Opioid Peptide Gene Expression in the Primary Hereditary Cardiomyopathy of the Syrian Hamster

I. REGULATION OF PRODYNORPHIN GENE EXPRESSION BY NUCLEAR PROTEIN KINASE C*

(Received for publication, July 5, 1996, and in revised form, November 18, 1996)

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Prodynorphin gene expression was investigated in adult ventricular myocytes isolated from normal (F1B) or cardiomyopathic (BIO 14.6) hamsters. Prodynorphin mRNA levels were higher in cardiomyopathic than in control myocytes and were stimulated by treatment of control cells with the protein kinase C (PKC) activator 1,2-dioctanoyl-sn-glycerol. Both chelerythrine and calphostin C, two PKC inhibitors, abolished the stimulatory effect of the diglyceride and significantly reduced prodynorphin gene expression in cardiomyopathic myocytes. Nuclear run-off experiments indicated that the prodynorphin gene was regulated at the transcriptional level and that treatment of nuclei isolated from control cells with 1,2-dioctanoyl-sn-glycerol increased prodynorphin gene transcription, whereas chelerythrine or calphostin C abolished this transcriptional effect. Direct exposure of nuclei isolated from cardiomyopathic myocytes to these inhibitors markedly down-regulated the rate of gene transcription. The expression of PKC-α, -δ, and -ε, as well as PKC activity, were increased in nuclei of cardiomyopathic myocytes compared with nuclei from control cells. The levels of both intracellular and secreted dynorphin B, a biologically active product of the gene, were higher in cardiomyopathic than in control cells and were stimulated or inhibited by cell treatment with 1,2-dioctanoyl-sn-glycerol or PKC inhibitors, respectively.

Cardiomyopathies are a major cause of mortality and morbidity, and this spectrum of disorders tops the list of diseases leading to cardiac transplantation. Hypertrophic cardiomyopathy is a disease of unknown etiology which is characterized by cardiac hypertrophy and disarray of myocardial fiber and fibrils (1, 2). Although half of the patients with hypertrophic cardiomyopathy show an apparent family history, and mutations in the β-myosin heavy chain gene have been identified in these patients (2–4), the molecular and genetic bases of the disease remain unclear. In particular, the identification of factors that may be involved in reprogramming myocardial growth and may lead to impaired contractility is in the beginning stages. It is increasingly becoming evident that the myocardial cell besides, being a target for the action of different hormones and growth factors, also acts as a source of peptides that may play a crucial role in regulating signal transduction at myocyte level. In this regard, we have provided evidence that the myocardial cell expresses the prodynorphin gene (5) and that this opioid gene is transcriptionally stimulated by protein kinase C (PKC) activation (6). Furthermore, cardiomyocytes are able to synthesize and release dynorphin B (5, 6), a biologically active end product of the prodynorphin gene that binds selectively to δ opioid receptors (7). Our previous studies have shown that the stimulation of myocardial opioid receptors affects phosphoinositide turnover (8, 9), depleting Ca2+ of the sarcoplasmic reticulum, and leading to a marked decrease in the amplitude of the cytosolic Ca2+ transient and in that of the associated contraction (9). In addition to affecting cytosolic Ca2+ homeostasis, δ opioid receptor stimulation also elicited intracellular alkalosis and changes in myocardial responsiveness to Ca2+ through a PKC-dependent activation of the Na+ /H+ antiporter (10). Interestingly, in several tissues endogenous opioids have been shown to inhibit cell proliferation and promote a mass increment by increasing the size of a fixed number of pre-existing cells (hypertrophy) (11, 12). These findings indicate that the myocardial function may be affected in an autocrine or paracrine fashion by an opioid gene and by the intracellular pathways that regulate its expression. They suggest consideration of the prodynorphin gene as a candidate gene for pathological processes involving an impairment of myocardial cell contractility, growth, and differentiation.

In the present study, we used BIO 14.6 cardiomyopathic Syrian hamsters as an experimental model of hypertrophic cardiomyopathy and investigated the expression of the prodynorphin gene in cardiac myocytes that have been isolated at various stages during the progression of the cardiomyopathy. The finding that PKC is involved in different models of cardiac hypertrophy as well as in prodynorphin gene transcription led us to evaluate whether PKC activation may contribute to regulate the expression of this opioid gene throughout the cardiomyopathic process.

MATERIALS AND METHODS

Control (F1B) and cardiomyopathic (BIO 14.6) male Syrian hamsters were purchased from Bio Breeders (Fitchburg, MA). BannHI, EcoRI, NcoI, ATP, CTP, GTP, UTP, collagenase B, and the acrylic-labeled myristoylated alanine-rich protein kinase C substrate (MARCKS) peptide were from Boehringer Mannheim. RNAMatrix™ was from BIO 101, Inc. (Vista, CA). [32P]CTP, [α-32P]UTP, and the 32P-labeled donkey

1 The abbreviations used are: PKC, protein kinase C; MARCKS, myristoylated alanine-rich protein kinase C substrate; ir, immunoreactive; dyn B, dynorphin B; BSA, bovine serum albumin; TBS, Tris-buffered saline.
anti-rabbit IgG antibody were from Amersham International. Antiserum to PKC-α, PKC-δ, PKC-ε, and PKC-ζ were from Calbiochem. Cholerythrine, calphostin C, H7, and staurosporine were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Pronase E, 1,2-di-iodotyrosyl-3-syn-glycerol, and all the other chemicals were from Sigma.

Isolation of Myocardial Nuclear Material—Briefly, each heart was retrogradely perfused at 37°C through the aorta with 25 ml of a nominally Ca2+-free bicarbonate buffer (perfusion buffer) of the following composition (mM): 116.4 NaCl, 5.4 KCl, 1.6 MgCl2, 6.2 NaHCO3, 1.0 NaH2PO4, 5.6 D-glucose, and 0.3 M sucrose, 5 mM MgCl2, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 μM β-mercaptoethanol. The myocardial nuclei were isolated from the perfusion buffer containing 25 μM CaCl2 and 4% bovine serum albumin (BSA). The nuclei were then allowed to sediment under gravity. The resulting nuclear pellet was resuspended in 20 ml of perfusion buffer containing 20 μM CaCl2, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 μM β-mercaptoethanol. The purity of the myocardial nuclei was assessed by estimating in the nuclear fraction the activity of marker enzymes of other selected cellular myocyte fractions. In particular, the activity of 5'-nucleotidase and that of the ouabain-sensitive Na⁺-K⁺ ATPase, two marker enzymes of the sarcoplasmic membranes, were measured by using the method described by Edwards and Maguire (23) and the procedure reported by Lamers and Stinis (24), respectively. Ouabain-sensitive Na⁺-K⁺ ATPase activity was taken as the activity inhibitable by 1 mM ouabain.

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 dyn B and capable of recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dyn B in their sequence (20, 21). Acetic acid extracts from control or cardiomyopathic cardiomyocytes or pooled samples from the incubation medium were processed by reverse-phase high performance liquid chromatography. The collected fractions were radioimmunoadsorbed, and the immunoreactivity was attributed to authentic dyn B by comparison with the elution position of a synthetic standard, according to a previously described procedure (5, 22).

Isolation of Nuclear and Cytosolic Fractions—Nuclear samples, to- tal myocardial nuclear material, and the cytosolic fraction were isolated from cardiac myocytes or cardiomyocytes by using a previ- ously described method (6). Briefly, at the end of the isolation procedure the myocardial cells, resuspended in perfusion buffer containing 1.0 mM CaCl2, were allowed to sediment under gravity. The resulting pellet was resuspended in a hypo-osmotic buffer (Buffer 1) of the follow- ing composition: 10 mM Tris/HCl, pH 7.4, 1 mM MgCl2, 10 mM NaCl, 5 mM CaCl2, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluo- ride, 1 μM leupeptin, and 10 mM β-mercaptoethanol. The resuspended cells were incubated for 30 min at 4°C and then sedimented at 1000 × g for 10 min at 4°C. The pellet was resuspended in 20 ml of Buffer I and sonicated at setting 2 in a Branson sonifier W-350. The sonicated preparation was added with Triton X-100 at a final concentration of 0.3% and that of the ouabain-sensitive Na⁺-K⁺ ATPase activity was measured in the cytosolic fraction.

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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 (ImageQuant 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 cardiomyopathic cells relative to the intensity in the corresponding sample obtained from control cells (considered as 100%).

Measurement of Nuclear PKC Activity—PKC activity from isolated myocardial nuclei was measured according to a previously described procedure (6), which utilized a continuous fluorescence assay in the presence of the acrylodan-labeled MARCKS peptide, a high affinity fluorescent substrate in vitro for PKC (31–34). This substrate consists of a conserved sequence of 24 amino acids from the MARCKS protein, which includes four sites for PKC phosphorylation (35). In the presence of PKC activators, maximum fluorescence is measured at 480 nm with excitation at 370 nm. In the course of phosphorylation by PKC, the intensity of the fluorescence decreases about 20% (35). 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 mM Tris/HCl, pH 7.0, 90 mM KCl, 3 mM MgCl₂, 0.3 mM CaCl₂, 0.1 mM EGTA, 100 μM ATP, 10% ethylene glycol, 0.5 μg of phosphatidylserine, 0.1 μg of 1,2-diacetanoyl-sn-glycero, and 75 nM acrylodan-labeled MARCKS peptide. The phosphorylation of the acrylodan-labeled peptide was started by adding 10 μg of nuclear protein.

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.

RESULTS

This investigation began as an attempt to determine whether prodynorphin mRNA could be detected in hamster ventricular myocytes and whether, in the affirmative, its levels may be altered under pathological heart conditions. Fig. 1 shows that the levels of prodynorphin mRNA were markedly increased in myocytes isolated from the heart of 60-day-old cardiomyopathic hamsters compared with cells obtained from age-matched control animals. No further increase in prodynorphin mRNA expression was observed in cardiomyopathic myocytes from 120- or 180-day-old BIO 14.6 hamsters (Fig. 1). We have previously shown that phorbol ester-mediated activation of PKC enhances prodynorphin gene expression in rat myocardial cells (6). In the present study, we investigated whether PKC may be part of the signal transduction pathway involved in the stimulation of prodynorphin mRNA expression observed in cardiomyopathic myocytes. To assess the capability of hamster ventricular myocytes to increase prodynorphin mRNA expression in response to an intervention that may lead to PKC activation, cardiac myocytes from control hamsters were incubated for 4 h in the presence of 1,2-dioctanoyl-sn-glycerol, a cell permeant diglyceride that acts as a potent PKC activator (37). This treatment induced a 5-fold increase in prodynorphin mRNA levels compared with untreated control myocytes (Fig. 2). Both chelerythrine or calphostin C, two novel and highly selective PKC inhibitors (38–41), counteracted this stimulatory effect in a dose-dependent manner and completely abolished the diglyceride-induced increase in prodynorphin mRNA expression at a concentration of 5 or 1 μM, respectively (Fig. 2). The effect induced by 1,2-dioctanoyl-sn-glycerol was also completely abolished by cell treatment with H7 (25 μM) or staurosporine (2 nM), two putative PKC inhibitors which have been reported to affect the enzyme activity with different degrees of potency and selectivity (42, 43) (Fig. 2). The incubation of cardiomyopathic myocytes in the presence of 5 μM chelerythrine or 1 μM calphostin C resulted in a marked decline in prodynorphin mRNA expression, although the mRNA level in cardiomyopathic myocytes remained significantly higher than in control cells (Fig. 3). Similar results were observed when cardiomyopathic myocytes were treated with 25 μM H7 or 2 nM staurosporine (not shown). The incubation of cardiomyopathic myocytes in the presence of 1,2-dioctanoyl-sn-glycero produced a significant increase in prodynorphin mRNA expression compared with untreated cardiomyopathic cells (Fig. 3). Under these experimental conditions, the level of prodynorphin mRNA in cardiomyopathic myocytes was significantly higher than that in diglyceride-treated control cells. When cardiomyopathic myocytes were exposed to 1,2-dioctanoyl-sn-glycerol, either in the presence of chelerythrine or in the presence of calphostin C, prodynorphin mRNA levels did not differ significantly from those observed in cardiomyopathic cells that had been incubated with each PKC inhibitor in the absence of the diglyceride (Fig. 3).

To investigate whether the observed changes in prodynorphin mRNA expression may have been induced at the transcriptional level, we assessed the rate of transcription of the prodynorphin gene by using an in vitro run-off transcription assay. Table I shows the activity of marker enzymes of selected subcellular fractions in nuclei prepared from normal hamster myocytes. It is evident that the activity of the ouabain-sensitive Na⁺/K⁺-ATPase and that of 5’-nucleotidase were both undetectable in the nuclear fraction, excluding a contamination by sarcosomal 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 undetect-
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Myocytes were isolated from 60-day-old control hamsters. Representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA are shown in the upper panel. Autoradiographic exposure was carried out as described in Fig. 1. A, untreated myocytes; B, 0.2 µg/ml 1,2-dioctanoyl-sn-glycerol for 4 h; C, D, E, and F, 4 h of exposure to 0.2 µg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 0.5, 1.0, 2.5, and 5 µM chelerythrine, respectively; G, H, I, and L, 4 h of exposure to 0.2 µg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 0.05, 0.1, 0.5, and 1.0 µM calphostin C, respectively; M, 4 h of exposure to 0.2 µg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 25 µM H7; N, 4 h of exposure to 0.2 µg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 2 mM staurosporine. Averaged values of prodynorphin mRNA levels are reported in the lower panel. The data are expressed as mean values ± S.E. (n = 6). *, significantly different from the control value; significant differences were observed throughout groups C–E or groups G–I, but not between groups E and F, groups I and L, or groups M and N (one-way analysis of variance, Newman Keul’s test).

TABLE I
Specific enzymatic activities in the homogenate and nuclear fraction of isolated cardiac myocytes

| Enzyme activities                          | Homogenate | Nuclear fraction |
|--------------------------------------------|------------|-----------------|
| Na’-K’ ATPase (ouabain-sensitive)          | 2.040 ± 0.060 | ND*             |
| 5’-Nucleotidase                            | 0.680 ± 0.080 | ND*             |
| NADH cytochrome c reductase (rotenone-insensitive) | 0.360 ± 0.110 | ND*             |
| Succinate dehydrogenase                    | 6.000 ± 0.300 | ND*             |
| NADPH cytochrome c reductase (rotenone-insensitive) | 0.250 ± 0.012 | 0.010 ± 0.007  |
| K’-EDTA ATPase                             | 4.800 ± 0.100 | ND*             |

* ND, nondetectable.

creased in nuclei isolated from cardiomyopathic myocytes compared with nuclei obtained from control cells. A decrease in the transcription rate of the prodynorphin gene was observed in nuclei isolated from cardiomyopathic myocytes that had been exposed both to chelerythrine or to calphostin C, although in these nuclei the rate of gene transcription was still higher than that observed in nuclei of the control cells (Fig. 4). A similar
the expression of both PKC-nuclear fraction of control cells (Fig. 5). On the contrary, PKC-
against the anti-PKC-
pathic cells (Figs. 5 and 6). Only a slight immunoreactivity was similar to that observed in total extracts from cardiomyo-
expression of PKC-(Fig. 5). PKC-
were not detected (not shown). The
immunoreactivity as confirmed in peptide antigen competition experiments (results not shown). The
PKC inhibitors were added to the reaction mixture (Fig. 7). Similar results were obtained when each PKC inhibitor was added to nuclei isolated from untreated control cells (not shown).

We next investigated whether the increase in the expression of PKC-α, -δ, and -ε observed in the nucleus of cardiomyopathic
the presence of nuclei isolated from cardiomyopathic myocytes than in the presence of nuclei obtained from control cells. No significant change in acrylodan-peptide fluorescence was observed in the presence of nuclei that had been isolated from cardiomyopathic cells and then pretreated with 5 μM chelerythrine. In both groups of cells (Figs. 5 and 6). Western blot analysis also indicated that the immunoreactivity against anti-PKC-δ and anti PKC-ε-specific antibodies was mainly detected in the nuclear fraction (Fig. 5) and was higher in nuclei isolated from cardiomyopathic
PKC activity. Fig. 7 shows that the phosphorylation rate of the acrylodan-labeled MARCKS peptide was significantly higher in
the presence of nuclei isolated from cardiomyopathic myocytes as well as in their incubation media (Fig. 8). In both
groups of myocytes the amount of secreted ir-dyn B was signif-

PKC αPKC δPKC εPKC ζ

1 2 3 4 5 6

PKC αPKC δPKC εPKC ζ

T C N T C N T C N T C N

Relative immunoreactivity (%)
significant higher than that observed at cellular level. A significant increase in the level of both intracellular and secreted ir-dyn B was observed in cardiomyopathic myocytes compared with control cells (Fig. 8). Fig. 8 shows that 1,2-dioctanoyl-sn-glycerol increased dyn B expression in both groups of myocytes as compared with untreated cells. Moreover, in the presence of the diglyceride, the amount of both intracellular and secreted dyn B was higher in cardiomyopathic than in control cells (Fig. 8). Both chelerythrine and calphostin C completely abolished the effect induced by 1,2-dioctanoyl-sn-glycerol in control cells and significantly inhibited both basal and diglyceride-stimulated dyn B expression in cardiomyopathic myocytes (Fig. 8).

**DISCUSSION**

Our data show that the expression of the prodynorphin gene was markedly increased in myocytes isolated from cardiomyopathic hearts compared with cells obtained from normal hearts. A number of experimental results in the present study suggest that PKC may be involved, at least in part, in mediating the observed increase in prodynorphin mRNA expression. First, prodynorphin mRNA levels could be increased by exposing both control and cardiomyopathic myocytes to a PKC activator. Second, calphostin C, respectively; H, cardiomyopathic myocytes exposed to 0.2 μg/ml 1,2-dioctanoyl-sn-glycerol for 4 h; I and L, cardiomyopathic cells treated for 4 h with 0.2 μg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively. Each single value in the medium was calculated in a final volume of 15 ml, corresponding to the volume of pooled samples of the incubation medium from 10⁶ cells. Each experiment was performed in the presence of a peptidase inhibitor mixture containing 20 μM bestatin, 1 mM leucyl-L-leucine, 3 μM poly-L-lysine, 0.3 μM thiorphan, 30 μM 1–10-phenanthroline, 6 μM 1,4-dithiothreitol. The data were expressed as mean values ± S.E. (n = 6). $\$, the value of the white bar is significantly different from that of the hatched bar. $^\ast$, significantly different from the control value; $^\ast\ast$, significant difference between two groups (one-way analysis of variance, Newman Keul’s test).

**FIG. 7.** Nuclear PKC activity in isolated cardiac myocytes from normal and cardiomyopathic hamsters. Myocytes were isolated from 60-day-old control or BIO 14.6 hamsters. Nuclear PKC activity was measured as described under “Materials and Methods.” The phosphorylation of the acrylodan-labeled MARCKS peptide was started by the addition of 10 μM of nuclear protein at the time indicated by the arrow. As the acrylodan-peptide becomes phosphorylated, it undergoes a time-dependent decrease in its fluorescence at 480 nm. Shown is the time course of the acrylodan-peptide fluorescence observed following the addition of nuclei isolated from normal (●) or cardiomyopathic ( ○) myocytes or in the presence of nuclei isolated from cardiomyopathic myocytes and subsequently pretreated for 30 min with 5 μM chelerythrine (△) or 1 μM calphostin C (○), before being added to the reaction mixture. The time course of the fluorescence of the acrylodan-peptide alone (■) is also reported. The data are expressed as mean values ± S.E. (n = 6). From 600 to 1200 s, ● or ○ were significantly different from ■, △, or ○; from 600 to 900 s, ● was significantly different from ○; no significant difference was observed between △ or ○ and ■ (one-way analysis of variance, Newman Keul’s test).

**FIG. 8.** Analysis of ir-dyn B expression in myocellular cells and in their incubation media. Cardiac myocytes were isolated from 60-day-old control or cardiomyopathic hamsters. Hatched bars, ir-dyn B in cells; white bars, ir-dyn B in the medium. A, untreated control myocytes; B, control myocytes exposed to 0.2 μg/ml 1,2-dioctanoyl-sn-glycerol for 4 h; C and D, control myocytes treated for 4 h with 0.2 μg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively; E, untreated cardiomyopathic cells; F and G, cardiomyopathic myocytes treated for 4 h with 0.2 μg/ml 1,2-dioctanoyl-sn-glycerol for 4 h; I and L, cardiomyopathic cells treated for 4 h with 0.2 μg/ml 1,2-dioctanoyl-sn-glycerol in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively. Each single value in the medium was calculated in a final volume of 15 ml, corresponding to the volume of pooled samples of the incubation medium from 10⁶ cells. Each experiment was performed in the presence of a peptidase inhibitor mixture containing 20 μM bestatin, 1 mM leucyl-L-leucine, 3 μM poly-L-lysine, 0.3 μM thiorphan, 30 μM 1–10-phenanthroline, 6 μM 1,4-dithiothreitol. The data were expressed as mean values ± S.E. (n = 6). $\$, the value of the white bar is significantly different from that of the hatched bar. $^\ast$, significantly different from the control value; $^\ast\ast$, significant difference between two groups (one-way analysis of variance, Newman Keul’s test).
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cardiovascular disease. Third, the treatment of isolated control nuclei either with chelerythrine or with calphostin C completely abolished the diglyceride-induced increase in opioid gene transcription. The fourth observation is that the exposure of nuclei isolated from cardiomyopathic myocytes to specific PKC inhibitors markedly down-regulated prodynorphin gene transcription. The fifth experimental observation is that PKC-α, -δ, and -ε were detectable in nuclei that had been isolated from both untreated control myocytes and cardiomyopathic cells and that the expression of these PKC isoforms was increased in nuclei from cardiomyopathic myocytes compared with nuclei obtained from control cells. The high degree of purity of the nuclear preparation used in the present study seems to exclude that PKC might have been associated with isolated nuclei merely as a result of cross-contamination by non-nuclear subcellular fractions. Here we report that, both in control myocytes and in cardiomyopathic cells, PKC-δ and PKC-ε appeared to be mainly expressed at the nuclear level. These results are in agreement with our previous immunoblot analysis of PKC isotype expression in adult rat ventricular myocytes, showing that both PKC-δ and PKC-ε were almost entirely expressed at the nuclear level (6). The results presented here are also in agreement with other studies that used immunofluorescent and confocal microscopy techniques to determine the subcellular localization of different PKC isoforms in intact cardiac myocytes, demonstrating that PKC-δ and -ε immunostaining patterns were mainly detectable in the nucleus of unstimulated cells (44). Similar to the present study, these studies failed to detect PKC-δ and PKC-ε in the cytosol (44). On the other hand, we cannot exclude that due to the extremely low expression of PKC isoforms in myocardial cells (45, 46) a cytosolic expression of both PKC-δ and PKC-ε might occur at a level below the sensitivity of the methods used in the present and in other studies. The molecular mechanism(s) underlying the increase in PKC-α, PKC-δ, and PKC-ε observed in the nucleus of cardiomyopathic cells remain to be elucidated. However, PKC-α was only slightly expressed in the nucleus of control myocytes, and its increase in the nuclear fraction of cardiomyopathic cells appeared to depend on a translocation of the isozyme from the cytosolic compartment. On the contrary, the increase in the expression of both PKC-δ and PKC-ε in the nucleus of cardiomyopathic cells seemed to occur independently of enzyme translocation and appeared to reflect the increase in the expression of these isozymes observed in total extracts from cardiomyopathic cells compared with control myocytes. Therefore, we cannot exclude that such an increase may result from changes in isozyme turnover and/or mRNA expression occurring during the cardiomyopathic process.

Further evidence correlating nuclear PKC to the regulation of prodynorphin gene transcription in the experimental model of cardiac hypertrophy currently investigated is provided by the observation that: (i) an enzyme activity capable of phosphorylating a specific PKC substrate is present in isolated control nuclei, (ii) the phosphorylation of this substrate occurred at a higher rate in the presence of nuclei isolated from cardiomyopathic cells than in the presence of control nuclei, and (iii) the enzyme activity in the nucleus could be suppressed by the same specific PKC inhibitors that abolished the transcriptional effect elicited by a PKC activator in isolated control nuclei and down-regulated prodynorphin gene transcription in nuclei obtained from cardiomyopathic cells.

The analysis of dyn B expression in normal and cardiomyopathic myocytes provides a variety of information on the possible sequelae of events resulting from the increase in prodynorphin gene expression observed in the B10 14.6 cardiomyopathic hamster heart. The current experimental data show that consistent amounts of ir-dyn B could be detected in hamster ventricular myocytes and in their incubation media and that the levels of both intracellular and secreted dyn B were significantly increased in cardiomyopathic myocytes compared with normal cells. In addition, we found that, in both control and cardiomyopathic myocytes, dyn B expression closely paralleled the changes in prodynorphin mRNA expression and gene transcription elicited in each cell type by interventions aiming at activating or inhibiting PKC. These observations indicate that in the cardiomyopathic cell, PKC-mediated events leading to the induction of the prodynorphin gene were associated with an increase in mRNA translation into a biologically active end product of the gene. Particularly interesting is the finding that, under all the experimental conditions tested in this study, the levels of ir-dyn B were significantly higher in the incubation medium than in the cardiac cell. This observation suggests that, in the ventricular myocardial cell, which lacks secretory granules (47), the prodynorphin-derived peptides may be constitutively released shortly after synthesis. In this regard, dyn B is known to bind selectively the κ opioid receptor (7), and cardiac myocytes have been shown to express this opioid receptor subtype (48). Therefore, the present findings may indicate that the increase in the synthesis and release of ir-dyn B observed in cardiomyopathic cells may be part of an autocrine circuit including the increase in the expression of an opioid gene and the interaction of the released peptide with an opioid receptor at the cell surface.

Despite the experimental evidence indicating that nuclear PKC may play a crucial role in the induction of the prodynorphin gene in cardiomyopathic myocytes, the current experimental data also show that both chelerythrine and calphostin C failed to completely abolish the increase in prodynorphin mRNA and dyn B expression in cardiomyopathic myocytes, while being effective in suppressing the diglyceride-induced increase in prodynorphin gene expression observed in control cells. Moreover, the same PKC inhibitors failed to completely abolish the increase in prodynorphin gene transcription when applied to nuclei isolated from cardiomyopathic cells, while being effective in suppressing both nuclear PKC activity and the increase in gene transcription elicited by a PKC activator in isolated control nuclei. These observations indicate that PKC activation may not represent the only signaling mechanism involved in the stimulation of prodynorphin gene expression in cardiomyopathic cells. It has been shown that intracellular Ca²⁺ overload and abnormalities in intracellular Ca²⁺ handling represent a prominent feature in the B10 14.6 strain of cardiomyopathic hamsters (49–53). In previous studies we have shown that in adult rat cardiac myocytes the expression of the prodynorphin gene can be stimulated by an increase in intracellular Ca²⁺ loading (5). The comparative analysis of intracellular Ca²⁺ homeostasis and prodynorphin gene expression both in normal and in cardiomyopathic hamster myocytes is the main subject in the following study.

The possible implications of the results of the present report remain to be elucidated. Nevertheless, a number of experimental data seem to indicate that the induction of the prodynorphin gene and the increase in dyn B expression may be involved in the cardiomyopathic process. Our first note in this study is that prodynorphin mRNA and ir-dyn B levels were already increased in myocytes isolated from cardiomyopathic animals of 60 days, an age which corresponds to an early phase in the cardiomyopathy. At this time, the heart of cardiomyopathic animals is still at a stage of multifocal necrosis (1), which precedes a period of compensatory hypertrophy, then ends in the stage of heart failure. Second, in adult rat ventricular myocytes, the stimulation of κ opioid receptors has been shown...
to produce a marked decrease in the amplitude of the cytosolic Ca\(^{2+}\) transient and in that of the associated contraction, along with a prolongation in the time course of either signal (9). Third, a significant reduction in both cytosolic Ca\(^{2+}\) transient and twitch amplitudes and an increase in the duration of cell contraction have been observed in ventricular myocytes isolated from 60- and 120-day-old BIO 14.6 cardiomyopathic Syrian hamsters compared with normal cells obtained from F1B controls (54). The fourth major observation is that the exposure of normal hamster myocytes to \(\kappa\) opioid receptor agonists reduced the amplitude of the cytosolic Ca\(^{2+}\) transient and that of the cell twitch to values approaching those observed in BIO 14.6 cardiomyopathic cells (54). Moreover, in this study the amplitude of either signal was significantly less in cardiomyopathic myocytes than in untreated cardiomyopathic cells (54). On the other hand, an alternative interpretation of the present results might suggest a link between the increase in prodynorphin gene expression observed here in cardiomyopathic myocytes and the onset of the contractile dysfunction associated with the cardiomyopathy. On the other hand, a helpful phenomenon counteracting the detrimental effects of calcium loading in the cardiac myocytes of BIO 14.6 hamsters.

**Acknowledgment**—We thank Giuseppe Delogu for technical assistance.

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