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Protein Kinase C Signaling Transduces Endorphin-Primed Cardiogenesis in GTR1 Embryonic Stem Cells

Carlo Ventura, Elisabetta Zinellu, Emiliana Maninchedda, Marina Fadda, Margherita Maioli

***Abstract*—**The prodynorphin gene and its product, dynorphin B, have been found to promote cardiogenesis in embryonic cells by inducing the expression of GATA-4 and Nkx-2.5, two transcription factor–encoding genes essential for cardiogenesis. The molecular mechanism(s) underlying endorphin-induced cardiogenesis remain unknown. In the present study, we found that GTR1 embryonic stem (ES) cells expressed cell surface κ opioid receptors, as well as protein kinase C (PKC)-α, -β_1, -β_2, -δ, -ϵ, and -ζ. Cardiac differentiation was associated with a marked increase in the B_{max} value for a selective opioid receptor ligand and complex subcellular redistribution of selected PKC isozymes. PKC-α, -β_1, -β_2, -δ, and -ϵ all increased in the nucleus of ES-derived cardiac myocytes, compared with nuclei from undifferentiated cells. In both groups of cells, PKC-δ and -ϵ were mainly expressed at the nuclear level. The nuclear increase of PKC-α, -β_1, and -β_2 was due to a translocation from the cytosolic compartment. In contrast, the increase of both PKC-δ and PKC-ϵ in the nucleus of ES-derived cardiomyocytes occurred independently of enzyme translocation, suggesting changes in isozyme turnover and/or gene expression during cardiogenesis. No change in PKC-ζ expression was observed during cardiac differentiation. Opioid receptor antagonists prevented the nuclear increase of PKC-α, PKC-β_1, and PKC-β_2 and reduced cardiomyocyte yield but failed to affect the nuclear increase in PKC-δ and -ϵ. PKC inhibitors prevented the expression of cardiogenic genes and dynorphin B in ES cells and abolished their development into beating cardiomyocytes. **(*Circ Res*. 2003;92:617-622.)**

Key Words: protein kinase C ■ cardiac differentiation ■ embryonic stem cells ■ gene expression ■ endorphins

Embryonic stem (ES) cells have been shown to differentiate in vitro into spontaneously beating cardiomyocytes exhibiting structural, biochemical, and electrophysiological features mimicking those detected during cardiogenesis in vivo.[1–6] In mice, ES cell-derived cardiomyocytes have been shown to form stable intracardiac grafts,[7] and pluripotent bone marrow cells have been recently found to differentiate into myocardial cells when injected into infarcted hearts.[8] These findings indicate that ES cells may represent an optimal renewable source for donor cardiac myocytes. The identification of signaling events that control the process of cardiogenesis is now a major area of inquiry. In a previous study, we have shown that P19 embryonal pluripotent cells express the prodynorphin gene and are able to synthesize and secrete dynorphin B, a biologically active end-product of the gene acting as a natural agonist of κ opioid receptors.[9] Exposure of P19 cells to dynorphin B primed the expression of GATA-4 and Nkx-2.5 genes, which encode for tissue-specific transcription factors essential for cardiogenesis in different animal species, including humans.[10–13] This cardiogenic program of gene expression was followed by the transcription of the cardiac-specific genes α-myosin heavy chain (MHC) and myosin light chain-2V (MLC-2V), and ultimately ensued in the appearance of beating colonies of cardiac myocytes. These findings indicate that the prodynorphin gene and its related peptide products are potential conductors of cardiogenesis in ES cells. Nevertheless, the molecular mechanism(s) underlying the cardiogenic role of this endorphinergic system remain to be elucidated. Within this context, unraveling the molecular patterning linking opioid peptide interaction with ES cells to the activation of cardiogenic gene transcription may represent a relevant step in the development of novel strategies for attaining the highest throughput of cardiogenesis from suitable multipotent cells.

We have previously provided evidence that protein kinase C (PKC) signaling transduced opioid receptor activation into cellular and transcriptional responses in adult ventricular cardiac myocytes.[14–16] In the present study, we investigated whether κ opioid receptors may be developmentally expressed in multipotent GTR1 ES cells and whether PKC signaling and subcellular redistribution of selected PKC isozymes may be coupled to opioid receptor activation throughout cell commitment to the cardiac lineage. We also

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assessed whether PKC activation may be involved in the transcription of cardiogenic genes, leading to the appearance of a myocardial phenotype.

Materials and Methods

Cardiac Differentiation of ES Cells

GTR1, a derivative of R1 ES cells[17] bearing the puromycin-resistance gene driven by the cardiomyocyte-specific MHC promoter, were kindly provided by Dr William L. Stanford (University of Toronto and Center for Modeling Human Disease, Canada). Cells were maintained in the undifferentiated state by culturing onto a layer of mitotically inactivated mouse embryo fibroblasts in the presence of KNOCKOUT D-MEM containing 15% fetal bovine serum (FBS), supplemented with a final concentration of 1000 U/mL Leukemia Inhibitory Factor (LIF). Before embryoid bodies (EBs) can be made, subconfluent undifferentiated ES cells were harvested from feeder layers by trypsinization, transferred onto 0.1% gelatin-coated plates, and grown to about 70% to 80% confluence in the presence of LIF-supplemented KNOCKOUT D-MEM, containing 15% FBS. These cells were used as undifferentiated cells in each experiment. The undifferentiated state of cells cultured with LIF on both feeder layers or gelatinized plates was inferred from the high percentage of cells positively stained for alkaline phosphatase activity and from the lack of nestin-positive cells (a detailed characterization of GTR1 ES cells is provided in an expanded Materials and Methods section, available in the online data supplement at http://www.circresaha.org). To induce cardiac differentiation, cells were plated onto specialty plates (Costar ultra-low attachment clusters), containing the culture medium lacking supplemental LIF. After 2 days of culture, the resulting EBs were plated onto tissue culture dishes. When spontaneous contractile activity was noticed (7 days after LIF removal), puromycin (2 μg/mL) was added to eliminate noncardiomyocytes and puromycin-selected cells were cultured for an additional period of 7 days. Analysis of MHC immunoreactivity revealed that at this stage cardiomyocytes comprised more than 99% of selected cells. Analysis of MLC-2V and MLC-2A mRNA expression indicated that puromycin-selected cells encompassed both ventricular- and atrial-like lineages (comparative assessment of the cardiomyocyte yield after a physical enrichment protocol or puromycin selection, as well as characterization of puromycin-selected cardiomyocytes are described in detail in the online data supplement).

EBs, collected at several stages after plating, as well as puromycin-selected cells, were processed for gene expression and immunofluorescence analyses. After LIF removal and throughout puromycin selection, GTR1 cells were also exposed to different selective protein kinase C inhibitors or opioid receptor antagonists.

Opioid Binding Assay

[^{3}H]U-69,593 (55.0 Ci/mmol) (Amersham International) was used as a selective κ opioid receptor ligand for binding assays in a plasma membrane-enriched fraction (referred to as F_{40}). Each sample (300 μg of protein) was incubated with the radiolabeled ligand in 0.25 mL of a binding buffer containing 50 mmol/L Tris HCl, pH 7.4, 5 mmol/L $MgCl_2$, 250 mmol/L sucrose, 0.1 mmol/L DTT, 0.5 mmol/L PMSF, 1 μmol/L leupeptin, and 10 mmol/L β-mercaptoethanol. The specific binding was measured as the difference between binding in the absence and presence of 10 μmol/L of the unlabeled ligand. The incubation media were filtered over vacuum on Whatman GF/B glass fiber filters. Filters were finally counted for radioactivity by liquid scintillation spectrometry. K_d and B_{max} values were calculated with the LIGAND program.[18]

Immunoblotting Analysis of PKC

Total cell lysates, cytosolic, or nuclear fractions were electrophoresed on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose using a semidry transfer cell. Immunoreactions were performed overnight at 4°C in the presence of the primary antibody (antisera to PKC-α, PKC-β_1, PKC-β_2, PKC-δ, PKC-ϵ, or PKC-ζ) diluted 1:100. The membranes were then incubated with ^{125}I-labeled donkey anti-rabbit IgG antibodies (10^6 cpm/mL) for 1 hour at room temperature, dried, and exposed to Kodak X-OMAT AR films with an intensifying screen for 48 hours at −70°C. The intensities of the autoradiographic bands were measured with a laser densitometer and, for each PKC isozyme, the data were expressed as percentage changes in the autoradiographic intensity in each sample (total lysates, cytosolic fraction, or nuclear fraction) from cardiac myocytes relative to the intensity in the corresponding sample obtained from undifferentiated cells (considered as 100%).

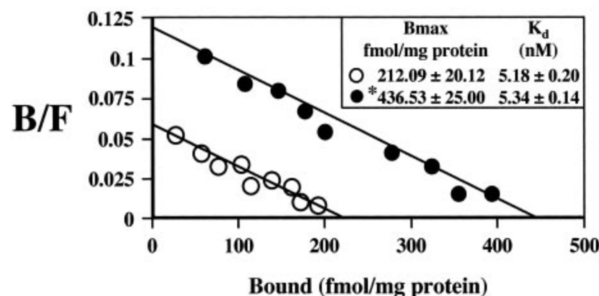

Figure 1. Scatchard analysis of the specific binding of [^{3}H]U-69,593 to F_{40} membranes. Samples were isolated from LIF-supplemented cells (○) or from ES-derived cardiomyocytes 4 days after puromycin addition (●). Data are expressed as mean±SE (n=6). *Significantly different from (○).

Gene Expression

Total RNA extraction, reverse transcription, and PCR conditions were previously described.[9] Prodynorphin, GATA-4, and Nkx-2.5 mRNAs were assessed by RNase protection assay, as detailed elsewhere.[19] Fragments of the main exon of mouse prodynorphin gene (424 bp), GATA-4 (292 bp), or Nkx-2.5 (414 bp) genes were inserted into pCRII-TOPO. Transcription of the plasmid linearized with *Apa*I, *Bam*HI, or *Xba*I generated sense strands of prodynorphin, GATA-4, or Nkx-2.5 mRNA, respectively, which were used to construct a standard mRNA curve. Transcription in the presence of [^{32}P]CTP of plasmids linearized with *Bam*HI generated antisense strands of prodynorphin and Nkx-2.5 mRNA, whereas transcription of plasmids linearized with *Xba*I produced an antisense strand of GATA-4 mRNA.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Expression of κ opioid receptors was assessed by the aid of [^{3}H]U-69,593 ([^{3}H]U-69), a selective radiolabeled ligand,[20] in a plasma membrane-enriched fraction (F_{40}) isolated from undifferentiated ES cells or puromycin-selected cells. ES-derived cardiomyocytes resulted to represent a mixed population of different cardiac lineages (ie, atrial- and ventricular-like; see online data supplement). The binding experiments revealed the presence of highly specific κ opioid-binding sites in the F_{40} fraction obtained from undifferentiated GTR1 cells (Figure 1), with specific binding ranging between 75% and 85% of the total bound. The Scatchard plots of [^{3}H]U-69 binding were linear and were characterized by a single dissociation constant (K_d) in the low nmol/L range (Figure 1). Analysis of [^{3}H]U-69 binding in the F_{40} fraction isolated from undifferentiated parental R1 cells yielded similar results (see online data supplement). A marked increase in the maximal binding capacity (B_{max}) for [^{3}H]U-69 was evident in plasma membranes that had been isolated from puromycin-selected cardiac myocytes, as com-

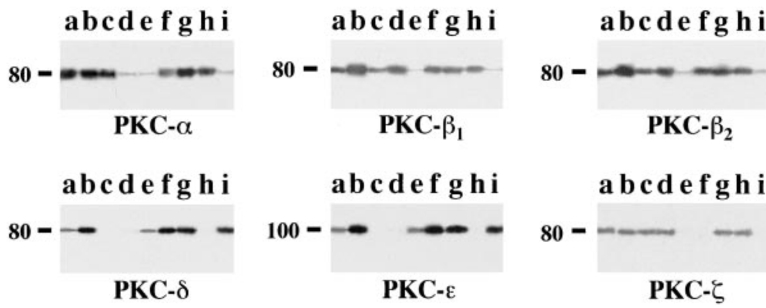

Figure 2. Subcellular distribution of PKC isozymes during cardiomyogenesis. Samples were prepared from LIF-supplemented cells or ES-derived cardiomyocytes 4 days after puromycin addition. Equal amounts of protein (20 μg) were subjected to 8% SDS-polyacrylamide gels and analyzed by immunoblotting as described in Materials and Methods. Autoradiograms are representative of 6 separate experiments. Sizes of the bands were determined by calibration with prestained marker proteins. Lanes a, c, and e indicate total cell lysates, cytosolic, or nuclear fractions, respectively, isolated from undifferentiated ES cells; lanes b, d and f, Total cell lysates, cytosolic, and nuclear fractions from ES-derived cardiomyocytes; lanes g, h and i, Total cell lysates, cytosolic, and nuclear fractions were isolated from ES-derived cells exposed to 1 μmol/L Mr-1452 after LIF removal and throughout puromycin selection.

pared with the corresponding fraction from undifferentiated GTR1 cells (Figure 1). No significant difference in the K_d values was found among the F_{40} fractions obtained from undifferentiated ES cells or ES-derived cardiomyocytes (Figure 1).

Immunoblot analysis of total extracts from both undifferentiated ES cells and ES-derived cardiac myocytes revealed the expression of PKC-α (80 kDa), PKC-β_1 and -β_2 (80 kDa), PKC-δ (78 kDa), PKC-ϵ (97 kDa), and PKC-ζ. (75 kDa) (Figure 2). A similar expression of PKC-α was observed in total extracts from undifferentiated ES cells and ES-derived cardiomyocytes, whereas PKC-β_1 and -β_2 expression was increased in total extracts from cardiac myocytes, as compared with undifferentiated cells (Figures 2 and 3). Only a faint immunoreactivity against anti-PKC-α–, -PKC-β_1–, and PKC-β_2–specific antibodies was detected in the nuclear fraction of undifferentiated ES cells. On the contrary, the expression of these isozymes was markedly increased in the nucleus of ES-derived cardiomyocytes (Figures 2 and 3). Concomitantly, PKC-α was downregulated, whereas PKC-β_1 and -β_2 were overexpressed in their cytosolic fraction. Western blot analysis also indicated that the immunoreactivity against anti-PKC-δ– and anti-PKC-ϵ–specific antibodies was mainly detectable in the nuclear fraction and was higher in nuclei that had been isolated from ES-derived cardiomyocytes than in nuclei obtained from undifferentiated GTR1 cells (Figures 2 and 3). The same figure shows that the expression of PKC-ζ appeared to be similar in both groups of cells. No evidence for a nuclear localization of this isozyme was provided (Figures 2 and 3).

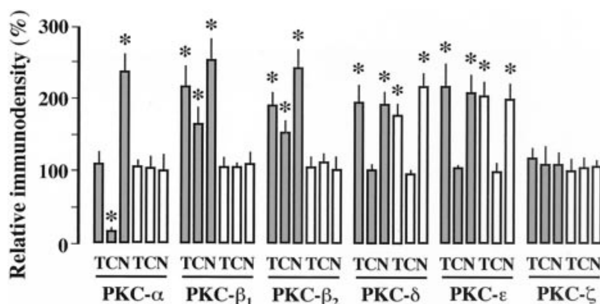

Figure 3. Quantitative analysis of the subcellular distribution of PKC isozymes in undifferentiated ES cells and ES-derived cardiomyocytes. Data are expressed as percentage changes in the intensity of autoradiographic bands of total extracts (T), cytosolic (C), and nuclear (N) fractions from ES-derived cardiomyocytes selected in the absence (gray bars) or presence (white bars) of 1 μmol/L Mr-1452, relative to the intensity in the autoradiographs of the corresponding samples from undifferentiated cells (considered as 100%). Mean±SE (n=6). *Significantly different from the control value.

Similar to P19 cells,[9] GTR1 ES cells expressed the prodynorphin gene and were able to synthesize and secrete dynorphin B. Moreover, after LIF removal their cardiac differentiation was associated with a remarkable increase in prodynorphin gene and dynorphin B expression (see online data supplement). We assessed whether secreted dynorphin B and cell surface opioid receptors may be responsible for the changes in subcellular PKC patterning observed during the cardiac differentiation of ES cells. After LIF withdrawal and throughout puromycin selection, GTR1 cells were exposed to Mr-1452, a selective κ opioid receptor antagonist.[21] Under these experimental conditions, ES-derived cardiomyocytes failed to exhibit an overexpression of PKC-β_1 and PKC-β_2 in total cellular extracts, as well as an increase of PKC-α and PKC-β_1/β_2 in the nuclear fraction (Figures 2 and 3). However, culturing undifferentiated ES cells with Mr-1452 did not appreciably affect the overexpression of PKC-δ and PKC-ϵ in the nucleus of ES-derived cardiomyocytes (Figures 2 and 3). Similar responses were observed in cardiomyocytes derived from undifferentiated ES cells that had been exposed to 1 μmol/L norbinaltorphimine (norBNI), another selective κ opioid receptor antagonist,[22] after LIF withdrawal, and during puromycin selection (not shown).

We next investigated whether PKC signaling may be involved in the modulation of a cardiogenic program of gene expression in the experimental model under investigation. Exposure of GTR1 cells to chelerythrine or calphostin C, two selective PKC inhibitors,[23,24] nearly abrogated the expression of the two cardiac lineage-promoting genes GATA-4 and Nkx-2.5 (Figure 4A). Expression of the cardiac-specific transcripts MHC and MLC-2V was concomitantly suppressed (Figure 4B). ES cell treatment with chelerythrine and calphostin C also downregulated prodynorphin gene expression (Figure 5A), as well as the synthesis and secretion of dynorphin B occurring throughout ES cell cardiogenesis (Figure 5B).

Cell treatment with 5 μmol/L chelerythrine or 1 μmol/L calphostin C after LIF removal and throughout 4 days of puromycin selection resulted in a remarkable decrease in the number of puromycin-resistant cells, representing 2% to 3%

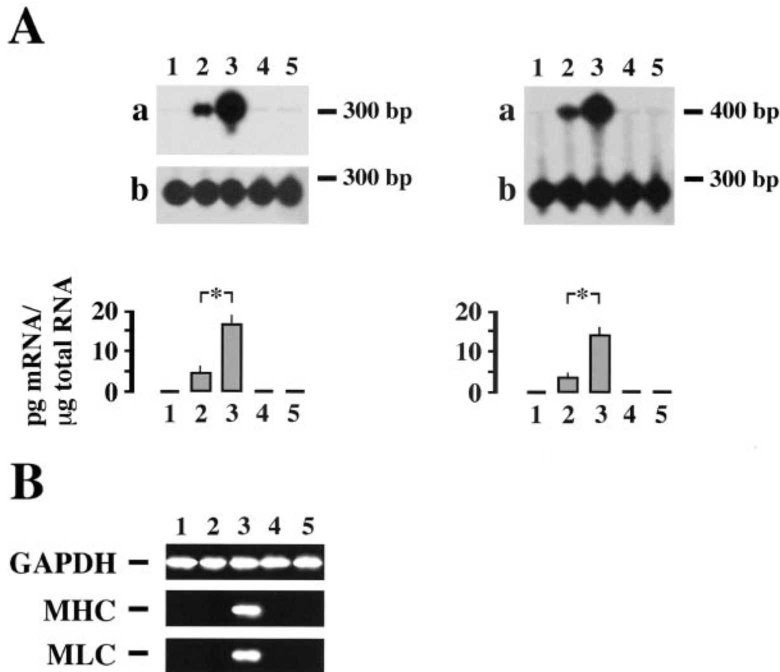

Figure 4. Effect of PKC inhibitors on a cardiac program of gene expression. Lane 1, Undifferentiated LIF-supplemented cells. Lane 2, Embryoid bodies collected 5 days after LIF removal. Lane 3, ES-derived cardiomyocytes 4 days after puromycin addition. After LIF removal and throughout puromycin selection cells were exposed to 5 μmol/L chelerythrine (lane 4) or 1 μmol/L calphostin C (lane 5). Four days after puromycin addition, cells were processed for gene expression analysis. A, RNase protection of GATA-4 (left, a) and Nkx-2.5 (right, a) mRNA. b, Cyclophilin mRNA. Equal amounts of total RNA (4 μg) from each sample were used in each RNase protection analysis. Autoradiographic exposure was for 2 days on Kodak X-Omat film with an intensifying screen. Right side of each panel reports the position of radiolabeled DNA markers, showing that the single protected fragments migrated with a molecular size comparable to GATA-4 (292 bases), Nkx-2.5 (414 bases), or cyclophilin (270 bases) mRNA. Due to the similar size of GATA-4– and cyclophilin-protected fragments, RNA samples were hybridized separately with GATA-4 and cyclophilin cRNA probes and the corresponding hybrids were run onto different gels. Averaged mRNA levels (mean±SE; n=6) are reported in the lower part of each panel. — indicates nondetectable. *Significant difference between values of bars included by the symbol (one-way analysis of variance, Newman Keul's test). B, RT-PCR of cardiac specific transcripts. MLC indicates myosin light chain-2V.

of the cell population yielded in the absence of PKC inhibitors. Immunocytochemical experiments indicated that cells surviving puromycin selection in the presence of PKC inhibitors failed to express MHC, as indicated by the lack of staining with MF20, a mouse monoclonal antibody raised against MHC (Figure 6). In this regard, a small percentage (about 1%) of MF20-negative cells was still observed under standard conditions (ie, without PKC inhibitors) in puromycin-selected cells (see online data supplement).

Culturing GTR1 cells in the presence of each PKC inhibitor also prevented the appearance of spontaneously beating colonies among the few cells resistant to puromycin treatment (Figure 7). Both Mr-1452 and norBNI significantly reduced, but did not abolish cardiomyocyte yield (Figure 7). Mr-1453, an inactive enantiomer of the corresponding opioid receptor antagonist, failed to affect cardiomyocyte yield (Figure 7). Cell treatment with 1 μmol/L Mr-1452 or norBNI after LIF removal and throughout 4 days of puromycin selection,

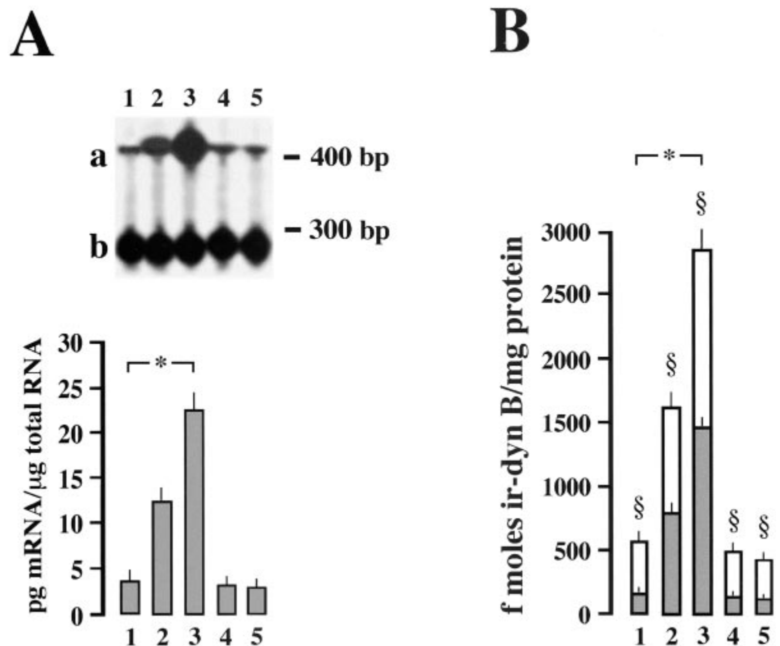

Figure 5. Effect of PKC inhibitors on prodynorphin gene and dynorphin B expression in ES cells. Lane 1, Undifferentiated LIF-supplemented cells. Lane 2, Embryoid bodies collected 5 days after LIF removal. Lane 3, ES-derived cardiomyocytes 4 days after puromycin addition. After LIF removal and throughout puromycin selection cells were exposed to 5 μmol/L chelerythrine (4) or 1 μmol/L calphostin C (5). Four days after puromycin addition, gene expression and radioimmunoassay analyses were performed. A, RNase protection of prodynorphin mRNA (a). b, Cyclophilin mRNA. Equal amounts of total RNA (4 μg) from each sample were used in each RNase protection analysis. Autoradiographic exposure was as described in the legend of Figure 4. Right side of the panel reports the position of radiolabeled DNA markers, showing that the single protected fragments migrated with a molecular size comparable to prodynorphin (424 bases) or cyclophilin (270 bases) mRNA. Averaged mRNA levels (mean±SE; n=6) are reported in the lower part of the panel. *Significant difference between values of bars included by the symbol (one-way analysis of variance, Newman Keul's test). B, Immunoreactive dynorphin B (ir-dyn B) was assessed in cells (gray bars) or medium (white bars), by the aid of a previously described radioimmunoassay procedure.[19] Each single value corresponds to the immunoreactivity normalized per milligram of cellular protein. Mean±SE (n=6). *Significant difference between values included by the symbol (one-way analysis of variance, Newman Keul's test). §Value of white bar is significantly different from that of the gray bar.

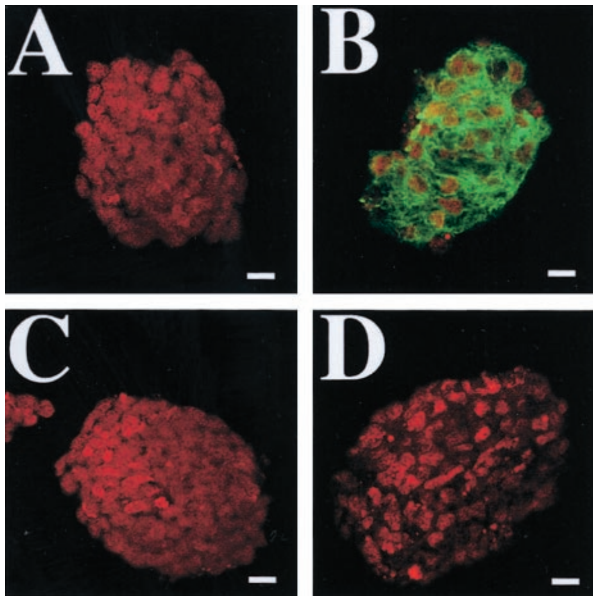

Figure 6. Immunofluorescence analysis of MHC. A, Undifferentiated LIF-supplemented cells. B, ES-derived cardiomyocytes 4 days after puromycin addition. After LIF removal and throughout puromycin selection, cells were exposed to 5 μmol/L chelerythrine (C) or 1 μmol/L calphostin C (D). In these experiments, immunocytochemical analyses were performed on the few cells surviving puromycin selection (2% to 3% of the population of puromycin resistant cells yielded in the absence of PKC inhibitors). Four days after puromycin addition, cells were fixed with 4% paraformaldehyde and α-myosin heavy chain expression was assessed by the aid of the MF20 mouse antimyosin monoclonal antibody. All microscopy was performed with a Biorad Microradians confocal microscope. DNA was visualized with propidium iodide (1 μg/mL). Bar=40 μm.

respectively, reduced the percentage of MF20-positive cells to 48.9±9.7% or 50.3±11.4% of the control value, estimated in the absence of opioid receptor antagonists (mean±SEM of 6 separate experiments).

Discussion

The present study identifies κ opioid binding sites in plasma membranes isolated from both undifferentiated GTR1 cells and ES-derived cardiac myocytes. In adult cardiomyocytes, κ opioid receptors have been extensively detected at the sarcolemmal level.[25–27] The finding that the B_{max} value for plasma membrane κ opioid receptors was markedly increased in ES-derived cardiomyocytes indicates that these receptors may be related to myocardial ontogeny and suggests that their overexpression may reinforce the cardiogenic potential associated with the increase in dynorphin B secretion occurring after LIF removal.

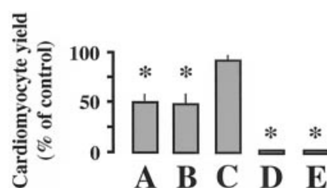

Figure 7. Analysis of cardiomyocyte yield from ES cells cultured in the absence or presence of opioid receptor antagonists or PKC inhibitors. After LIF removal and throughout puromycin selection, cells were cultured in the absence or presence of 1 μmol/L each of Mr-1452 (A), norBNI (B), or Mr-1453 (C), or exposed to 5 mol/L chelerythrine (D) or 1 μmol/L calphostin C (E). Four days after puromycin addition, the number of beating clusters was assessed as an estimate of cardiomyocyte yield. Data (mean±SE; n=6) are expressed as percentage change in the number of beating colonies, relative to the control baseline yield in ES-derived cardiac myocytes, considered as 100%. *Significantly different from the control.

The experiments described in this study also indicate that PKC activation is tightly associated with the cardiac differentiation of ES cells. PKC-α, -β_1, -β_2, -δ, and -ϵ were all increased in the nucleus of ES-derived cardiac myocytes, as compared with nuclei from undifferentiated cells. In both groups of cells, PKC-δ and -ϵ were mainly expressed at nuclear level. This finding is in agreement with our previous immunoblot analysis of PKC isotype expression in adult myocardial cells, showing that both PKC-δ and PKC-ϵ were almost entirely expressed at nuclear level.[19,28] The present results are also in agreement with other studies that used immunofluorescent and confocal microscopy techniques to determine the subcellular localization of different PKC isozymes in intact myocytes, showing that PKC-δ and -ϵ immunostaining patterns were mainly detectable in the nucleus of unstimulated cells.[29] The molecular mechanism(s) underlying the increase in PKC-α, -β_1, -β_2, -δ, and -ϵ currently observed in the nucleus of ES-derived cardiomyocytes remain to be elucidated. However, PKC-α was only slightly expressed in the nucleus of undifferentiated cells and its increase in the cardiomyocyte nucleus depended on a translocation from the cytosolic compartment. On the contrary, the increase of both PKC-δ and PKC-ϵ in the nucleus of ES-derived cardiomyocytes occurred independently of enzyme translocation and appeared to reflect the overexpression of these isozymes detected in total cellular extracts from myocardial cells. We cannot exclude that such an increase may result from changes in isozyme turnover and/or gene expression occurring during the commitment to the cardiac lineage. Differently from PKC-δ and -ϵ, the nuclear increase in both PKC-β_1 and -β_2 observed in ES-derived cardiac myocytes was associated with enhanced isozyme expression in the cytosolic fraction of these cells, suggesting a complex interplay between selected PKC mRNA expression and isozyme redistribution within the cytosolic and nuclear compartments.

Coupling of cell-surface κ opioid receptors and secreted dynorphin B to PKC signaling within the cardiogenic process is supported by the observation that exposure of GTR1 ES cells to opioid receptor antagonists reduced the amount of cells committed to the cardiac lineage and prevented the nuclear increase of PKC-α, PKC-β_1, and PKC-β_2. Failure of Mr-1452 to affect the amount of PKC-δ and -ϵ detectable in the nucleus excludes an involvement of cell surface opioid receptors in enhancing the expression of these isozymes during cardiac differentiation. This may also explain why exposure of GTR1 ES cells to opioid receptor antagonists failed to abolish completely their capability to develop into cardiomyocytes. Whether the overexpression of PKC-δ and -ϵ may involve an intracellular action of dynorphin peptides remains to be elucidated. A causal role of PKC signaling in the activation of a cardiogenic program of differentiation is substantiated by the finding that both chelerythrine and calphostin C prevented the overexpression of the prodynorphin gene, as well as the onset of GATA-4 and Nkx-2.5

transcripts, after LIF withdrawal. Requirement for PKC activation in cardiogenesis is further inferred from the fact that ES cell treatment with specific PKC inhibitors counteracted the expression of the cardiac specific genes MHC and MLC-2V and suppressed ES cell differentiation into beating cardiomyoctyes. Downregulation of prodynorphin gene and dynorphin B expression by PKC inhibitors also prompts the hypothesis that changes in subcellular profiling of PKC isozymes may orchestrate an autocrine circuit of cardiogenesis involving a feed-forward stimulation of opioid gene expression sustained by coupling of secreted dynorphin B with plasma membrane opioid receptors.

On the whole, the present study indicates that recruitment of PKC signaling by a dynorphinergic system and changes in expression and subcellular distribution of selected PKC isozymes may be deeply involved in the cardiac differentiation of ES cells. Nevertheless, cardiogenesis is a complex phenomenon within ES cell commitment toward multiple developmental fates. Although puromycin selection led to a virtually pure population of cardiomyocytes, we cannot exclude that, during the early stage of EB differentiation, autocrine/paracrine signals essential for cardiogenesis may also originate from endorphin- and/or PKC-related patterning in cells committed to a nonmyocardial lineage. Additionally, puromycin-selected cells resulted to be a mixture of ventricular and atrial cardiomyocytes. Within this context, combinatorial approaches using multiple promoters and targeted markers may be envisioned to refine the selection procedure and generate ventricular versus atrial cardiomyocytes. Clarification of these issues must await further functional and molecular approaches and is the subject for future investigations.

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