THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 10, Issue of March 7, pp. 6685–6692, 1997
© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

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)

Carlo Ventura‡§¶, Gianfranco Pintus‡§, Maria Giovanna Fiori‡, Federico Bennardini‖, Giangavino Pinna‡, and Leonardo Gaspa§

From the ‡Institute of Biological Chemistry "A. Bonsignore," School of Medicine, University of Sassari, Viale San Pietro 43/B, 07100 Sassari, Italy, the §National Laboratory of the National Institute of Biostructures and Biosystems, Osilo, Italy, and the ‖Institute of Biological Chemistry, School of Pharmacy, University of Sassari, Via Muroni 23/A, 07100 Sassari, Italy

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 disarrays 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)[1] activation (6). Furthermore, cardiac myocytes are able to synthesize and release dynorphin B (5, 6), a biologically active end product of the prodynorphin gene that binds selectively to κ opioid receptors (7). Our previous studies have shown that the stimulation of myocardial opioid receptors affects phosphoinositide turnover (8, 9), depleting Ca^{2+} of the sarcoplasmic reticulum, and leading to a marked decrease in the amplitude of the cytosolic Ca^{2+} transient and in that of the associated contraction (9). In addition to affecting cytosolic Ca^{2+} homeostasis, κ opioid receptor stimulation also elicited intracellular alkalosis and changes in myofilament responsiveness to Ca^{2+} 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). *Bam*HI, *Eco*RI, *Nco*I, ATP, CTP, GTP, UTP, collagenase B, and the acrylodan-labeled myristoylated alanine-rich protein kinase C substrate (MARCKS) peptide were from Boehringher Mannheim. RNAMATRIX™ was from BIO 101, Inc. (Vista, CA). [^{32}P]CTP, [α-^{32}P]UTP, and the ^{125}I-labeled donkey

* This work was supported by Telethon-Italy Grant 593. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 39-79-228278 or 39-79-228279; Fax: 39-79-228120.

[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.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

anti-rabbit IgG antibody were from Amersham International. Antisera to PKC-α, PKC-δ, PKC-ε, and PKC-ζ were from Calbiochem. Chelerythrine, calphostin C, H7, and staurosporine were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Pronase E, 1,2-dioctanoyl-*sn*-glycerol, and all the other chemicals were from Sigma.

Cell Isolation—Ventricular cardiac myocytes were isolated from control (F1B) or cardiomyopathic (BIO 14.6) male Syrian hamsters of 60, 120, or 180 days according to a technique previously described (13). Briefly, each heart was retrogradely perfused at 37 °C through the aorta with 25 ml of a nominally Ca^{2+}-free bicarbonate buffer (perfusion buffer) of the following composition (mM): 116.4 NaCl, 5.4 KCl, 1.6 $MgSO_4$, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, 5.6 D-glucose; this medium was continuously gassed with 95% O_2, 5% CO_2 (pH 7.36 ± 0.05). The perfusate was then switched to a similar solution containing 0.5 mg/ml collagenase B (0.7 unit/mg), 0.05 mg/ml Pronase E, and 25 μM $CaCl_2$. After approximately 20 min, the heart tissue became soft and was then minced and mechanically dispersed by gentle pipetteting. The resulting myocyte suspension was filtered through nylon gauze, and the cells were allowed to settle under gravity for 6–10 min. The supernatant was removed by aspiration, and the pellet of cells was resuspended in 20 ml of perfusion buffer containing 250 μM $CaCl_2$ and 4% bovine serum albumin (BSA). The myocytes were again allowed to sediment under gravity and the pellet was resuspended in 20 ml of perfusion buffer containing 4% BSA and 0.5 mM $CaCl_2$. Sedimented myocytes were finally resuspended in perfusion buffer containing 1.0 mM $CaCl_2$.

Under these experimental conditions, 80–90% of the cardiac myocytes resuspended in 1.0 mM $CaCl_2$ had a rod-shaped appearance, clear striations, sharp edges, and no evidence of granulations or blebs. Additionally, density sedimentation of newly isolated myocytes in the presence of BSA, prior to final resuspension, has been shown to represent one of the methods of choice to control the presence of non-myocytes in preparations of adult cardiomyocytes (14–16). Due to the filtering effect of repeated passages through 4% BSA, smaller cells or small cell pieces were almost completely excluded from the final preparation of myocytes used in the present study, and contamination by non-myocytes was negligible (less than 2%). Only freshly isolated myocytes were used in each experiment.

RNA Extraction and Determination of Prodynorphin mRNA—RNA was extracted from cardiac myocytes by using the method described by Chomczynsky and Sacchi (17), and the levels of prodynorphin mRNA were assessed by the aid of a sensitive solution hybridization RNase protection assay as described in detail elsewhere (6, 18, 19). Briefly, a 400-base pair *Hin*dIII-*Bam*HI fragment of the main exon of rat genomic prodynorphin clone was inserted into pGEM3. Transcription of the plasmid linearized with *Bam*HI generated a sense strand of prodynorphin mRNA used to construct a standard curve of prodynorphin mRNA, while transcription of the plasmid linearized with *Eco*RI in the presence of [^{32}P]CTP (800 Ci/mmol) gave an antisense strand used to hybridize cellular prodynorphin mRNA. Unlabeled antisense prodynorphin mRNA was also synthesized from the plasmid and was used in nuclear run-off experiments to hybridize ^{32}P-labeled RNA synthesized by isolated myocardial nuclei (see below in this section). The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide nondenaturing gel. Autoradiographic exposure was performed for 48 h. The individual bands were counted for radioactivity by liquid scintillation spectrometry, and counts/min were translated to picogram values on a correlated standard curve. Data were expressed as picograms 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 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 radioimmunoassayed, 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—Nuclei were isolated from myocytes of control or cardiomyopathic hamsters by using a previously described method (6). Briefly, at the end of the isolation procedure the myocardial cells, resuspended in perfusion buffer containing 1.0 mM $CaCl_2$, were allowed to sediment under gravity. The resulting pellet was resuspended in a hypo-osmotic buffer (Buffer I) of the following composition: 10 mM Tris/HCl, pH 7.4, 1 mM $MgCl_2$, 10 mM NaCl, 5 mM $CaCl_2$, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 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 0.1% and then centrifuged at 1000 × *g* for 10 min at 4 °C. The pellet was resuspended in a buffer (Buffer II) containing 10 mM Tris/HCl, pH 8.0, 0.3 M sucrose, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 mM β-mercaptoethanol. The resuspended material was layered over an equal volume of Buffer II containing 0.6 M sucrose and centrifuged at 1500 × *g* for 10 min at 4 °C. The resulting nuclear pellet was resuspended in a buffer (Buffer III) containing 50 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 mM β-mercaptoethanol.

The purity of the myocardial nuclei was assessed by estimating in the nuclear fraction the activity of marker enzymes of other selected subcellular myocyte fractions. In particular, the activity of 5′-nucleotidase and that of the ouabain-sensitive Na^+-K^+ ATPase, two marker enzymes of the sarcolemmal membranes, were assessed 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. The extent of ATP hydrolysis was followed by measuring the release of inorganic phosphate, according to the method of LeBel *et al.* (25). Succinate dehydrogenase activity and rotenone-insensitive NADH cytochrome *c* reductase activity were assessed as marker enzymes of inner and outer mitochondrial membranes, respectively, and were measured according to the procedures described by King (26) and by Sottocasa *et al.* (27). The activity of rotenone-insensitive NADPH cytochrome *c* reductase and that of K^+-EDTA ATPase were measured as marker enzymes of the sarcoplasmic reticular membranes and of the myofibrillar fraction, respectively, according to the methods described by Sottocasa *et al.* (27) and by Scholte (28).

In order to prepare the cytosolic fraction, cardiac myocytes that had been isolated from both control or cardiomyopathic hamsters were first homogenated with a Dounce homogenizer (three strokes of an A pestle) in a medium containing: 50 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 1000 × *g* at 4 °C for 15 min. The supernatant was further centrifuged at 100,000 × *g* at 4 °C for 60 min. The resulting supernatant was designated the cytosolic fraction.

Nuclear Run-off Transcription Assay—Nuclear run-off experiments were performed as described in detail elsewhere (6, 29). Briefly, nuclei resuspended in 90 μl of Buffer III were added with 100 μl of 2 × reaction buffer (10 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, 1 mM each of ATP, GTP, and CTP), and 5 μl of [α-^{32}P]UTP (3000 Ci/mmol), followed by incubation at room temperature for 15 min. DNA was digested by incubating the transcription mixture for 5 min at room temperature in the presence of 1 μl of 20,000 units/ml RNase-free DNase. Nuclear RNA was isolated by using guanidine thiocyanate and acid phenol extraction (17), followed by purification on RNAMATRIX™. Equal counts of ^{32}P-labeled RNA (about 5 × 10^6 cpm) were then subjected to a solution hybridization RNase protection assay and were hybridized for 12 h at 55 °C in the presence of unlabeled antisense prodynorphin mRNA. Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K, as described by Krause *et al.* (19). The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide nondenaturing gel. Autoradiographic exposure was for 48 h. ^{32}P-Labeled nuclear RNA was also hybridized with unlabeled antisense cyclophilin mRNA synthesized from a *Nco*I-linearized pBS vector containing a 270-base pair fragment of plB15, a cDNA clone encoding for rat cyclophilin (30). Cyclophilin mRNA was utilized as a constant mRNA for control.

Immunoblotting Analysis of Protein Kinase C—Nuclear samples, total cell lysates, or cytosolic fractions from control or cardiomyopathic myocytes were electrophoresed on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose using a semidry transfer cell (BIO 101, Inc.). After transfer, the blot was saturated for 1 h at room temperature with 3% BSA in Tris-buffered saline containing Tween (TBS-T) (50 mM Tris/HCl, pH 7.4, 150 mM 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-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 ^{125}I-labeled donkey anti-rabbit IgG antibodies (10^6 cpm/ml)

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

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 $MgCl_2$, 0.3 mM $CaCl_2$, 0.1 mM EGTA, 100 μM ATP, 10% ethylene glycol, 0.5 μg of phosphatidylserine, 0.1 μg of 1,2-dioctanoyl-*sn*-glycerol, and 75 nM acrylodan-labeled MARCKS peptide. The phosphorylation of the acrylodan-labeled peptide was started by adding 10 μg of nuclear protein.

Proteins—Protein concentration was determined by the method of Lowry *et al.* (36), using BSA 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.

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

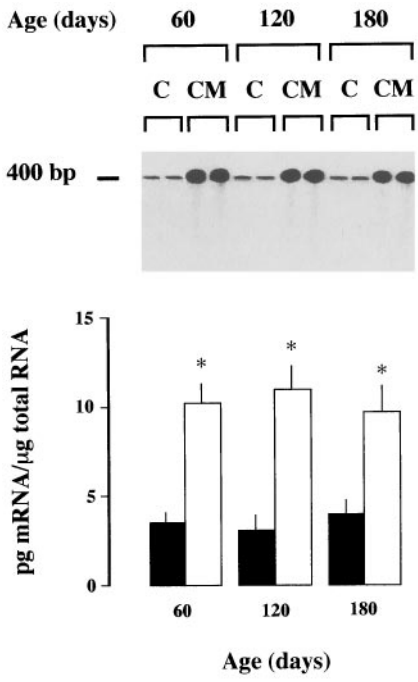

FIG. 1. **Expression of prodynorphin mRNA in isolated cardiac myocytes from normal and cardiomyopathic hamsters.** The myocardial cells were isolated from F1B (control, *C*) or from BIO 14.6 (cardiomyopathic, *CM*) hamsters of the age of 60, 120, or 180 days. The *upper panel* shows representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA. Autoradiographic exposure was for 2 days on Kodak X-Omat film with an intensifying screen. The *bar* indicates the position of a 400-base pair radiolabeled DNA marker, showing that the single protected fragment migrates with a molecular size of 400 bases, corresponding to prodynorphin mRNA. The *lower panel* shows the levels of prodynorphin mRNA in control myocytes (*black bars*) or in cardiomyopathic cells (*white bars*). The data are expressed as mean values ± S.E. (n = 6). *, significantly different from the control value.

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*-glycerol 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 sarcolemmal 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-

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

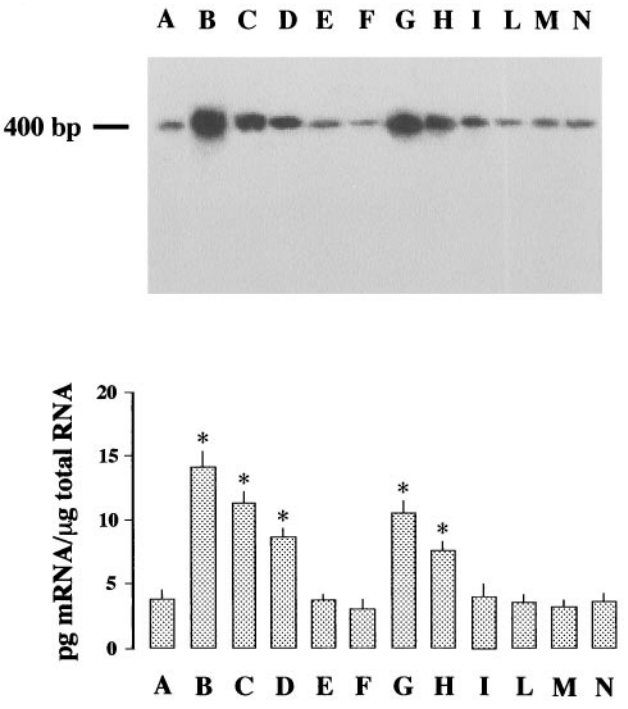

FIG. 2. **Prodynorphin mRNA expression in normal hamster myocytes exposed to 1,2-dioctanoyl-*sn*-glycerol in the absence or presence of different PKC inhibitors.** Myocytes were isolated from 60-day-old control hamsters. Representative autoradiograms of the ribonuclease protection analysis of myocardial 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 nM 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).

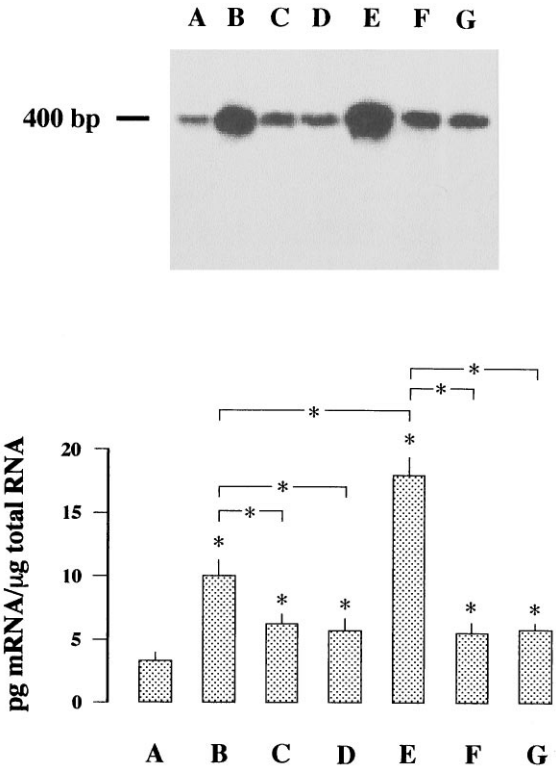

FIG. 3. **Prodynorphin mRNA expression in cardiomyopathic myocytes treated with PKC inhibitors or 1,2-dioctanoyl-*sn*-glycerol.** The myocardial cells were isolated from 60-day-old cardiomyopathic hamsters. Representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA are shown in the *upper panel*. Autoradiographic exposure was carried out as described in Fig. 1. *A*, untreated control cells; *B*, untreated cardiomyopathic myocytes; *C* and *D*, 4 h of treatment of cardiomyopathic myocytes with 5 μM chelerythrine or 1 μM calphostin C, respectively; *E*, 4 h of treatment of cardiomyopathic cells with 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol; *F* and *G*, 4 h of exposure of cardiomyopathic myocytes to 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively. 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 difference between two groups (one-way analysis of variance, Newman Keul's test).

able in the nuclear fraction. Moreover, the nuclear preparation appeared to be essentially free of sarcoplasmic reticular membranes and myofibrils as revealed by the measure of the corresponding marker enzymes rotenone-insensitive NADPH cytochrome *c* reductase and K^+-EDTA ATPase. The absence of contamination in nuclei prepared from cardiomyopathic myocytes was also confirmed (not shown).

Prodynorphin gene transcription was increased in nuclei isolated from control myocytes that had been exposed for 4 h to 1,2-dioctanoyl-*sn*-glycerol, compared with nuclei from untreated control cells (Fig. 4). Such a stimulatory effect was abolished by cell treatment with 5 μM chelerythrine or with 1 μM calphostin C (Fig. 4). We have previously shown that PKC isozymes are expressed in isolated rat myocardial nuclei and that the activation of nuclear PKC may be involved in the stimulation of prodynorphin gene transcription in rat myocytes (6). In this present study, the exposure of nuclei isolated from normal hamster myocytes to 1,2-dioctanoyl-*sn*-glycerol elicited an increase in prodynorphin gene transcription that was similar to that observed in nuclei from diglyceride-treated control cells. The incubation of isolated nuclei with 5 μM chelerythrine or with 1 μM calphostin C abolished this direct effect of 1,2-dioctanoyl-*sn*-glycerol on transcriptional activity (Fig. 4). Fig. 4 shows that prodynorphin gene transcription was markedly increased 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

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

The myocardial cells were isolated from 60-day-old control hamsters. Enzymatic activities are expressed as mean values ± S.E. (n = 6).

Enzyme activities	Homogenate	Nuclear fraction
	μmoles product formed/mg protein/h	
Na^+-K^+ ATPase (ouabain-sensitive)	2.040 ± 0.060	ND[a]
5′-Nucleotidase	0.680 ± 0.080	ND[a]
NADH cytochrome *c* reductase (rotenone-insensitive)	0.360 ± 0.110	ND[a]
Succinate dehydrogenase	6.000 ± 0.300	ND[a]
NADPH cytochrome *c* reductase (rotenone-insensitive)	0.250 ± 0.012	0.010 ± 0.007
K^+-EDTA ATPase	4.800 ± 0.100	ND[a]

[a] ND, nondetectable.

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

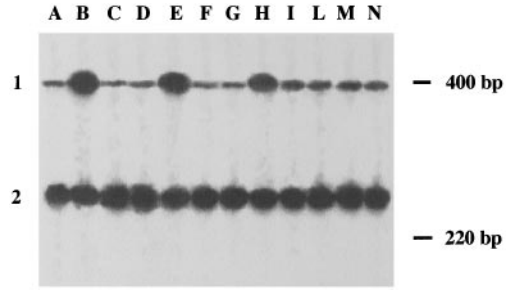

FIG. 4. **Analysis of the rate of transcription of the prodynorphin gene in isolated myocardial nuclei.** Myocardial nuclei were isolated from myocytes of both control or cardiomyopathic hamsters of 60 days and the nuclear run-off assay was performed according to the procedure described under "Materials and Methods." Autoradiograms are representative of six separate experiments. *1*, transcription of the podynorphin gene; *2*, cyclophilin mRNA. Autoradiographic exposure was for 2 days on Kodak X-Omat film with an intensifying screen. On the right are indicated the position of 400- or 220-base pair radiolabeled DNA markers, showing that the single protected fragments migrated with a molecular size of 400 or 270 bases, corresponding to prodynorphin or cyclophilin mRNA, respectively. *A*, nuclei were isolated from untreated control myocytes; *B*, nuclei were isolated from control myocytes that have been exposed to 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol for 4 h; *C* and *D*, nuclei were isolated from control 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; *E*, nuclei, isolated from untreated control cells, were subsequently exposed to 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol for 4 h; *F* and *G*, nuclei, isolated from untreated control cells, were subsequently incubated 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; *H*, nuclei were isolated from untreated cardiomyopathic myocytes; *I* and *L*, nuclei were isolated from cardiomyopathic myocytes that have been treated for 4 h with 5 μM chelerythrine or 1 μM calphostin C, respectively; *M* and *N*, nuclei, isolated from untreated cardiomyopathic cells, were then exposed for 4 h to 5 μM chelerythrine or 1 μM calphostin C, respectively.

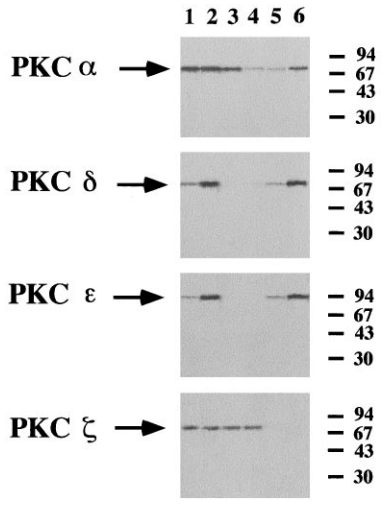

FIG. 5. **Subcellular distribution of PKC isozymes in normal and cardiomyopathic myocytes.** Total cell lysates and cytosolic and nuclear fractions were prepared from myocytes isolated from 60-day-old control or cardiomyopathic hamsters. Equal amounts of protein (20 μg) from each sample were subjected to 8% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting as described under "Materials and Methods." Autoradiograms are representative of six separate experiments. The *arrows* to the left of each panel indicate PKC immunoreactivity as confirmed in peptide antigen competition experiments (results not shown). The numbers to the right of each panel refer to the molecular mass (kilodaltons) of marker proteins. *Lanes 1*, *3*, and *5* correspond, respectively, to total cell lysates and cytosolic or nuclear fractions isolated from normal cells; *lanes 2*, *4*, and *6* correspond, respectively, to total cell lysates and cytosolic or nuclear fractions isolated from cardiomyopathic myocytes.

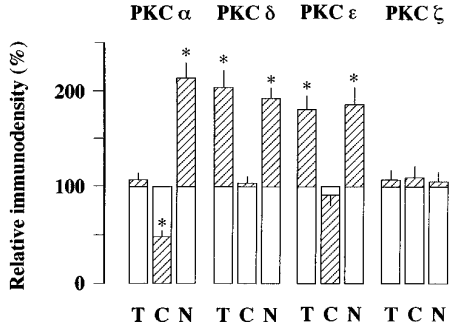

FIG. 6. **Quantitative analysis of the subcellular distribution of PKC isozymes in normal and cardiomyopathic myocytes.** Data are expressed as percentage changes in the intensity of autoradiographic bands of total extracts (*T*), cytosolic (*C*), or nuclear (*N*) fractions from cardiomyopathic myocytes (*hatched bars*) relative to the intensity in the autoradiographs of the corresponding samples from normal cells (*white bars*, 100%). The data are expressed as mean values ± S.E. (n = 6). *, significantly different from the control value.

decrease in prodynorphin gene transcription was observed when either chelerythrine or calphostin C were directly applied to nuclei isolated from cardiomyopathic cells (Fig. 4).

Immunoblot analysis of total extracts from both control and cardiomyopathic myocytes revealed the expression of PKC-α (80 kDa), PKC-δ (78 kDa), PKC-ϵ (97 kDa), and PKC-ζ (75 kDa) (Fig. 5). PKC-β and PKC-γ were not detected (not shown). The expression of PKC-α in total extracts from control myocytes was similar to that observed in total extracts from cardiomyopathic cells (Figs. 5 and 6). Only a slight immunoreactivity against the anti-PKC-α-specific antibody was detected in the nuclear fraction of control cells (Fig. 5). On the contrary, PKC-α expression was increased in nuclei of cardiomyopathic myocytes compared to the nuclear fraction from control cells (Figs. 5 and 6). Such an increase was associated with a concomitant reduction in isozyme expression in the cytosolic fraction from cardiomyopathic cells (Figs. 5 and 6). Different from PKC-α, the expression of both PKC-δ and PKC-ϵ was increased in total cellular extracts from cardiomyopathic myocytes compared with control 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 myocytes than in nuclei obtained from control cells (Figs. 5 and 6). The expression of PKC-ζ appeared to be similar in cardiomyopathic myocytes and in control cells (Figs. 5 and 6). No evidence for PKC-ζ was found in myocardial nuclei isolated from both groups of cells (Fig. 5).

We next investigated whether the increase in the expression of PKC-α, -δ, and -ϵ observed in the nucleus of cardiomyopathic myocytes may be associated with an increase in nuclear 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 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 or 1 μM calphostin C for 30 min before being 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 finally investigated whether the increase in prodynorphin mRNA levels observed in cardiomyopathic myocytes may result in an increase in the expression of a biologically active end product of the prodynorphin gene. Consistent amounts of ir-dyn B were found both in control and in 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-

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

icantly 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).

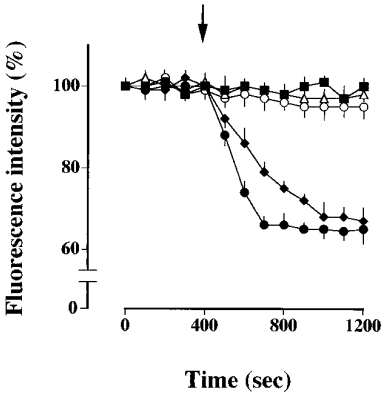

FIG. 7. **Nuclear PKC activity in isolated cardiac myocytes from normal and cardiomyopathic hamsters.** Myocytes were isolated from 60-day-old control or BIO 14.6 hamsters. Nuclear PKC activity was measured as described under "Materials and Methods." The phosphorylation of the acrylodan-labeled MARCKS peptide was started by the addition of 10 μg 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).

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, in both groups of cells the stimulatory effect elicited by 1,2-dioctanoyl-*sn*-glycerol was suppressed when either chelerythrine or calphostin C were added. The third observation is that prodynorphin mRNA levels were markedly reduced in cardiomyopathic myocytes exposed to these specific PKC inhibitors compared with untreated cardiomyopathic cells.

The run-off experiments performed in isolated myocardial nuclei revealed that both the increase in mRNA levels induced by treatment of control cells with 1,2-dioctanoyl-*sn*-glycerol and the increase in prodynorphin mRNA expression observed in cardiomyopathic myocytes occurred at the transcriptional level. The involvement of PKC in these transcriptional events appears to be inferred by the observation that cell treatment with both chelerythrine or calphostin C completely abolished the stimulatory effect of the diglyceride and caused a marked decrease in prodynorphin gene transcription in cardiomyopathic myocytes. The experiments described here yielded a number of interrelated observations suggesting that nuclei isolated from hamster myocytes may harbor PKC and that nuclear PKC activation may play an important role in the transcriptional regulation of the prodynorphin gene under normal or pathological heart conditions. Our first observation was that the exposure of isolated control nuclei to 1,2-dioctanoyl-*sn*-glycerol caused a marked increase in prodynorphin gene transcription. Second, the transcriptional response elicited by the diglyceride in isolated control nuclei was similar in magnitude to that observed in nuclei obtained from diglyceride-treated

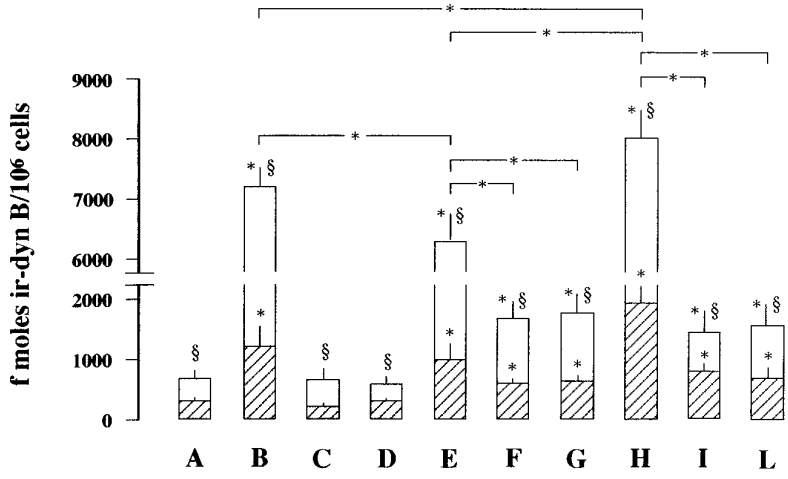

FIG. 8. **Analysis of ir-dyn B expression in myocardial cells and in their incubation media.** Cardiac myocytes were isolated from 60-day-old control or cardiomyopathic hamsters. *Hatched bars*, ir-dyn B in cells; *white bars*, ir-dyn B in the medium. *A*, untreated control myocytes; *B*, control myocytes 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 5 μM chelerythrine or 1 μM 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 10^6 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 are expressed as mean values ± S.E. (n = 6). §, the value of the *white bar* is significantly different from that of the *hatched bar*. *, significantly different from the control value; ⌜*⌝, significant difference between two groups (one-way analysis of variance, Newman Keul's test).

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

control cells. 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 isozymes 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 isozymes 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 isozymes 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 BIO 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 Ca^{2+} overload and abnormalities in intracellular Ca^{2+} handling represent a prominent feature in the BIO 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 Ca^{2+} loading (5). The comparative analysis of intracellular Ca^{2+} 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

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

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 κ 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 that have been treated with a κ opioid receptor agonist than in untreated cardiomyopathic cells (54). On the whole, these observations might suggest a link between the increase in prodynorphin gene expression observed here in cardiomyopathic myocytes and the onset of the contractile dysfunctions associated with the cardiomyopathy. On the other hand, an alternative interpretation of the present results might be inferred from the finding that no significant increase in prodynorphin mRNA level could be detected in cardiomyopathic myocytes from 120- or 180-day-old BIO 14.6 hamsters compared with cardiomyopathic cells obtained from 60-day-old animals. This observation may lead to the conclusion that the overexpression of the prodynorphin gene observed in an early phase of the cardiomyopathy may not be related to the progressive worsening of the myocardial function. In this regard, a number of experimental results may instead suggest that the increase in prodynorphin gene and dyn B expression may represent an adaptive phenomenon counteracting the progression of the cardiomyopathy. First, there is a substantial body of experimental evidence supporting the existence of abnormal calcium loading in the cardiac myocytes of BIO 14.6 hamsters (49–53) and suggesting that the altered intracellular Ca^{2+} homeostasis may account for most of abnormalities in excitation-contraction coupling, as well as for the depressed inotropic state observed in these cells (52, 53). Second, the negative inotropic effects induced by κ opioid receptor stimulation in myocardial cells are largely attributable to the depletion of Ca^{2+} from the sarcoplasmic reticulum (9). Therefore, decreasing the sarcoplasmic reticulum Ca^{2+} loading may be viewed as a helpful phenomenon counteracting the detrimental effects of intracellular Ca^{2+} overload. The third observation is that an additional consequence of κ opioid receptor stimulation in myocardial cells is a PKC-dependent activation of the Na^+/H^+ exchanger (10). This has been shown to elicit cytosolic alkalosis, an event that shifts the dynamic equilibrium between Ca^{2+} bound to myofilaments and cytosolic Ca^{2+} concentration and leads to an increase in myofilament sensitivity to Ca^{2+} (10). Such a mechanism of action, by increasing the contractile response to a given cytosolic Ca^{2+} concentration, may blunt the negative inotropic effect due to the opioid-induced depletion of Ca^{2+} from the sarcoplasmic reticulum. In the cardiomyopathic cell, it might have the appealing property of sustaining cell inotropism despite a marked reduction in the amplitude of the cytosolic Ca^{2+} transient.

Further studies are needed to establish the exact role played by the increase in the expression of the prodynorphin gene in cardiomyopathic myocytes and to understand whether the manipulation of this endogenous dynorphinergic system might prove beneficial in the cardiomyopathic process.

Acknowledgment—We thank Giuseppe Delogu for technical assistance.

REFERENCES

1. Azari, J., Brumbaugh, P., and Huxtable, R. (1980) *J. Mol. Cell. Cardiol.* **12,** 1353–1366
2. Hengstenberg, C., and Schwartz, K. (1994) *J. Mol. Cell. Cardiol.* **26,** 3–10
3. Elstein, E., Liew, C. C., and Sole, M. J. (1992) *J. Mol. Cell. Cardiol.* **24,** 1471–1477
4. Hengstenberg, C., Carrier, L., Schwartz, K., and Maisch, B. (1994) *Herz* **19,** 84–90
5. Ventura, C., Guarnieri, C., Vaona, I., Campana, G., Pintus, G., and Spampinato, S. (1994) *J. Biol. Chem.* **269,** 5384–5386
6. Ventura, C., Pintus, G., Vaona, I., Bennardini, F., Pinna, G., and Tadolini, B. (1995) *J. Biol. Chem.* **270,** 30115–30120
7. Chavkin, C., James, J. F., and Goldstein, A. (1982) *Science* **215,** 413–415
8. Ventura, C., Guarnieri, C., Stefanelli, C., Cirielli, C., Lakatta, E. G., and Capogrossi, M. C. (1991) *Biochem. Biophys. Res. Commun.* **179,** 972–978
9. Ventura, C., Spurgeon, H. A., Lakatta, E. G., Guarnieri, C., and Capogrossi, M. C. (1992) *Circ. Res.* **70,** 66–81
10. Ventura, C., Capogrossi, M. C., Spurgeon, H. A., and Lakatta, E. G. (1991) *Am. J. Physiol.* **261,** H1671–H1674
11. Zagon, I. S., Rhodes, R. E., and McLaughlin, P. J. (1985) *Science* **227,** 1049–1051
12. Zagon, I. S., Rhodes, R. E., and McLaughlin, P. J. (1986) *Cell Tissue Res.* **246,** 561–565
13. Capogrossi, M. C., Kort, A. A., Spurgeon, H. A., and Lakatta, E. G. (1986) *J. Gen. Physiol.* **88,** 589–613
14. Piper, H. M., Probst, I., Schwartz, P., Hutter, F. J., and Spieckermann, P. G. (1982) *J. Mol. Cell. Cardiol.* **14,** 397–412
15. Jacobson, S. L., and Piper, H. M. (1986) *J. Mol. Cell. Cardiol.* **18,** 661–678
16. Piper, H. M., Jacobson, S. L., and Schwartz, P. (1988) *J. Mol. Cell. Cardiol.* **20,** 825–835
17. Chomczynsky, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
18. Blum, M. (1989) *Methods Enzymol.* **168,** 618–633
19. Krause, J. E., Cremins, J. D., Carter, M. S., Brown, E. R., and MacDonald, M. R. (1989) *Methods Enzymol.* **168,** 634–652
20. Cone, R. I., Weber, E., Barchas, J. D., and Goldstein, A. J. (1983) *J. Neurosci.* **3,** 2146–2152
21. Xie, G. X., and Goldstein, A. J. (1987) *J. Neurosci.* **7,** 2049–2055
22. Spampinato, S., Canossa, M., Bachetti, T., Campana, G., Murari, G., and Ferri, S. (1992) *Brain Res.* **580,** 225–232
23. Edwards, M. J., and Maguire, M. H. (1970) *Mol. Pharmacol.* **6,** 641–648
24. Lamers, J. M. J., and Stinis, J. T. (1981) *Biochim. Biophys. Acta* **640,** 521–534
25. LeBel, D., Poirier, G. C., and Beaudain, A. R. (1976) *Anal. Biochem.* **85,** 86–99
26. King, T. E. (1967) *Methods Enzymol.* **10,** 322–331
27. Sottocasa, G. L., Kuylenstierna, B., Ernester, L., and Bergstrand, A. (1967) *J. Cell Biol.* **32,** 415–437
28. Scholte, H. R. (1973) *Biochim. Biophys. Acta* **305,** 413–427
29. Greenberg, M. E. (1987) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K., eds) pp. 4.10.1–4.10.8, John Wiley & Sons, New York
30. Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. J., and Sutclife, J. G. (1988) *DNA* (*N. Y.*) **7,** 261–267
31. Aderem, A. A., Albert, K. A., Keum, M. M., Wang, J. K. T., Greengard, P., and Cohn, Z. A. (1988) *Nature* **332,** 362–364
32. Blackshear, P. J., Wen, L., Glynn, B. P., and Witters, L. A. (1986) *J. Biol. Chem.* **261,** 1459–1469
33. Patel, J., and Kligman, D. (1987) *J. Biol. Chem.* **262,** 16686–16691
34. Thelen, M., Rosen, A., Nairn, A. C., and Aderem, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5603–5607
35. McIlroy, B. K., Walters, J. D., and Johnson, J. D. (1991) *Anal. Biochem.* **195,** 148–152
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
37. Lapetina, E. G., Reep, B., Ganong, B. R., and Bell, R. M. (1985) *J. Biol. Chem.* **260,** 1358–1361
38. Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **172,** 993–999
39. Ko, F. N., Chen, I. S., Wu, S. J., Lee, L. G., Haung, T. F., and Teng, C. M. (1990) *Biochim. Biophys. Acta* **1052,** 360–365
40. Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989) *Biochem. Biophys. Res. Commun.* **159,** 548–553
41. Tamaoki, T. (1991) *Methods Enzymol.* **201,** 340–347
42. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) *Biochemistry* **23,** 5036–5041
43. Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedwick, A. D., Wadsworth, J., Westmacott, D., and Wilkinson, S. E. (1989) *FEBS Lett.* **259,** 61–63
44. Disatnik, M. E., Buraggi, G., and Mochly-Rosen, D. (1994) *Exp. Cell. Res.* **210,** 287–297
45. Allen, B. G. and Katz, S. (1991) *Biochemistry* **30,** 4334–4343
46. Kohout, T. A., and Rogers, T. B. (1993) *Am. J. Physiol.* **264,** C1350–C1359
47. Jamieson, J. D., and Palade, G. E. (1964) *J. Cell Biol.* **23,** 151–172
48. Ventura, C., Bastagli, L., Bernardi, P., Caldarera, C. M., and Guarnieri, C. (1989) *Biochim. Biophys. Acta* **987,** 69–74
49. Proschek, L., and Jasmin, G. (1982) *Muscle Nerve* **5,** 26–32
50. Wagner, J. A., Reynolds, I. J., Weisman, H. F., Dudeck, P., Weisfeldt, M. L., and Snyder, S. H. (1986) *Science* **232,** 515–518
51. Weisman, H. F., and Weisfeldt, M. L. (1987) *J. Am. Coll. Cardiol.* **10,** 1135–138
52. Sen, L., O'Neill, M., Marsh, J. D., and Smith, T. W. (1990) *Circ. Res.* **67,** 599–608
53. Sen, L., O'Neill, M., Marsh, J. D., and Smith, T. W. (1990) *Am. J. Physiol.* **259,** H1533–H1543
54. Kasper, E., Ventura, C., Ziman, B. D., Lakatta, E. G., Weisman, H., and Capogrossi, M. C. (1992) *Life Sci.* **50,** 2029–2035

Downloaded from http://www.jbc.org/ by guest on July 24, 2018

Opioid Peptide Gene Expression in the Primary Hereditary Cardiomyopathy of the Syrian Hamster: I. REGULATION OF PRODYNORPHIN GENE EXPRESSION BY NUCLEAR PROTEIN KINASE C

Carlo Ventura, Gianfranco Pintus, Maria Giovanna Fiori, Federico Bennardini, Giangavino Pinna and Leonardo Gaspa

J. Biol. Chem. 1997, 272:6685-6692.
doi: 10.1074/jbc.272.10.6685

Access the most updated version of this article at http://www.jbc.org/content/272/10/6685

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 18 of which can be accessed free at
http://www.jbc.org/content/272/10/6685.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 24, 2018