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# Dynorphin B Is an Agonist of Nuclear Opioid Receptors Coupling Nuclear Protein Kinase C Activation to the Transcription of Cardiogenic Genes in GTR1 Embryonic Stem Cells

Carlo Ventura, Elisabetta Zinellu, Emiliana Maninchedda, Margherita Maioli

**Abstract**—The cardiac differentiation of embryonic stem (ES) cells was found to involve prodynorphin gene and dynorphin B expression and was associated with the interaction of secreted dynorphin B with cell surface opioid receptors coupled with protein kinase C (PKC) signaling and complex subcellular redistribution patterning of selected PKC isozymes. Here, confocal microscopy revealed the presence of immunoreactive dynorphin B-like material in GTR1 ES cells, suggesting that dynorphin peptides may also act intracellularly. Opioid binding sites were identified in ES cell nuclei, with a single dissociation constant in the low nanomolar range. A significant increase in  $B_{\max}$  for a  $\kappa$  opioid receptor ligand was observed in nuclei isolated from ES-derived cardiomyocytes compared with nuclei from undifferentiated cells. Direct exposure of nuclei isolated from undifferentiated ES cells to dynorphin B or U-50,488H, a synthetic  $\kappa$  opioid receptor agonist, time- and dose-dependently activated the transcription of GATA-4 and Nkx-2.5, 2 cardiac lineage-promoting genes. Nuclear exposure to dynorphin B also enhanced the rate of prodynorphin gene transcription. These responses were abolished in a stereospecific fashion by the incubation of isolated nuclei with selective opioid receptor antagonists. Nuclei isolated from undifferentiated cells were able to phosphorylate the acrylodan-labeled MARCKS peptide, a high-affinity fluorescent PKC substrate. Exposure of isolated nuclei to dynorphin B induced a remarkable increase in nuclear PKC activity, which was suppressed by opioid receptor antagonists. Nuclear treatment with PKC inhibitors abolished the capability of dynorphin B to prime the transcription of cardiogenic genes. (*Circ Res.* 2003;92:623-629.)

**Key Words:** nuclear receptors ■ nuclear signaling ■ endorphins ■ embryonic stem cells ■ gene transcription

Opioid peptides govern important physiological responses, including pain,<sup>1</sup> behavior,<sup>2</sup> and learning and memory,<sup>3,4</sup> and have also been shown to affect cell growth in a wide variety of normal and malignant tissues.<sup>5,6</sup> Dynorphin B, an end product of the prodynorphin gene acting as a natural agonist of  $\kappa$  opioid receptors, has been recently shown to prime cardiogenesis in P19 embryonal pluripotent cells,<sup>7</sup> indicating that endorphinergic systems may be involved in myocardial growth and differentiation. In a companion article<sup>8</sup> in this issue of *Circulation Research*, we reported that the cardiac differentiation of GTR1 embryonic stem (ES) cells was tightly entangled with the interaction of secreted dynorphin B with cell surface opioid receptors and was coupled with protein kinase C (PKC) signaling and complex subcellular redistribution patterning of selected PKC isozymes. However, radioimmunoassay of immunoreactive dynorphin B in ES cells revealed that, though most of the peptide was targeted for secretion, consistent amounts of dynorphin B are also found at the cellular level, with a progressive increase

throughout the cardiogenic process.<sup>7,8</sup> These observations prompt the hypothesis that the cardiac differentiation of ES cells may also result from an intracellular action of the opioid peptide.

Within this context, it is increasingly becoming evident that cell nuclei harbor the potential for an intrinsic signal transduction pathway(s), as indicated by the presence of nuclear enzymes and substrates associated with the synthesis of diacylglycerol and inositol phospholipids. In isolated rat liver nuclei, the nuclear envelope was first found to synthesize in vitro phosphatidic acid, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-diphosphate.<sup>9,10</sup> Moreover, nuclear inositol phospholipids have been consistently shown to serve as regulators of chromatin structure,<sup>11,12</sup> and nuclear phosphoinositide-related signaling has been reported to operate in conjunction with the activation of nuclear PKC isozymes, affording gene transcription regulation.<sup>13,14</sup> Consonant with these findings is our recent observation that PKC- $\delta$  and PKC- $\epsilon$  are constitutively

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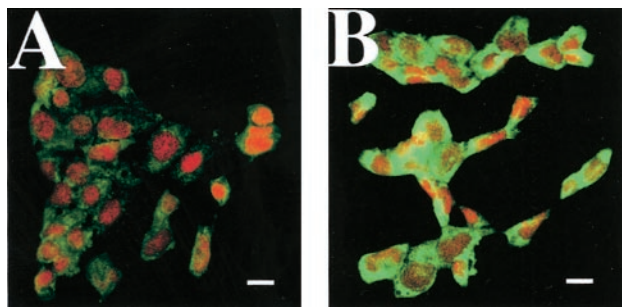
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**Figure 1.** Immunofluorescence analysis of immunoreactive dynorphin B in ES cells. A, Undifferentiated LIF-supplemented cells. B, ES-derived cardiomyocytes 4 days after puromycin addition. All microscopy was performed with a Bio-Rad Micro-Radiations confocal microscope. DNA was visualized with propidium iodide (1  $\mu\text{g}/\text{mL}$ ). Bar=40  $\mu\text{m}$ .

expressed in the nucleus of undifferentiated ES cells and that their expression is remarkably enhanced in nuclei isolated from ES-derived cardiac myocytes.<sup>8</sup> These data raise the question of whether nuclear signaling may have a role in ES cell commitment to the cardiac lineage and whether cardiogenesis may also involve the intervention of an intracellular agonist coupling nuclear-embedded PKC isozymes to the activation of a gene program of cardiac differentiation.

In the present study, we investigated whether opioid receptors might be expressed in the nucleus of ES cells and whether, in the affirmative, a nuclear endorphinergic system might modulate the cardiogenic potential of ES cells. In this regard, we assessed whether a direct exposure of isolated ES cell nuclei to dynorphin B or a synthetic ligand of  $\kappa$  opioid receptors might affect the transcription rate of cardiac lineage-promoting genes. Opioid-treated nuclei were also processed for the analysis of nuclear PKC activity.

## Materials and Methods

### Cardiac Differentiation of GTR1 ES Cells

GTR1, a derivative of R1 ES cells<sup>15</sup> bearing the puromycin-resistance gene driven by the cardiomyocyte-specific  $\alpha$ -myosin heavy chain promoter, were kindly provided by Dr William L. Stanford (University of Toronto and Center for Modeling Human Disease, Toronto, Canada). Cardiac differentiation and puromycin selection of ES-derived cardiomyocytes were performed as previously described.<sup>8</sup>

### Immunofluorescence

When processed for fluorescence microscopy, the cells were fixed with 4% paraformaldehyde. Immunoreactive dynorphin B-like ma-

terial was identified by the aid of 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.<sup>16,17</sup> All microscopy was performed with a Bio-Rad Micro-Radiations confocal microscope. DNA was visualized with propidium iodide (1  $\mu\text{g}/\text{mL}$ ).

### Isolation of ES Cell Nuclei and Opioid Binding Assay

Isolation of nuclei and assessment of nuclear purity were performed as detailed elsewhere.<sup>14</sup> Only freshly isolated nuclei were used in each experiment.

[<sup>3</sup>H]U-69,593 ([<sup>3</sup>H]U-69, 55.0 Ci/mmol, Amersham International) was used as a selective  $\kappa$  opioid receptor ligand. Each sample (300  $\mu\text{g}$  of protein) was incubated with the radiolabeled ligand, and binding assays were conducted as previously described.<sup>8</sup>  $K_d$  and  $B_{\text{max}}$  values were calculated with the LIGAND program.<sup>18</sup>

### Nuclear Runoff Transcription Assay

Nuclear runoff experiments were carried out as described.<sup>13,14</sup> Nuclear RNA was isolated using guanidine thiocyanate and acid phenol extraction, followed by purification on RNAMATRIX (BIO 101, Inc [Vista, Calif]). Equal counts of <sup>32</sup>P-labeled RNA ( $\approx 5 \times 10^6$  cpm) were then subjected to a solution hybridization RNase protection assay and were hybridized for 12 hours at 55°C in the presence of unlabeled prodynorphin, GATA-4, Nkx-2.5, MyoD, or neurogenin1 cRNA probes. Cyclophilin mRNA was used as a constant mRNA for control.

### Nuclear PKC Activity

PKC activity from isolated nuclei was measured according to a previously described procedure,<sup>13,14</sup> which used a continuous fluorescence assay in the presence of the acrylodan-labeled myristoylated alanine-rich PKC substrate (MARCKS peptide, Bio-synthesis Inc), a high-affinity fluorescent substrate in vitro for PKC.<sup>19,20</sup> This substrate consists of a conserved sequence of 24 amino acids from the MARCKS protein, which includes 4 sites for PKC phosphorylation.<sup>21</sup> In the presence of PKC activators, maximum fluorescence is measured at 480 nm, with excitation at 370 nm, and PKC-dependent phosphorylation is highlighted by a decrease in fluorescence intensity.<sup>21</sup> In the present study, the fluorescence changes occurring during the phosphorylation of the MARCKS peptide were monitored at 37°C. The reaction mixture contained, in a final volume of 1 mL, 10 mmol/L Tris-HCl, pH 7.0, 90 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, 0.3 mmol/L CaCl<sub>2</sub>, 0.1 mmol/L EGTA, 100  $\mu\text{mol}/\text{L}$  ATP, 10% ethylene glycol, 0.5  $\mu\text{g}$  phosphatidylserine, 0.1  $\mu\text{g}$  1,2-dioctanoyl-*sn*-glycerol, and 75 nmol/L acrylodan-labeled MARCKS peptide. The phosphorylation of the acrylodan-labeled peptide was started by adding 10  $\mu\text{g}$  of nuclear protein.

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

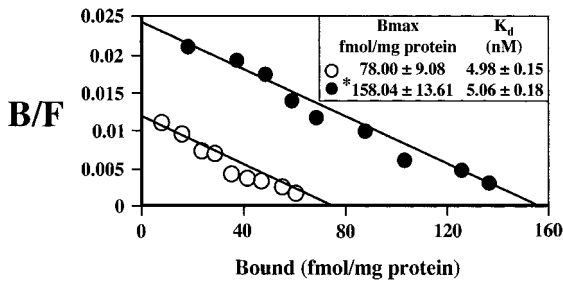
## Results

Immunoreactive dynorphin B-like material was previously detected by a radioimmunoassay procedure in acetic acid extracts

### Specific Enzymatic Activities in the Homogenate and Nuclear Fraction Isolated From GTR1 ES Cells

Enzyme Activities	Homogenate	Nuclear Fraction
Na <sup>+</sup> ,K <sup>+</sup> -ATPase (ouabain-sensitive)	2.310±0.070	ND
5'-nucleotidase	0.825±0.064	ND
NADH cytochrome <i>c</i> reductase (rotenone-insensitive)	0.165±0.100	ND
Succinate dehydrogenase	4.950±0.420	ND
NADPH cytochrome <i>c</i> reductase (rotenone-insensitive)	0.305±0.013	0.009±0.006

Each fraction was prepared from undifferentiated LIF-supplemented cells. Values for enzymatic activities ( $\mu\text{mol}$  product formed/mg protein per hour) are mean±SE (n=6). ND indicates nondetectable.



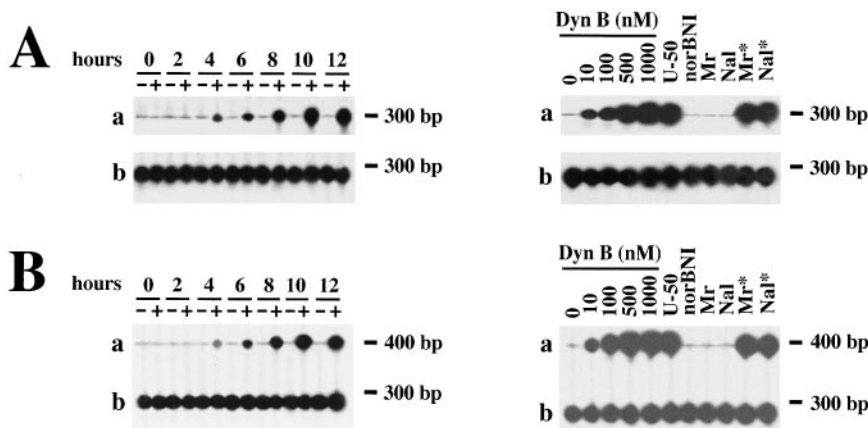
**Figure 2.** Scatchard analysis of the specific binding of [<sup>3</sup>H]U-69 to ES cell nuclei. Samples were isolated from undifferentiated LIF-supplemented cells (open circles) or from ES-derived cardiomyocytes 4 days after puromycin addition (filled circles). The data are expressed as mean ± SE values (n=6). B/F indicates bound/free ratio. \*Significantly different from LIF-supplemented cells (by 1-way ANOVA, Newman-Keuls test), P<0.05.

from undifferentiated or cardiac lineage-committed ES cells.<sup>7,8</sup> In the present study, the intracellular patterning of immunoreactive dynorphin B was investigated by the aid of confocal microscopy in GTR1 ES cells. Confirming the results from radioimmunoassay analysis, immunostaining of dynorphin B-like material was faintly detected in undifferentiated ES cells and was highly enhanced in puromycin-selected cardiomyocytes (Figure 1). This observation prompted us to verify whether the opioid peptide may act intracellularly. In a companion study,<sup>8</sup> we detected κ opioid receptors in a plasma membrane preparation from GTR1 ES cells and provided evidence that the expression of these cell surface receptors is increased in ES-derived cardiac myocytes. In the present study, we determined whether nuclear opioid binding sites may also be detectable in ES cells. The nuclear preparation used in the present study exhibited a high degree of purity. The Table shows the activity of marker enzymes of selected subcellular fractions in nuclei prepared from undifferentiated GTR1 cells. It is evident that the activity of the ouabain-sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase and the activity of 5' nucleotidase were both undetectable in the nuclear fraction, excluding a contamination by plasma membranes. The

contamination by inner or outer mitochondrial membranes was excluded by the measure of succinate dehydrogenase and rotenone-insensitive NADH cytochrome c reductase activities, both of which were undetectable in the nuclear fraction. Moreover, the nuclear preparation appeared to be essentially free of endoplasmic reticular membranes, as revealed by the measure of the corresponding marker enzyme, rotenone-insensitive NADPH cytochrome c reductase.

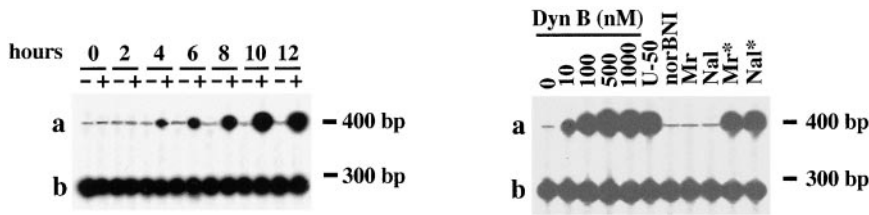
Nuclei isolated from undifferentiated ES cells or ES-derived cardiomyocytes were incubated in the presence of [<sup>3</sup>H]U-69, a selective radiolabeled κ opioid receptor ligand.<sup>22</sup> The binding experiments revealed the presence of highly specific κ opioid binding sites in nuclei obtained from undifferentiated GTR1 cells (Figure 2). Specific binding ranged between 75% and 85% of the total bound. The Scatchard plots of [<sup>3</sup>H]U-69 binding were linear, with a single dissociation constant (K<sub>d</sub>) in the low nanomolar range (Figure 2). A marked increase in the maximal binding capacity (B<sub>max</sub>) for [<sup>3</sup>H]U-69 was evident in nuclei that had been isolated from ES-derived cardiac myocytes compared with the corresponding fraction from undifferentiated cells (Figure 2). No significant difference in the K<sub>d</sub> values was found among nuclear fractions obtained from undifferentiated ES cells or ES-derived cardiomyocytes (Figure 2). On the whole, the B<sub>max</sub> values currently observed in nuclei isolated from either undifferentiated or cardiac lineage-committed cells were ≈3-fold lower than the values previously observed in a plasma membrane preparation isolated from the corresponding group of cells.<sup>8</sup>

By the aid of nuclear runoff experiments, we show that a direct exposure of nuclei isolated from undifferentiated ES cells to dynorphin B leads to a marked increase in the transcription rate of the 2 cardiac lineage-promoting genes, GATA-4 and Nkx-2.5 (Figures 3A and 3B). Interestingly, dynorphin B was also able to increase the transcription rate of the prodynorphin gene (Figure 4). These transcriptional responses were evident after 4 hours of incubation in the presence of 10<sup>-8</sup> mol/L dynorphin B and time-dependently



**Figure 3.** Dynorphin B (Dyn B) primes the transcription of GATA-4 and Nkx-2.5 genes in isolated ES cell nuclei. Nuclei were isolated from undifferentiated LIF-supplemented cells and exposed for the indicated times to 10 nmol/L Dyn B (left panels) or treated for 8 hours with increasing concentrations of the opioid peptide (Dyn B) or with 1 μmol/L U-50 (right panels). Isolated nuclei were also separately exposed for 8 hours to 1 μmol/L Dyn B in the presence of 1 μmol/L norBNI, 1 μmol/L Mr-1452 (Mr), 1 μmol/L naloxone (Nal), 1 μmol/L Mr-1453 (Mr\*), or 1 μmol/L (+)-naloxone (Nal\*). Panels A and B correspond to the nuclear runoff assays of GATA-4 and Nkx-2.5 genes, respectively. Autoradiographic exposure was for 2 days on

Kodak X-Omat film with an intensifying screen. Row a shows transcription of GATA-4 or Nkx-2.5 genes; row b, cyclophilin mRNA. The 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-base), Nkx-2.5 (414-base), or cyclophilin (270-base) mRNA. Because of the similar size of GATA-4-protected and cyclophilin-protected fragments, <sup>32</sup>P-labeled nuclear RNA was hybridized separately with GATA-4 and cyclophilin cRNA probes, and the corresponding hybrids were run onto different gels. Autoradiograms are representative of 6 separate experiments.



**Figure 4.** Exposure of isolated ES nuclei to Dyn B enhances prodynorphin gene transcription. Nuclei were isolated from undifferentiated LIF-supplemented cells. Experimental conditions were those described in the legend of Figure 3. Row a shows transcription of the prodynorphin gene; row b, cyclophilin mRNA. The 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 prodynorphin (424-base) or cyclophilin (270-base) mRNA. Autoradiograms are representative of 6 separate experiments.

ing that the single protected fragments migrated with a molecular size comparable to prodynorphin (424-base) or cyclophilin (270-base) mRNA. Autoradiograms are representative of 6 separate experiments.

increased up to 8 to 12 hours of exposure (Figures 3 and 4). Dynorphin B-induced gene transcription occurred in a dose-dependent fashion, being primed by a concentration of the opioid peptide as low as  $10^{-8}$  mol/L and reaching a maximum when nuclei were incubated with concentrations of dynorphin B ranging between 0.5 and 1.0  $\mu$ mol/L (Figures 3 and 4). Similar to dynorphin B, U-50,488H (U-50), a selective synthetic  $\kappa$  opioid receptor agonist,<sup>23</sup> markedly stimulated the transcription rate of GATA-4, Nkx-2.5, and prodynorphin genes in isolated nuclei (Figures 3 and 4). Incubation with Mr-1452 or nor-binaltorphimine (norBNI), 2 selective  $\kappa$  opioid receptor antagonists,<sup>24,25</sup> or with the broader opioid receptor antagonist naloxone completely suppressed the stimulatory effects elicited by dynorphin B (Figures 3 and 4) or U-50 (not shown). On the contrary, the inactive enantiomers Mr-1453 or (+)-naloxone did not affect the transcriptional responses mediated by dynorphin B (Figures 3 and 4) or U-50 (not shown).

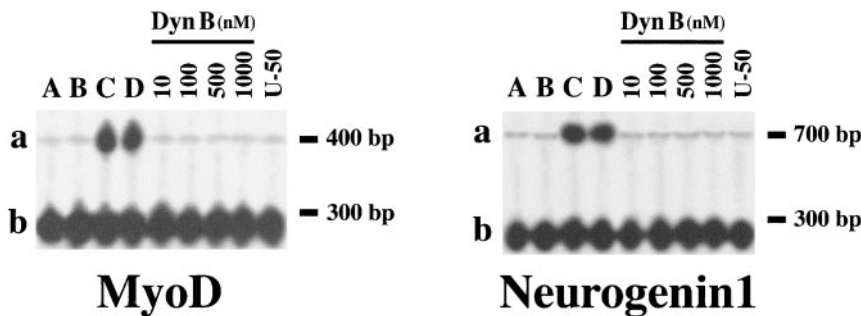
We investigated whether endorphin-induced transcription might also encompass genes that are essential for the specification of nonmyocardial lineages. As a positive control, GTR1 or the parental R1 ES cells were committed to myogenesis or neurogenesis in the presence of defined media (see online data supplement). As expected, the transcription rate of MyoD, a gene involved in skeletal myogenesis,<sup>26</sup> was enhanced in nuclei isolated from embryoid bodies (EBs) collected at day 7 after they were plated on tissue culture dishes (Figure 5). Similarly, the transcription of neurogenin1, a vertebrate neuronal determination gene,<sup>27,28</sup> was enhanced in nuclei isolated from EBs that had been subjected to neurogenic induction in the presence of retinoic acid (Figure 5). It is noteworthy that the incubation of nuclei isolated from undifferentiated GTR1 cells with doses of dynorphin B

ranging from  $10^{-8}$  mol/L to 1.0  $\mu$ mol/L failed to affect the transcription rate of both MyoD and neurogenin1 genes (Figure 5). Transcription of both genes was similarly unaffected by U-50 (Figure 5).

In a companion study,<sup>8</sup> we have provided evidence that PKC signaling is involved in the cardiogenic commitment of GTR1 ES cells and that selected PKC isozymes are constitutively expressed in the nucleus of undifferentiated GTR1 cells. In the present study, we determined whether PKC activity could be detected in the nucleus of undifferentiated ES cells and whether this enzyme activity might be enhanced after a direct exposure of isolated nuclei to dynorphin B.

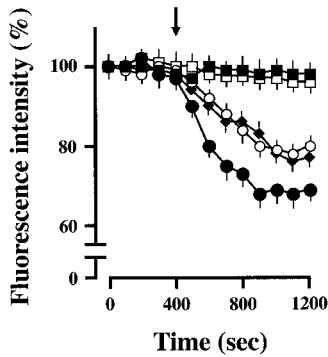
Figure 6 shows that nuclei were able to phosphorylate the high-affinity fluorescent PKC substrate acrylodan-labeled MARCKS peptide. Interestingly, exposure of isolated nuclei to 1  $\mu$ mol/L dynorphin B induced a significant time-dependent increase in nuclear PKC activity. Such a response was antagonized by treatment in the presence of Mr-1452 (Figure 6) or norBNI (not shown). Nuclear PKC activity was completely abolished when ES cell nuclei were exposed for 30 minutes to 5  $\mu$ mol/L chelerythrine (Figure 6) or 1  $\mu$ mol/L calphostin C (not shown), 2 selective PKC inhibitors,<sup>29,30</sup> before being added to the reaction mixture.

We finally examined whether the transcriptional responses triggered by dynorphin B in isolated nuclei might also be elicited in intact cells throughout their commitment to the cardiac lineage and whether, in the affirmative, the activation of an endorphin-primed program of cardiogenic gene transcription might be prevented by PKC inhibitors. We have previously shown that the levels of GATA-4, Nkx-2.5, and prodynorphin mRNA progressively increased in EBs and puromycin-selected cardiomyocytes obtained from GTR1 cells.<sup>8</sup> In the present study, an 8-hour treatment in the



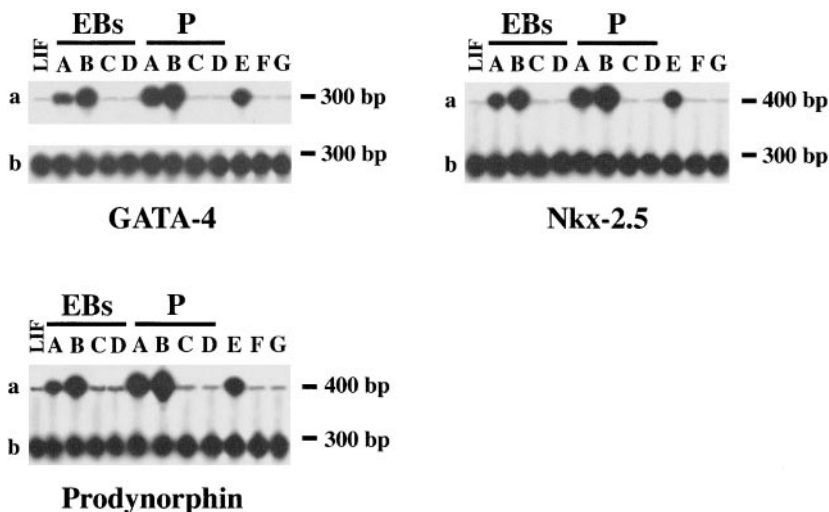
**Figure 5.** Dyn B and U-50 fail to activate the transcription of genes promoting nonmyocardial lineages. Nuclei were isolated from undifferentiated LIF-supplemented GTR1 (A) or R1 ES (B) cells. As a positive control, nuclear runoff assays were performed in nuclei isolated from GTR1 (C) or R1 (D) cells previously committed to myogenesis or neurogenesis in the presence of defined media (online data supplement). In separate experiments, nuclei isolated from undifferentiated LIF-supplemented GTR1 cells were exposed for 8 hours to the indicated concentrations of Dyn B or 1  $\mu$ mol/L U-50. Row a shows transcription of MyoD or neurogenin1 genes; row b, cyclophilin mRNA. The 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 that of MyoD (425-base), neurogenin1 (742-base), or cyclophilin (270-base) mRNA. Autoradiograms are representative of 6 separate experiments.

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**Figure 6.** PKC activity in isolated nuclei. Nuclei were isolated from undifferentiated LIF-supplemented cells. MARCKS peptide phosphorylation was started by adding 10  $\mu\text{g}$  of nuclear protein (arrow) and was followed at 37°C by monitoring time-dependent decreases in its fluorescence at 480 nm. Filled squares indicate MARCKS peptide alone; open and filled circles, nuclei exposed for 30 minutes in the absence or presence of 1  $\mu\text{mol/L}$  Dyn B, respectively; filled diamonds, nuclei treated for 30 minutes with 1  $\mu\text{mol/L}$  Dyn B in the presence of 1  $\mu\text{mol/L}$  Mr; and open squares, nuclei incubated for 30 minutes with 1  $\mu\text{mol/L}$  Dyn B in the presence of 5  $\mu\text{mol/L}$  chelerythrine. Data are expressed as mean  $\pm$  SE values ( $n=6$ ). From 600 to 1200 seconds, values indicated by open and filled circles and filled diamonds were significantly different from values indicated by open and filled squares; from 600 to 1000 seconds, values indicated by filled circles were significantly different from values indicated by open circles and filled diamonds. No significant difference was observed between values indicated by open circles and filled diamonds (1-way ANOVA, Newman-Keuls test).

presence of 1  $\mu\text{mol/L}$  dynorphin B further enhanced GATA-4 and Nkx-2.5 gene transcription, as well as the transcription rate of the prodynorphin gene, in both EBs and ES-derived cardiomyocytes (Figure 7). Figure 7 shows that both the transcriptional effects promoted by dynorphin B in intact cells and those observed after a direct application of the opioid agonist to nuclei isolated from undifferentiated cells were completely abolished by the treatment in the presence of PKC inhibitors.



**Figure 7.** Effect of PKC inhibitors on transcriptional responses elicited by Dyn B in intact cells and isolated nuclei. Lanes are as follows: LIF, nuclei were isolated from undifferentiated cells; EBs and P, nuclei were isolated from EBs collected 5 days after LIF removal or ES-derived cardiomyocytes 4 days after puromycin (P) addition, respectively. For lanes A through D, during the last 8 hours, both EBs and P-selected cells were treated in the absence (A) or presence (B) of 1  $\mu\text{mol/L}$  Dyn B or exposed to 1  $\mu\text{mol/L}$  Dyn B in the presence of 5  $\mu\text{mol/L}$  chelerythrine (C) or 1  $\mu\text{mol/L}$  calphostin C (D). Nuclei were then isolated from each group of cells and processed for the nuclear runoff analysis of the indicated transcripts. For lane E, nuclei isolated from undifferentiated cells were directly exposed for 8 hours to 1  $\mu\text{mol/L}$  Dyn B. For lanes F and G, nuclei of undifferentiated cells were incubated for 8 hours with 1  $\mu\text{mol/L}$  Dyn B in the presence of 5  $\mu\text{mol/L}$  chelerythrine (F) or 1  $\mu\text{mol/L}$  calphostin C (G). Row a shows transcription of GATA-4, Nkx-2.5, or prodynorphin genes; row b, cyclophilin mRNA. The right side of each panel reports the position of radiolabeled DNA markers, as described in the legend of Figures 3 and 4. Autoradiograms are representative of 6 separate experiments.

chelerythrine (F) or 1  $\mu\text{mol/L}$  calphostin C (G). Row a shows transcription of GATA-4, Nkx-2.5, or prodynorphin genes; row b, cyclophilin mRNA. The right side of each panel reports the position of radiolabeled DNA markers, as described in the legend of Figures 3 and 4. Autoradiograms are representative of 6 separate experiments.

In separate experiments, cell treatment with 1  $\mu\text{mol/L}$  dynorphin B after leukemia inhibitory factor (LIF) removal and throughout 4 days of puromycin selection also increased cardiomyocyte yield (the number of beating colonies reached  $220.34 \pm 18.0\%$  of the control value, estimated in cardiomyocytes selected from untreated cells; mean  $\pm$  SEM of 5 separate experiments).

### Discussion

In previous studies, we have provided evidence that prodynorphin gene expression and dynorphin B expression orchestrate cardiac differentiation in P19 embryonal carcinoma cells.<sup>7</sup> The present investigation has revealed that dynorphin B-like material is detectable in undifferentiated GTR1 ES cells and that a substantial increase in dynorphin B-related immunostaining occurs in ES-derived cardiomyocytes. The possibility that the process of cardiogenesis may require the intracellular action of dynorphin peptides is supported by the observation that  $\kappa$  opioid binding sites are expressed in a highly purified preparation of ES cell nuclei. Such a hypothesis is further inferred from the finding that a direct exposure to dynorphin B of nuclei isolated from undifferentiated cells results in a remarkable activation of the transcription rate of GATA-4, Nkx-2.5, and prodynorphin genes. Within this context, the observed increase in the  $B_{\text{max}}$  value for the selective  $\kappa$  opioid receptor ligand [<sup>3</sup>H]U-69 in nuclei obtained from ES-derived myocardial cells suggests that the availability of nuclear opioid binding sites for intracellular endorphins may be a developmentally regulated process and that nuclear opioid receptors may be part of the molecular machinery coaxing ES cells to a cardiac lineage commitment.

In our previous investigations,  $\kappa$  opioid receptors were uncovered in the nucleus of adult ventricular myocytes and were found to be overexpressed in nuclei of cardiomyocytes isolated from Syrian hamsters of the BIO 14.6 strain, an experimental model of primary hereditary hypertrophic cardiomyopathy.<sup>31</sup> However, the possibility that these nuclear

receptors may be involved in the modulation of a gene program of myocardial growth and/or differentiation remained an open issue. The present finding that dynorphin B activates the transcription of cardiogenic genes in isolated nuclei for the first time provides evidence for a functional role of nuclear opioid receptors in developmental decisions. The capability of the synthetic agonist U-50 to mimic the effects of dynorphin B and the finding that these effects are suppressed by selective opioid receptor antagonists suggest that the nuclear responses triggered by dynorphin B are specific in nature. Such a view is further supported by the observation that dynorphin B-mediated gene transcription was suppressed in a stereospecific manner by nuclear exposure to Mr-1452 or naloxone. The failure of both dynorphin B and U-50 to affect the transcription of genes promoting skeletal muscle or neuronal specification seems to exclude a generalized activation of repressed genes and suggests that coupling of nuclear opioid receptors to GATA-4 and Nkx-2.5 gene transcription may represent a mechanism pertaining to ES cell cardiogenesis. Within this context, the observation that exposure of ES cell nuclei to dynorphin B enhanced prodynorphin gene transcription indicates that dynorphin B-mediated induction of cardiogenic genes may involve a tonic feed-forward stimulation elicited at the nuclear level by the opioid peptide on its coding gene.

The experiments described in the present study indicate that nuclear PKC activation may represent a major signal transduction pathway in the cardiac differentiation of ES cells. In the present study, we show that an enzyme activity capable of phosphorylating a specific PKC substrate was present in the nucleus of undifferentiated GTR1 cells. Evidence indicating that nuclear opioid receptors and nuclear PKC may be coupled to the cardiac differentiation of ES cells results from the following findings: (1) the induction of GATA-4, Nkx-2.5, and prodynorphin gene transcription triggered by dynorphin B in isolated nuclei was abolished both by opioid receptor antagonists and by PKC inhibitors; (2) the phosphorylation rate of the MARCKS peptide was increased in the presence of nuclei that had been isolated from undifferentiated ES cells and then exposed to dynorphin B; and (3) the enzyme activity in the nucleus could be suppressed by the same specific PKC inhibitors that abolished the transcriptional responses primed by dynorphin B in isolated nuclei and that were previously found to abrogate the cardiac differentiation in ES cells.<sup>8</sup>

In a companion study,<sup>8</sup> we show that culturing ES cells with opioid receptor antagonists does not affect the overexpression of PKC- $\delta$  and PKC- $\epsilon$  observed in the nucleus of ES-derived cardiac myocytes and fails to abolish completely the capability of ES cells to develop into cardiomyocytes. These results may now be explained by the present observations, which show that dynorphin B may also act intracellularly at nuclear level. The involvement of dynorphin B and endorphin-mediated PKC activation in the cardiac differentiation of ES cells is additionally supported by the capability of the opioid peptide to further increase cardiogenic gene transcription in both EBs and ES-derived cardiomyocytes and by the observation that these responses were abolished by PKC inhibitors. Akin to a cardiogenic role of the prodynor-

phin gene and its related peptide product(s) is the increase in cardiomyocyte yield elicited by the exposure of GTR1 cells to dynorphin B after LIF removal and throughout puromycin selection.

On the whole, the present study indicates that intracrine signals for myocardial differentiation may be fashioned by a nuclear endorphinergic system in ES cells. This system encompasses dynorphin B-mediated stimulation of nuclear opioid receptors, the activation of nuclear PKC signaling, and the onset of a cardiogenic program of gene expression. Further studies are in progress to elucidate whether additional transcriptional profiling may be recruited by the activation of nuclear opioid receptors in ES cells.

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