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, -β1, -β2, -δ, -ε, and -ζ. Cardiac differentiation was associated with a marked increase in the Bmax value for a selective opioid receptor ligand and complex subcellular redistribution of selected PKC isozymes. PKC-α, -β1, -β2, -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, -β1, and -β2 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-β1, and PKC-β2 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.10-11 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
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 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 observed, 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 observed, cells were plated onto specialty plates (Costar ultra-low attachment clusters), containing the culture medium lacking supplemental LIF. After 7 days of culture, 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

[3H]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 F40). 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 MgCl2, 250 mmol/L sucrose, 0.1 mmol/L DTT, 0.5 mmol/L PMSF, 1 mmol/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. Kd and Bmax values were calculated with the LIGAND program.

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 semidyry transfer cell. Immunoreactions were performed overnight at 4°C in the presence of the primary antibody (antisera to PKC-α, PKC-β1, PKC-β2, PKC-δ, PKC-ε, or PKC-ζ) diluted 1:100. The membranes were then incubated with 125I-labeled donkey anti-rabbit IgG antibodies (105 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%).

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.10 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 Apal, BamHI, or Xbal 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 [32P]CTP of plasmids linearized with BamHI generated antisense strands of prodynorphin and Nkx-2.5 mRNA, whereas transcription of plasmids linearized with Xbal 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 [3H]U-69,593 ([3H]U-69), a selective radiolabeled ligand,20 in a plasma membrane-enriched fraction (F40) 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 F40 fraction obtained from undifferentiated GTR1 cells (Figure 1), with specific binding ranging between 75% and 85% of the total bound. The Scatchard plots of [3H]U-69 binding were linear and were characterized by a single dissociation constant (Kd) in the low nmol/L range (Figure 1). Analysis of [3H]U-69 binding in the F40 fraction isolated from undifferentiated parental R1 cells yielded similar results (see online data supplement). A marked increase in the maximal binding capacity (Bmax) for [3H]U-69 was evident in plasma membranes that had been isolated from puromycin-selected cardiac myocytes, as com-

Figure 1. Scatchard analysis of the specific binding of [3H]U-69,593 to F40 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 (○).
pared with the corresponding fraction from undifferentiated GTR1 cells (Figure 1). No significant difference in the $K_d$ values was found among the $F_{\alpha}$ 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-\(\alpha\) (80 kDa), PKC-\(\beta_1\), and PKC-\(\beta_2\) (80 kDa), PKC-\(\delta\) (78 kDa), PKC-\(\epsilon\) (97 kDa), and PKC-\(\zeta\) (75 kDa) (Figure 2). A similar expression of PKC-\(\alpha\) was observed in total extracts from undifferentiated ES cells and ES-derived cardiomyocytes, whereas PKC-\(\beta_1\) and PKC-\(\beta_2\) 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-\(\alpha\), anti-PKC-\(\beta_1\), and PKC-\(\beta_2\)-specific antibodies was detected in the nuclear fraction of undifferentiated ES cells. On the contrary, the expression of these isoforms was markedly increased in the nucleus of ES-derived cardiomyocytes (Figures 2 and 3). Concomitantly, PKC-\(\alpha\) was downregulated, whereas PKC-\(\beta_1\) and PKC-\(\beta_2\) were overexpressed in their cytosolic fraction. Western blot analysis also indicated that the immunoreactivity against anti-PKC-\(\delta\) and anti-PKC-\(\epsilon\)-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-\(\zeta\) appeared to be similar in both groups of cells. No evidence for a nuclear localization of this isozyme was provided (Figures 2 and 3).

Similar to P19 cells, 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 \(\kappa\) opioid receptor antagonist. Under these experimental conditions, ES-derived cardiomyocytes failed to exhibit an overexpression of PKC-\(\beta_1\) and PKC-\(\beta_2\) in total cellular extracts, as well as an increase of PKC-\(\alpha\) and PKC-\(\beta_1/\beta_2\) in the nuclear fraction (Figures 2 and 3). However, culturing undifferentiated ES cells with Mr-1452 did not appreciably affect the overexpression of PKC-\(\delta\) and PKC-\(\epsilon\) 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 \(\mu\)mol/L norbinaltorphimine (nortBNI), another selective \(\kappa\) opioid receptor antagonist, 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, 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 \(\mu\)mol/L chelerythrine or 1 \(\mu\)mol/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% of the corresponding samples from undifferentiated ES cells; lanes b, d and f, Total cell lysates, cytosolic, and nuclear fractions were isolated from ES-derived cardiomyocytes.
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).

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 Nix-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 3 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), Nix-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. *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.

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.
receptors have been extensively detected at the sarcolemmal level. The finding that the Bmax 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.

The experiments described in this study also indicate that PKC activation is tightly associated with the cardiac differentiation of ES cells. PKC-α, -β1, -β2, -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. 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. The molecular mechanism(s) underlying the increase in PKC-α, -β1, -β2, -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-β1 and -β2 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-β1, and PKC-β2. 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 cardiomyocytes. Downregulation of prodynorphin gene and dynorphin B expression by PKC inhibitors also prompts the hypothesis that changes in subcellular profiling of PKC isoforms 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 dynorpheristic system and changes in expression and subcellular distribution of selected PKC isoforms 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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References

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Expanded Materials and Methods

Materials

KNOCKOUT D-MEM, foetal bovine serum (FBS), M-MLV reverse transcriptase, Taq polymerase, DNA molecular weight markers and dNTPs were purchased from Gibco. Restriction enzymes were from Boehringer Mannheim. pCRII-TOPO was from Invitrogen (CA). $^{32}\text{P}]\text{CTP}$, and $[^3\text{H}]\text{U-69,593}$ were from Amersham International. Unlabeled U-69,593 was from The Upjohn Co. (Kalamazoo, MI). $(—)-(3\text{-Furylmethyl})\alpha $-normetazocine methanesulfonate (Mr-1452) was a gift from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CO). RNAMATRIX™ was from BIO 101, Inc. (Vista, CA). Antisera to PKC-α, PKC-β1, PKC-β2, PKC-δ, PKC-ε, or PKC-ζ were from Calbiochem. Chelerythrine and calphostin C were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Monoclonal antibodies raised against sarcomeric myosin (MF 20) and nestin were from the Developmental Studies Hybridoma Bank (The University of Iowa, IA). Leukemia Inhibitory Factor (LIF) was from Chemicon. Ultra low attachment clusters were from Costar. All the other chemicals were from Sigma.
Differentiation and Selection of ES-Derived Cardiomyocytes

GTR1 ES cells, bearing a transgene encoding the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter driving the puromycin-resistance gene were kindly provided by Dr. William L. Stanford (University of Toronto and Centre for Modeling Human Disease, Canada). These cells are a derivative of R1 ES cells, previously shown to generate late-stage viable tetraploid embryos, as well as germ line transmitting chimeras at a remarkable rate (1). GTR1 ES cells were used at early passages (passage 6-8), and 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% FBS, supplemented with a final concentration of 1000 U/ml LIF. Culture medium also included penicillin/streptomycin, 2 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, sodium pyruvate, and non-essential aminoacids. Before embryoid bodies (EBs) can be made, subconfluent undifferentiated ES cells were recovered from feeder layers by trypsinization and transferred onto 0.1% gelatin-coated plates. In gelatinized plates, cells were grown to about 70-80% confluency in the presence of LIF-supplemented KNOCKOUT D-MEM containing 15% FBS. Based on characterization studies (see below), these cells were used as undifferentiated cells in each experiment. Then, 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 non-cardiomyocytes and puromycin-selected cells were cultured for an additional period of 7 days. Then, to determine the cardiomyocyte content, the selected cells were dissociated with trypsin, and processed for MHC immunoreactivity. In comparative analyses, the cardiomyocyte content was assessed following a physical enrichment protocol. Briefly, rhythmically contracting regions were microdissected with a sterile Pasteur pipette 14 days after LIF withdrawal, dissociated with trypsin and subjected to immunocytoLOGY.
EBs, collected at several stages after plating, as well as puromycin-selected cells were processed for gene expression and immunofluorescence analyses. Following LIF removal and throughout puromycin selection, GTR1 cells were also exposed to different selective protein kinase C inhibitors or opioid receptor antagonists.

**Immunofluorescence analysis of ES-derived cardiomyocytes**

Puromycin-selected cells were treated with trypsin, and the resulting suspension was cultured at low density to permit visualization of individual cells. The cultures were fixed with 4% paraformaldehyde. MHC was assessed by the aid of the MF 20 mouse antimyosin monoclonal antibody (2). All microscopy was performed with a Biorad Microradians confocal microscope. DNA was visualized with propidium iodide (1 µg/ml).

**Isolation of Subcellular Fractions**

In order to prepare a fraction (referred to as F₄₀) enriched in plasma membranes, undifferentiated or puromycin-selected cells were first homogenated with a Dounce homogenizer (three strokes of an A pestle) in a medium containing: 50 mmol/L Tris/HCl, pH 7.4, 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L DTT, 0.5 mmol/L PMSF, 1 µmol/L leupeptin, and 10 mmol/L β-mercaptoethanol. The homogenate was centrifuged at 1000 x g at 4 °C for 15 min. The supernatant was further centrifuged at 40000 x g at 4 °C for 30 min. The resulting pellet (F₄₀) was then resuspended in the binding buffer (see below in this section). In the F₄₀ fraction and in cell homogenates from undifferentiated cells, the ouabain-sensitive Na⁺-K⁺ ATPase activity was 18.3 ± 0.8 and 1.7 ± 0.06 µmoles/mg protein/hour, respectively, as estimated by the measure of the inorganic phosphate released (3).

Isolation of nuclei and assessment of nuclear purity were performed as detailed elsewhere (3). The nuclear preparation lacked contamination by endoplasmic reticular membranes, inner or
outer mitochondrial membranes or plasma membranes, as indicated by the measurement of the activity of the corresponding marker enzymes rotenone-insensitive NADPH cytochrome c reductase, succinate dehydrogenase, rotenone-insensitive NADH cytochrome c reductase, 5’ nucleotidase and Na⁺,K⁺-ATPase, which were all undetectable in the nuclear fraction (not shown).

To prepare the cytosolic fraction, ES cells were first homogenated with a Dounce homogenizer (Three strokes of an A pestle) in a medium containing: 50 mmol/L Tris/HCl, pH 7.4, 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1µmol/L leupeptin and 10 mmol/L β-mercaptoethanol. The homogenate was centrifuged at 1000 x g at 4 °C for 15 min. The supernatant was further centrifuged at 100,000 x g at 4 °C for 60 min. The resulting supernatant was designated the cytosolic fraction.
Opioid Binding Assay

[^3]H]U-69,593 (55.0 Ci/mmol) was used as a selective κ opioid receptor ligand in binding assays. Each sample (300 µg of protein) was incubated with the radiolabeled ligand (1, 2, 3, 4, 5, 10, 15, 20 or 30 nmol/L) in 0.25 ml of a binding buffer containing: 50 mmol/L Tris HCl, pH 7.4, 5 mmol/L MgCl₂, 250 mmol/L sucrose, 0.1 mmol/L DTT, 0.5 mmol/L PMSF, 1 µmol/L leupeptin, 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 binding reactions were allowed to run for 45 min at 25 °C and stopped by diluting 5-times with ice-cold binding buffer. The incubation media were then filtered over vacuum on Whatman GF/B glass fiber filters, followed by three washes with 10 ml of ice-cold binding buffer. Filters were finally counted for radioactivity by liquid scintillation spectrometry. K_d and B_max values were calculated with the LIGAND program (4).

Immunoblotting Analysis of Protein Kinase C (PKC)

Total cell lysates, cytosolic fractions or nuclear samples from undifferentiated ES cells or ES-derived cardiomyocytes were electrophoresed on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose using a semi-dry transfer cell (BIO 101, Inc. Vista, CA). After transfer, the blot was saturated for 1 h at room temperature with 3% BSA in Tris-buffered saline containing Tween (TBS-T) (50 mmol/L Tris/HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20). The immunoreaction was carried out overnight at 4 °C in TBS containing 1% BSA, 0.02% Tween 20 and the primary antibody (antisera to PKC-α, PKC-β₁, PKC-β₂, PKC-δ, PKC-ε, or PKC-ζ) diluted 1:100. After being washed three times with TBS-T (5 min each wash), the membranes were incubated with ¹²⁵I-labeled donkey anti-rabbit IgG antibodies (10⁶ cpm/ml) in TBS-T with 1% BSA for 1 h at room temperature. After additional washings with TBS-T, the nitrocellulose
membranes were dried and exposed to Kodak X-OMAT AR films with an intensifying screen for 48 h at -70 °C. The intensities of the autoradiographic bands were measured with a laser densitometer (Image Quant-Computing Densitometer 300/325, Molecular Dynamics, Sunnyvale, CA) 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%).

Analysis of Gene Expression

Total RNA extraction, reverse transcription, oligonucleotide sequences and RT-PCR conditions for the detection of α-myosin heavy chain (MHC), myosin light chain-2V (MLC-2V), myosin light chain-2A (MLC-2A), and GAPDH mRNAs were previously described (5,6). Prodynorphin, GATA-4 and Nkx-2.5 mRNAs were assessed by RNase protection assay, as described in detail elsewhere (3). Briefly, 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 (Invitrogen, CA). Transcription of the plasmid linearized with ApaI, BamHI, or XbaI 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 [32P]CTP (800 Ci/mmol) (Amersham International) of plasmids linearized with BamHI generated antisense strands of prodynorphin and Nkx-2.5 mRNA, whereas transcription of plasmids linearized with XbaI produced an antisense strand of GATA-4 mRNA. 32P-labeled antisense cyclophilin mRNA was synthesized from a NcoI-linearized pBS vector containing a 270-base pair fragment of pLB15, a cDNA clone encoding for rat cyclophilin (4). Cyclophilin mRNA was utilized as a constant mRNA for control. Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and
electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was performed for 48 h. The individual bands were counted for radioactivity by liquid scintillation spectrometry, and cpm values were translated to pg values on a correlated standard curve. Data were expressed as pg of mRNA/µg of total RNA.

**Identification of Dynorphin B-Like Material**
Immunoreactive dynorphin B (ir-dyn B) was measured by a radioimmunoassay procedure that utilized the 13 S antiserum raised against dynorphin B and capable of recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dynorphin B in their sequence (7,8). Acetic acid extracts from undifferentiated or cardiac-lineage-committed ES cells, or pooled samples from their incubation media were processed by reverse-phase high performance liquid chromatography. The collected fractions were radioimmunoassayed and the immunoreactivity was attributed to authentic dynorphin B by comparison with the elution position of a synthetic standard, according to a previously described procedure (3).

**Proteins**
Protein concentration was determined with the folin reagent (9), using bovine serum albumin as a standard.

**Data analysis**
The statistical analysis of the data was performed by using a one-way analysis of variance followed by Newman Keul’s test and assuming a $p$ value less than 0.05 as the limit of significance.
**Additional Figures and Supporting Information**

**Characterization of GTR1 ES cells**

ES cells seeded on feeder cells exhibited an undifferentiated morphology characterized by rounded colonies with distinct borders, tightly packed with very small ES cells (Fig. 1). The undifferentiated state was further inferred by assessment of alkaline phosphatase activity, according to a previously described protocol (10). As expected for undifferentiated cells, 4-day-old cultures on feeder layers stained intensely for alkaline phosphatase activity. Analysis of staining indicated a significantly higher percentage of undifferentiated ES cells in the presence of KNOCKOUT D-MEM, containing 15% FBS + LIF, than in the presence of D-MEM supplemented by 15% FBS + LIF (Fig. 2). No significant change in the amount of undifferentiated cells was observed in ES cells that had been recovered from feeder layers by trypsinization and grown onto gelatinized plates in LIF supplemented KNOCKOUT D-MEM, containing 15% FBS (Fig. 2). No evidence for nestin positive cells, an indication for the presence of potential precursors of neurons and glial cells (11), was detected in ES cells cultures grown in KNOCKOUT D-MEM supplemented with LIF (not shown).

Comparative analyses of the cardiomyocyte yield were performed in non-selected cells, as well as in GTR1 cells that had been subjected either to physical isolation or puromycin selection. To determine the cardiomyocyte content, the different cultures were digested with trypsin and replated at a lower density to assess immunoreactivity in individual cells. These studies indicated that cardiomyocytes (MF 20 immunoreactive cells) comprised less than 1% of the non-selected population (Table 1). Although selection by physical isolation yielded a remarkable enrichment in cardiomyocytes, these cells comprised only 4.2% of the cells detectable in microdissected cultures (Table 1). On the contrary, immunofluorescence analysis revealed that about 99% of the cells obtained with genetic selection expressed sarcomeric myosin (Table 1). The presence of sarcomeric myosin immunoreactivity coupled with the absence of nebulin immunoreactivity has been shown
to represent a valuable feature for the identification of cardiomyocytes in ES cell cultures (12). Akin to these observations, puromycin-selected GTR1 cells exhibited MF 20 immunoreactivity but not nebulin immunoreactivity, as assessed in the presence of the NB2 antibody (not shown).

Electrophysiologic analyses have identified action potentials typical for both atrial and ventricular cardiomyocytes in ES-isolated cell preparations (13,14). In GTR1 cells, RT-PCR analysis of MLC-2V and MLC-2A transcripts was performed in samples isolated from adult mouse atria or ventricles, undifferentiated GTR1 cells, and puromycin selected cardiomyocyte cultures. As expected, MLC-2A and MLC-2V mRNA were selectively expressed in atria and ventricles, respectively (Fig. 3). No signal arose from undifferentiated cultures. In contrast, both MLC-2V and MLC-2A mRNA were detectable in genetically selected cardiomyocytes (Fig. 3), suggesting that puromycin-selected cardiomyocytes may represent a mixed population of cells consisting of several different cardiac lineages (i.e. atrial, and ventricular-like).

**Prodynorphin gene expression during cardiogenesis in GTR1 cells**

Consonant with our previous findings in P19 cells (5), prodynorphin mRNA expression was detectable in undifferentiated LIF-supplemented cells and time-dependently increased following LIF removal in both EBs and puromycin-selected cardiomyocytes (Fig. 4 A). Immunoreactive dynorphin B was concomitantly detectable in both acetic acid extracts and incubation media from LIF-treated cells (Fig. 4 B). Following LIF withdrawal, both cellular and released dynorphin B progressively increased in EBs and puromycin-selected cardiomyocytes (Fig. 4 B).

**Comparative analysis of [³H]U-69,593 binding to F₄₀ membranes in GTR1 cells and R1 ES cells**

Binding experiments performed by the aid of the selective radiolabeled ligand [³H]U-69,593 ([³H]U-69) revealed the presence of highly specific κ opioid-binding sites in a plasma membrane-
enriched fraction (F_{40}) isolated from undifferentiated GTR1 cells (Fig. 5 A), with specific binding ranging between 75% and 85% of the total bound. The Scatchard plots of [^3H]U-69 binding were linear and were characterized by a single dissociation constant (K_d) in the low nM range (Fig. 5 A). Similar binding parameters were observed in the F_{40} fraction isolated from undifferentiated parental R1 cells (Fig. 5 B).
References


TABLE 1. Cardiomyocyte yield in non-selected, physically isolated, and puromycin selected differentiating GTR1 ES cells.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>MF 20 (+) cells</th>
<th>MF 20 (-) cells</th>
<th>cardiomyocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No selection (A)</td>
<td>20</td>
<td>3000</td>
<td>0.66</td>
</tr>
<tr>
<td>Physical isolation (B)</td>
<td>127</td>
<td>3000</td>
<td>4.2</td>
</tr>
<tr>
<td>Puromycin selection (C)</td>
<td>1012</td>
<td>10</td>
<td>99.0</td>
</tr>
</tbody>
</table>

Analysis was performed on multiple dishes obtained from the same differentiating culture. A, 14 days after LIF removal, plates were treated with trypsin and the resulting suspension was plated at low density to permit visualization of individual cells. After 24 hours of culture, sarcomeric myosin immunoreactivity was assessed (Methods). B, 14 days after cardiogenic induction, rhythmically contracting regions were microdissected with a sterile Pasteur pipette, dissociated with trypsin and processed as described for MF 20 staining. C, 7 days after LIF removal, upon the appearance of spontaneous contractile activity, puromycin (2 µg/ml) was added to eliminate non-cardiomyocytes and puromycin selected cells were cultured for 7 additional days. The selected cells were trypsinized and the resulting suspension was cultured at low density. After 24 hours, MF 20 positive cells were assessed.
Figure Legend

Figure 1. GTR1 ES cells grown for 4 days on a layer of mitotically inactivated mouse embryo fibroblasts. Phase contrast image using a 20X objective.

Figure 2. Analysis of alkaline phosphatase staining. The percentage of undifferentiated cells was estimated by assessing alkaline phosphatase activity in ES cells grown for 4 days on feeder cells with DMEM + LIF (A), or KNOCKOUT D-MEM + LIF (B), or in ES cells that had been recovered from feeder layers and grown to 70-80% confluency onto gelatinized plates in the presence of KNOCKOUT D-MEM + LIF (C). *, significantly different from A.

Figure 3. Analysis of MLC-2V and MLC-2A in myocardial tissues and ES cells. Total RNA was extracted from atria or ventricles of adult mice, as well as from undifferentiated ES cells harvested from feeder layers and grown to 70-80% confluency onto gelatinized plates, or from puromycin selected cells, 7 days after puromycin addition. RNA samples were then processed for RT-PCR analysis. Each panel shows ethidium bromide-stained agarose gels of the PCR amplification of the indicated transcripts. MLC-2V, myosin light chain-2V. MLC-2A, myosin light chain-2A.

Figure 4. Prodynorphin gene and dynorphin B expression in EBs and puromycin-selected cardiomyocytes. (A) RNase protection of prodynorphin mRNA. Time 0, undifferentiated LIF-supplemented cells. EBs, embryoid bodies collected 3, 5 or 7 days after LIF removal. P, puromycin-selected cardiomyocytes: puromycin was added at day 8 following LIF removal. Equal amounts of total RNA (4 µg) from each sample were used in each RNase protection analysis. The left side of the panel reports the position of a radiolabeled DNA marker, showing that the single protected fragment migrated with a molecular size comparable to prodynorphin mRNA (424 bases). Averaged mRNA levels (Mean ± S.E.; n = 6) are reported in the lower part of the panel. (B), immunoreactive dynorphin B (ir-dyn B) was assessed in cells (Grey bars) or medium (White bars). Each single value corresponds to the immunoreactivity normalized per milligram of cellular protein. Mean values ± S.E. (n = 6). *, significant difference between values included by the
symbol (one-way analysis of variance, Newman Keul’s test). §, the value of white bar is significantly different from that of the grey bar.

**Figure 5.** Scatchard analysis of the specific binding of $[^3$H]$U$-69,593 to F$_{40}$ membranes isolated from undifferentiated, LIF-supplemented GTR1 cells (A) or R1 ES cells (B). The data are expressed as mean values ± S.E. (n = 6).
Online fig 3

GAPDH -

MLC-2V -

MLC-2A -
A

B

B/F

Bound (fmol/mg protein)

Online fig 5

Bmax

Bmax

Kd

Kd

fmol/mg protein

fmol/mg protein

(nM)

(nM)

212.09 ± 20.12  5.18 ± 0.20

222.15 ± 30.18  4.98 ± 0.30