-acetate. No transcriptional effect was observed when cardiac myocytes or isolated nuclei where exposed to 4α-phorbol 12,13-didecanoate. The phorbol ester-induced increase in prodynorphin gene transcription was prevented by pretreatment of myocytes or isolated nuclei with staurosporine, suggesting that myocardial opioid gene expression may be regulated by nuclear protein kinase C. In this regard, cardiac myocytes expressed protein kinase C-α, -δ, -ε, and -ζ, as shown by immunoblotting. Only protein kinase C-α and protein kinase C-ε were expressed in nuclei that have been isolated from control myocytes, suggesting that these two isotypes of the enzyme may be part of the signal transduction pathway involved in the effect elicited by the phorbol ester on opioid gene transcription in isolated nuclei. The incubation of myocardial nuclei isolated from control cells in the presence of a protein kinase C activator induced the phosphorylation of the myristoylated alanine-rich protein kinase C substrate peptide, a specific fluorescent substrate of the enzyme. The possibility that prodynorphin gene expression may control the heart function through autocrine or paracrine mechanisms is discussed.

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‡ The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; dyn, dynorphin B; ir-dyn, immunoreactive dynorphin B; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MARCKS, myristoylated alanine-rich protein kinase C substrate.

The signal transduction pathway(s) involved in the expression of the prodynorphin gene in cardiac myocytes is currently unknown. However, the observed effects of k opioid receptor agonists on myocardial cytosolic Ca2+ and pH homeostasis were largely attributable to the capability of these opioids to increase the formation of inositol 1,4,5-triphosphate and inositol 1,3,4,5-tetraphosphate (8) and to elicit a protein kinase C-dependent stimulation of the Na+/H+ antiporter (5), indicating that myocardial opioid receptors are coupled to phosphoinositide turnover and protein kinase C (PKC). This enzyme is expressed in various forms and is widely distributed in different tissues (9–11). In the myocardial cell, PKC activation,
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besides representing an important step in the opioidergic pathway, has been reported to regulate the expression of different genes, including c-fos and skeletal α-actin genes (12). PKC activation by phorbol esters has also been shown to induce the human proenkephalin gene transcribed into CV 1 cells, and a DNA sequence on this opioid gene has been identified conferring the sensitivity to phorbol ester induction (13). The role of protein kinase C in the regulation of prodynorphin gene expression in cells naturally expressing this gene and, in particular, in the myocardial cell, has not been investigated, however.

In the present study, we aimed at elucidating whether PKC activation induced by phorbol 12-myristate 13-acetate (PMA) may influence the expression of prodynorphin mRNA as well as the synthesis and release of dynorphin B in adult cultured rat ventricular myocytes. The effect induced by the phorbol ester on the transcription of the prodynorphin gene and the presence of a PKC in isolated myocardial nuclei were also investigated.

MATERIALS AND METHODS

Isolation of Cardiac Myocytes—Hearts from 2–3-month-old male Wistar rats were retrogradely perfused through the aorta in the presence of a bicarbonate buffer, pH 7.4, containing low calcium and collagenase (14). Following the isolation procedure, the cardiomyocytes were subjected to a short term-serum-free primary culture, according to a previously described method (15). Briefly, the cardiomyocytes were cultured under 5% CO2 atmosphere at 37 °C in Petri dishes (60-mm Falcon dishes, Becton Dickinson) at a density of 2 × 10⁴ cells/dish in 3 ml of M-199 medium (with Earle’s salts) containing 0.2% bovine serum albumin, 10⁻⁴ M insulin, 2.5 × 10⁻⁴ M penicillin G, 2.5 × 10⁻⁴ M streptomycin, and 10⁻⁴ M cytosine arabinofuranoside. One day after plating, cells were treated under the experimental conditions described in the legend of each figure.

RNA Extraction and Determination of Prodynorphin mRNA—Following RNA isolation from the adult cultured myocytes (16), the levels of prodynorphin mRNA were determined by using a sensitive hybridization RNase protection assay (17, 18). Briefly, a 400-base pair HindIII-BamHI fragment of the main exon of rat genomic prodynorphin clone was inserted into pGEM3. Transcription of the plasmid linearized with BamHI generated a sense strand of prodynorphin mRNA used to construct a standard curve of prodynorphin mRNA, whereas transcription of the plasmid linearized with EcoRI in the presence of [³²P]UTP (80 Ci/mmol) gave an antisense strand used to hybridize cellular prodynorphin mRNA. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide nondenaturing gel. Autoradiographic exposure was performed for 48 h. The individual bands were counted for radioactivity by liquid scintillation spectrometry, and cpm values were transformed to pg values on a corresponding standard curve. Data were expressed as pg of mRNA/μg of total RNA.

Identification of Dynorphin B-like Material—Immunoactive dynorphin B (ir-dyn B) was measured by a radioimmunoassay procedure that utilized the 13 S antiserum raised against dyn B and capable of recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dyn B in their sequence (19, 20). Acetic acid extracts from cardiac myocytes or pooled samples from the culture medium were processed by reverse-phase high performance liquid chromatography. The collected fractions were radioimmunoassayed according to a previously described method (6, 21), and the immunoreactivity was attributed to authentic dyn B by comparison with the elution position of a standard.

Isolation of Myocardial Nuclei—Nuclei from control or PMA-treated cells were isolated by a modification of a previously described method (22). Cells were rinsed twice with a physiological saline solution (Buffer I) containing 137 mM NaCl, 1.2 mM MgSO₄, 5 mM KCl, 1.2 mM Na₂HPO₄, 20 mM HEPES, 16 mM ω-glucose, and 1 mM CaCl₂, pH 7.4. The nuclei were separated with Buffer I containing 90 μl of Buffer II. The nuclei of the samples were homogenized in Buffer II containing 1000 × g for 10 min at 4 °C. The cardiac myocytes were then resuspended in a hypo-osmotic buffer (Buffer II) of the following composition: 10 mM Tris/HCl, pH 7.4, 1 mM MgCl₂, 10 mM NaCl, 5 mM CaCl₂, 0.1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 μM β-mercaptoethanol. The resuspended cells were incubated for 30 min on ice and then sedimented at 1000 × g for 10 min at 4 °C. The pellet was resuspended in 20 ml of Buffer II and sonicated for 40 s at setting 2 in a Branson sonifier W-350. The sonicated preparation was added with Triton X-100 at a final concentration of 0.1% and then centrifuged at 1000 × g for 10 min at 4 °C. The pellet was resuspended in a buffer (Buffer III) containing 10 mM Tris, pH 8.0, 0.3 mM succrose, 5 mM MgCl₂, 0.1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 μM β-mercaptoethanol. The resuspended material was layered over an equal volume of Buffer III containing 0.6 mM succrose and centrifuged at 1500 × g for 15 min at 4 °C. The resulting nuclear pellet was finally resuspended in a buffer (Buffer IV) containing 50 mM Tris/HCl, pH 8.0, 5 mM MgCl₂, 0.1 mM EDTA, 40% glycerol, 0.1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 μM β-mercaptoethanol.

Nuclear Run-off Transcription Assay—Nuclear run-off experiments were carried out by using a modification of the method described by Livingstone et al. (23). Nuclear suspensions (10 ml) resuspended in 90 μl of Buffer II were hybridized with 100 μl of 2 × reaction buffer (10 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 0.3 mM KCl, 5 mM diithiothreitol, 1 mM each of ATP, GTP, and CTP), and 10 μl of [α-³²P]UTP (3000 Ci/mmol), followed by incubation at room temperature for 15 min. DNA was digested by incubating the transcription mixture for 5 min at room temperature in the presence of 1 μl of 20,000 units/ml RNase-free DNase. Nuclear RNA was isolated by using guanidine thiocyanate and acid phenol extraction (16), followed by purification on RNAMATRIX (Bio 101, Inc. Vista, CA). The ³²P-labeled RNA pellet was resuspended in 1 ml of TES solution containing 10 mM TES, pH 7.4, 10 mM EDTA, and 0.2% SDS. Samples were eventually diluted by adding TES solution to equalize the cpm of ³²P-labeled RNA/ml (about 5 × 10⁶). 1 ml of RNA solution was then mixed with 1 ml of TES/NaCl solution and heated at 80 °C for 10 min. A total volume of 2 ml of RNA solution was then hybridized to 1 μg of heat-denatured DNA insert corresponding to a 400-base pair fragment of the main exon of rat genomic prodynorphin clone that has been immobilized on nitrocellulose filters following isolation from a pGEM3 plasmid. In this regard, it has been shown that the use of a DNA insert versus the whole plasmid-containing insert can considerably increase the efficiency of hybridization (17).

The hybridization reaction was allowed to run for 36 h at 65 °C. After the hybridization, the filters were washed in 2 × SSC for 1 h at 65 °C with several changes and treated with RNase A for 30 min at 37 °C. The filters were washed again in 2 × SSC for 1 h at 37 °C and exposed to Kodak X-Omat film at -70 °C. The radioactivity was measured in the secondary antibody (1:5000 horseradish peroxidase-linked donkey anti-rabbit immunoglobulin) in PBST containing 1% fat-free milk powder. Finally, the nitrocellulose membranes were washed three times in PBST, developed by the ECL method, and exposed to films for 5 min (the secondary antibody as well as the ECL Western blotting detection reagents and the autoradiography film Hyperfilm MP were all from Amersham International).

Measurement of Nuclear PKC Activity—PKC activity from isolated myocardial nuclei was measured by using a continuous fluorescence assay (24–26). After transfer, the blot was saturated for 1 h at room temperature with 2% fat-free milk powder in PBST (80 mM NaH₂PO₄, 20 mM Na₂HPO₄, 100 mM NaCl containing 0.05% Tween 20, pH 7.5). The immunoreaction was carried out for 1 h at room temperature in PBST and 2% milk powder containing the primary antibody diluted 1:1000 in PBST with PKC-α, PKC-βII, or PKC-δ antibodies. After being washed three times with PBST (5 min each wash), the blot was incubated for 1 h at room temperature in the presence of the secondary antibody (1:5000 horseradish peroxidase-linked donkey anti-rabbit immunoglobulin) in PBST containing 1% fat-free milk powder. Finally, the nitrocellulose membranes were washed three times in PBST, developed by the ECL method, and exposed to films for 5 min (the secondary antibody as well as the ECL Western blotting detection reagents and the autoradiography film Hyperfilm MP were all from Amersham International).

Measurement of Nuclear PKC Activity—PKC activity from isolated myocardial nuclei was measured by using a continuous fluorescence assay (24–26) that utilized the acrylodan-labeled myristoylated alanine-rich protein kinase C substrate (MARKS) peptide (Boehringer Mannheim), a high affinity substrate in vitro for PKC (28–31). The MARKS peptide consists of a conserved sequence of 24 amino acids from the MARKS protein, which includes four sites for PKC phosphorylation (27). The fluorogenic acrylodan label is introduced N-terminally onto the MARKS peptide. In the presence of PKC activators, maximum fluorescence is measured at 480 nm with excitation at 370 nm. In the course of phosphorylation by PKC, the intensity of the fluorescence decreases about 20% (27). In the present study, the fluorescence changes during phosphorylation of the MARKS peptide were monitored at 25 °C. The reaction mixture contained, in a final volume of 1 ml, 10 mM Tris/HCl, pH 7.0, 90 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 100 μM ATP, 30% ethylene glycol, 0.5 μg phosphatidylserine, 0.1 μg 1,2-dioctanoyl-sn-glycerol, and 75 mM acrylodan-labeled MARKS peptide. Phosphorylation of the acrylodan-labeled peptide...
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RESULTS

RNA extracted from adult cultured rat ventricular cardiac myocytes was subjected to a solution hybridization RNase protection analysis using a synthetic 32P-antisense probe for detection. Incubation of the cultured myocytes in the presence of 100 nM PMA induced a marked increase in the amount of detectable mRNA. This stimulatory effect was evident after 1 h of exposure to the phorbol ester and peaked at 4 h after exposure to 100 nM PMA (Fig. 1). When the cardiac myocytes were pretreated for 30 min with 2 nM staurosporine, a putative PKC inhibitor (32), and subsequently treated for 4 h with PMA in the presence of this PKC inhibitor, the levels of prodynorphin mRNA did not differ significantly from those observed in control cardiac myocytes (Fig. 1). After 6 h of treatment with PMA, the levels of prodynorphin mRNA progressively declined and resulted in being down-regulated to the control value between 12 and 24 h (Fig. 1). Fig. 2 reports the dose-response curve of the effect of PMA on prodynorphin mRNA expression, following a 4-h exposure of the cultured myocytes to the phorbol ester. A significant increase in mRNA levels was observed at a concentration as low as 1 nM. The amount of prodynorphin mRNA was progressively increased in a dose-dependent manner by cell treatment in the presence of the phorbol ester, reaching a plateau when the myocytes where cultured in the presence of PMA concentrations ranging between 50 and 100 nM.

Consistent amounts of ir-dyn B were found under basal conditions in cardiac myocytes and in the culture medium (Fig. 3). Culturing of myocytes for 4 h in the presence of 100 nM PMA resulted in a marked increase in the level of this biologically active peptide product of the prodynorphin gene both in the cells and in the culture medium (Fig. 3). The same figure shows that the cellular levels of ir-dyn B were significantly lower than those detected in the medium, both in the absence and the presence of the phorbol ester. Myocyte treatment with 2 nM staurosporine suppressed the PMA-mediated increase in ir-dyn B both in the medium and in the myocardial cell (Fig. 3). No significant increase in ir-dyn B levels was observed in cells or medium when the myocytes were cultured for 4 h in the presence of 100 nM 4α-phorbol 12,13-didecanoate (Fig. 3).

To investigate whether the increase in prodynorphin mRNA expression elicited by PMA reflects changes in the transcriptional status of the myocyte nucleus, we assessed the rate of transcription of the pradynorphin gene by using an in vitro run-off transcription assay. The nuclear preparation used in the present study lacked contamination by sarcoplasmic reticular membranes, inner or outer mitochondrial membranes, or sarcosomal membranes, as indicated by the measurement of the activities of the corresponding marker enzymes rotenone-insensitive NADPH-cytochrome c reductase, succinate dehydrogenase, rotenone-insensitive NADH-cytochrome c reductase, and 5'-nucleotidase (33–35), which were all undetectable in the nuclear fraction (data not shown). We found that incubation of the cardiac myocytes in the presence of 100 nM PMA, a concentration that proved to be the most effective in increasing prodynorphin mRNA, resulted in a marked increase in nuclear transcription of the prodynorphin gene (Fig. 4). Interestingly, in nuclei isolated from untreated cells and subsequently exposed for 4 h to 100 nM PMA, prodynorphin gene transcription was found to be enhanced at a level similar to that observed in the nuclei from PMA-treated cardiomyocytes (Fig. 4). The pretreatment of cardiomyocytes or isolated nuclei with staurosporine abolished the PMA-induced increase in transcriptional activity. No increase in transcription of the prodynorphin gene was observed following incubation of myocytes or isolated nuclei in the presence of 100 nM 4α-phorbol 12,13-didecanoate (Fig. 4).

Immunoblot analyses of total extracts from untreated cardiac myocytes revealed the expression of PKC-α (80 kDa), PKC-δ (78 kDa), PKC-ε (97 kDa), and PKC-ζ (75 kDa) (Fig. 5). PKC-β and PKC-γ were not detected (not shown). Western blot analysis of nuclear samples from untreated cells revealed that only PKC-δ and PKC-ε were expressed in the myocardial nucleus (Fig. 5). The densitometric analysis of the bands corresponding to PKC-δ and PKC-ε indicated that each of these two PKC isotypes was almost completely expressed at nuclear level (not shown).

We next investigated whether the exposure of isolated myocardial nuclei to a PKC activator may result in the phosphorylation of a specific PKC substrate. Fig. 6 shows the effect induced on acrylodan-peptide phosphorylation by the incubation of myocardial nuclei, isolated from control cells, in the absence or the presence of 1,2-dioctanoyl-sn-glycerol, a diglyceride that acts as a potent PKC activator (36). When the nuclear fraction was incubated with the acrylodan peptide in the absence of the PKC activator, no significant change in the peptide fluorescence was observed (Fig. 6). On the contrary, exposure of the myocardial nuclei to 1,2-dioctanoyl-sn-glycerol for 10 min resulted in a time-dependent fluorescence decrease at the 480 nm emission maximum of the PKC substrate, corresponding to PKC-mediated acrylodan-peptide phosphorylation (Fig. 6). The maximal decrease in the intensity of fluorescence was achieved about 10 min from the addition of each nuclear sample to the reaction mixture.
Our data show that the prodynorphin gene was expressed in adult rat ventricular myocytes and that prodynorphin mRNA was translated into a biologically active end product. The observation that the levels of ir-dyn B were significantly higher in the culture medium than in the myocytes suggests that in the ventricular myocardial cell, which lacks secretory granules (37), the prodynorphin-derived peptides may be constitutively released shortly after synthesis. PMA elicited a marked increase in the expression of prodynorphin mRNA within the first 4 h of treatment, which was associated to a significant increase in levels of ir-dyn B both in the cells and in the culture medium. The finding that these effects of the phorbol ester were inhibited by staurosporine suggests that prodynorphin mRNA induction by PMA was a PKC-mediated event that was associated to an increase in mRNA translation into the biologically active peptide product. A role for PKC in prodynorphin mRNA induction is also supported by the observation that the levels of this mRNA were down-regulated to control values following prolonged exposures to PMA, a treatment that is known to result in depletion or down-regulation of PKC (38, 39). PKC involvement in the regulation of prodynorphin gene expression is further supported by the finding that the inactive phorbol ester 4α-phorbol 12,13-didecanoate did not alter both prodynorphin mRNA and dyn B levels. The nuclear run-off experiments performed in myocardial nuclei isolated from PMA-treated cells indicate that the level of PMA-mediated stimulation of prodynorphin mRNA expression was transcriptional. Specificity and PKC dependence of this stimulatory action of PMA are suggested by the lack of an effect by 4α-phorbol 12,13-didecanoate and by the capability of staurosporine to suppress the increase in transcription due to myocyte exposure to the phorbol ester. However, PMA increased nuclear transcription of the prodynorphin gene even when it was di-

**Discussion**

Our data show that the prodynorphin gene was expressed in adult rat ventricular myocytes and that prodynorphin mRNA was translated into a biologically active end product. The observation that the levels of ir-dyn B were significantly higher in the culture medium than in the myocytes suggests that in the ventricular myocardial cell, which lacks secretory granules (37), the prodynorphin-derived peptides may be constitutively released shortly after synthesis. PMA elicited a marked increase in the expression of prodynorphin mRNA within the first 4 h of treatment, which was associated to a significant increase in levels of ir-dyn B both in the cells and in the culture medium. The finding that these effects of the phorbol ester were inhibited by staurosporine suggests that prodynorphin mRNA induction by PMA was a PKC-mediated event that was associated to an increase in mRNA translation into the biologically active peptide product. A role for PKC in prodynorphin mRNA induction is also supported by the observation that the levels of this mRNA were down-regulated to control values following prolonged exposures to PMA, a treatment that is known to result in depletion or down-regulation of PKC (38, 39). PKC involvement in the regulation of prodynorphin gene expression is further supported by the finding that the inactive phorbol ester 4α-phorbol 12,13-didecanoate did not alter both prodynorphin mRNA and dyn B levels. The nuclear run-off experiments performed in myocardial nuclei isolated from PMA-treated cells indicate that the level of PMA-mediated stimulation of prodynorphin mRNA expression was transcriptional. Specificity and PKC dependence of this stimulatory action of PMA are suggested by the lack of an effect by 4α-phorbol 12,13-didecanoate and by the capability of staurosporine to suppress the increase in transcription due to myocyte exposure to the phorbol ester. However, PMA increased nuclear transcription of the prodynorphin gene even when it was di-

**FIG. 2.** Dose-response of PMA effect on the expression of prodynorphin mRNA. The myocardial cells were incubated in the presence of each concentration of PMA for a period of 4 h. [ ], control; ●, PMA. The data are expressed as the mean values ± S.E. (n = 6). *, significantly different from the control value. Representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA are shown in the inset. Autoradiographic exposure was carried out as described in Fig. 1.

**FIG. 3.** Regulation of ir-dyn B levels by PMA. Adult cultured rat ventricular myocytes were treated for 4 h with 100 nm PMA in the absence or presence of 2 nm staurosporine (ST) or exposed for a period of 4 h to 100 nm 4α-phorbol 12,13-didecanoate (4α-phorbol). Shaded bars, ir-dyn B in cultured myocytes; white bars, ir-dyn B in the medium. Each single value in the medium was calculated in a final volume of 15 ml corresponding to the volume of pooled samples of the culture medium from 10⁶ cells. Each experiment was performed in the presence of a peptidase inhibitor mixture containing 20 μM bestatin, 1 mM l-leucyl-l-leucine, 3 μM pepstatin A, 0.3 μM thiorphan, 30 μM 1,10-phenanthroline, and 6 μM 1,4-dithiothreitol. The data are expressed as the mean values ± S.E. (n = 6). *, significantly different from its own control value; §, significantly different from its own control value; $, the value of the white bar is significantly different from that of the shaded bar.

**FIG. 4.** Effect of PMA on the rate of transcription of the prodynorphin gene in isolated myocardial nuclei. Isolation of myocardial nuclei and run-off transcriptional analysis of the prodynorphin gene were performed as described under “Materials and Methods.” pB15, a cDNA encoding rat cyclophilin, and pBR322 plasmid DNA were included as positive and negative controls, respectively. The values are representative of six experiments. 1, transcription of the prodynorphin gene; 2, pBR322; 3, pB15. A, nuclei were isolated from control cardiac myocytes; B, nuclei were isolated from cardiac myocytes that have been exposed to 100 nm PMA for 4 h; C, nuclei were isolated from untreated myocytes and subsequently exposed to 100 nm PMA for 4 h; D, nuclei were isolated from cardiac myocytes cultured for 4 h in the presence of 100 nm PMA and 2 μM staurosporine; E, nuclei isolated from untreated cells were subsequently incubated for 4 h in the presence of 100 nm PMA and 2 μM staurosporine; F, nuclei were isolated from cardiac myocytes that have been exposed to 100 nm 4α-phorbol 12,13-didecanoate for 4 h; G, nuclei were isolated from untreated myocytes and then incubated in the presence of 100 nm 4α-phorbol 12,13-didecanoate for 4 h.
are representative of four separate experiments. Antisera to PKC-δ, PKC-ε, and PKC-γ were used to confirm the discovery of PKC-α in the myocardial nuclei. This direct effect of PMA also suggests that PKC-δ and PKC-ε were detected in the myocardial nuclei and may be part of the signal transduction pathway involved in the stimulation of prodynorphin gene transcription observed when PMA was directly added to intact isolated nuclei. The phosphorylation of the MARCKS peptide by "nuclear embedded" PKC also seems to indicate that the biochemical machinery necessary for activation of PKC is present in the myocardial nucleus. This hypothesis is in agreement with other studies showing that several enzymes and substrates associated with diacylglycerol production are present in the nuclei of rat liver (43) and that inositol phospholipids are synthesized in nuclei of Friend cells (44). Whether the PKC here described in isolated myocardial nuclei might be activated by endogenous factors remains to be defined. However, receptors for prolactin and various growth hormones have been identified in isolated nuclei from rat liver and spleen mononuclear cells, and these agonists have been shown to increase the activity of the PKC in a similar extent of that elicited by PMA (45), suggesting that the phospholipase effect might be mimicked by endogenous agonists and that PKC activation through specific nuclear receptors might be involved in the regulation of nuclear events, including gene transcription.

The implications of the results of the present report, showing an increase in prodynorphin gene expression following the activation of PKC in myocardial nuclei are still unknown. Nevertheless, the stimulation of nuclear PKC, by eliciting increased prodynorphin gene expression and release of a biologically active opioid peptide, may stimulate myocardial opioid receptors, resulting in the activation of autocrine or paracrine mechanisms that might trigger previously described effects of opioid peptides on myocardial Ca²⁺ signaling and pH homeostasis as well as on the intracellular state of the cardiac myocyte (4, 5). Moreover, in several tissues endogenous opioids have been shown to act on plasma membrane opioid receptors in such a way to inhibit cell proliferation and promote cell differentiation (46, 47). We cannot exclude that myocardial opioid gene expression and its stimulation via nuclear PKC may also play an autocrine role in the regulation of myocardial cell growth and differentiation.

The mechanisms by which PMA-mediated stimulation of nuclear PKC may affect the expression of the prodynorphin gene have not been investigated in the present study. It has been hypothesized that a potential mechanism by which PKC may affect gene transcription is the phosphorylation of RNA polymerase or the phosphorylation of specific nuclear proteins acting as transcription factors (48, 49). However, nuclear events initiated by PKC activation, including the activation of RNA polymerases, have been reported to be long-lived and to persist after the down-regulation of PKC (50), indicating that a role for the regulation of transcription by phosphorylation-dephosphorylation remains to be defined. The identification of possible molecular mechanisms involved in PMA effect on gene transcription is further complicated by the finding that differ-
ent consensus sequences that function as PKA-responsive elements and by the fact that DNA-binding proteins that are part of the PMA-activated signal transduction network have been only in part identified (51). Therefore, the mechanism(s) by which the expression of the prodynorphin gene is regulated by PKC in the myocardial cell remains to be elucidated.

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