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

II. ROLE OF INTRACELLULAR CALCIUM LOADING*

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We have previously shown that prodynorphin gene expression was markedly increased in adult myocytes of BIO 14.6 cardiomyopathic hamsters and that nuclear protein kinase C (PKC) may be involved in the induction of this opioid gene. Here we report that the cytosolic Ca^{2+} concentration was significantly increased in resting and in KCl-depolarized cardiomyopathic myocytes compared with normal cells. In normal and in cardiomyopathic cells, KCl significantly increased prodynorphin mRNA levels and prodynorphin gene transcription. These effects were abolished by the Ca^{2+} channel blocker verapamil. In control myocytes, the KCl-induced increase in prodynorphin mRNA expression was in part attenuated by chelerythrine or calphostin C, two selective PKC inhibitors. In these cells, KCl induced the translocation of PKC-α into the nucleus, increasing nuclear PKC activity. In resting cardiomyopathic myocytes, the increase in prodynorphin mRNA levels and gene transcription were significantly attenuated by the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethylester being completely abolished when the chelating agent was administered in the presence of PKC inhibitors. KCl and the PKC activator 1,2-dioctanoyl-*sn*-glycerol additively stimulated prodynorphin gene expression both in normal and in cardiomyopathic cells. Therefore, we conclude that PKC activation and intracellular Ca^{2+} overload may represent the two major signaling mechanisms involved in the induction of the prodynorphin gene in cardiomyopathic cells.

Hypertrophic cardiomyopathy is a disease which is characterized by abnormal thickening of the left ventricular wall in the absence of overt causes of myocardial hypertrophy. The Syrian hamster of the BIO 14.6 strain has been shown to represent a useful model of genetically determined hypertrophic cardiomyopathy and heart failure. In this strain, myocardial hypertrophy is also associated with myocardial fiber derangement, microvascular spasm, and focal necrosis (1–4). The physiopathology of the disease is not fully understood, but the observation that different calcium antagonists are able to reduce the degree of myocardial damage and may preserve the contractility of the cardiomyopathic hamster heart (5, 6) suggests the possibility that a disturbance in the intracellular Ca^{2+} homeostasis may play a major role in the cardiomyopathic process. Such a hypothesis appears to be supported by the finding that, in the heart of BIO 14.6 hamsters, the myocardial lesions are associated with intracellular Ca^{2+} overload and with abnormalities in cytosolic Ca^{2+} handling (7–12).

Intracellular Ca^{2+} loading has been reported to modulate the expression of different genes in the myocardial cell, including G protein genes and protooncogenes (13–15). We have previously shown that in adult rat cardiac myocytes, KCl-stimulated intracellular Ca^{2+} overload induced prodynorphin gene expression and increased the synthesis and the release of dynorphin B (16), an opioid peptide displaying selective binding activity to κ opioid receptors (17). These receptors are expressed in myocardial cells (18) and their stimulation has been shown to deplete of Ca^{2+} the sarcoplasmic reticulum and to produce a severe negative inotropic effect in isolated rat myocytes (19). These results suggest that an opioid gene may have an autocrine role in the regulation of intracellular Ca^{2+} homeostasis and may be overexpressed under pathological heart conditions associated with an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$).

In a companion study (44), we provided evidence that the expression of the prodynorphin gene was markedly enhanced in myocytes isolated from BIO 14.6 cardiomyopathic hamsters and that protein kinase C (PKC)[1] activation may be a signaling mechanism increasing prodynorphin gene transcription. To test the hypothesis that abnormal cytosolic Ca^{2+} homeostasis may also represent a critical defect with pathogenic importance in the induction of the prodynorphin gene in the BIO 14.6 hamster model of hypertrophic cardiomyopathy, we examined the relationships among prodynorphin mRNA levels, prodynorphin gene transcription, dynorphin B expression and $[Ca^{2+}]_i$ in myocytes isolated from both normal or cardiomyopathic hamster hearts.

MATERIALS AND METHODS

The acetoxy-methyl ester derivative of the Ca^{2+} probe fura-2 (fura-2/AM) was from Molecular Probes. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxy-methylester (BAPTA/AM) was from Biomol Research Laboratories, Inc (Plymouth Meeting, PA). Animals and all other chemicals were from the sources listed in a companion study (44).

Cardiac myocytes were isolated from 60-day-old control (F1B) or cardiomyopathic (BIO 14.6) hamsters by using the procedure described in a companion study (44). The extraction of RNA, the determination of prodynorphin mRNA, the isolation of myocardial nuclei, the assessment of purity of the nuclear fraction and the nuclear run-off transcription

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[1] The abbreviations used are: PKC, protein kinase C; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxy-methylester; MARCKS, myristoylated alanine-rich PKC substrate; ir, immunoreactive; dyn B, dynorphin B.

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assay were all performed as described (44), as were the immunoblotting analysis and the quantitative immunoautoradiography of PKC isozymes, the measurement of PKC activity and the identification of dynorphin B-like material.

Estimation of Cytosolic Calcium Level in Single Myocardial Cells—Isolated cardiac myocytes were resuspended in a medium (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, 1.0 $CaCl_2$; this medium was continuously gassed with 95% O_2, 5% CO_2 (pH 7.36 ± 0.05). The myocardial cells were loaded with fura-2/AM (3 μM) for 10 min at 25 °C and then washed for 5 min with the perfusion buffer. Fura-2-loaded myocytes were then transferred to a Lucite chamber on the stage of an inverted microscope (Olympus OSP-3 microscopic photometry system for intracellular Ca^{2+} measurement) and were continuously superfused at 37 °C with perfusion buffer, gassed with 95% O_2 and 5% CO_2. Excitation wavelengths were 340 and 380 nm, and emission wavelength was 505 nm. The switching capability of the two excitation wavelengths was 100 times/s (time resolution of 10 ms). Fluorescence signals from single cells were transmitted to a computer for on-line analysis. At the end of each experiment, background autofluorescence from cells not loaded with fura-2 (usually less than 1% of the fura-2 signal from the cell) was subtracted from the initial signals. A calibration of the fluorescent signal was performed to represent actual cytosolic Ca^{2+} ($[Ca^{2+}]_i$) values: first, the myocardial cells were perfused in the presence of 3 μM ionomycin until a peak 340 nm signal was evident; then, the myocytes were perfused with 3 mM EGTA to obtain a minimum value. The equation of Grynkiewicz *et al.* (20) determined the fluorescence ratio (340/380 nm), which was used to minimize problems related to variations in dye concentration and leakage as well as in cell thickness.

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

In a companion study (44), we have shown that prodynorphin mRNA levels were significantly higher in cardiomyopathic than in normal hamster myocytes. In this study, we show that such an increase in prodynorphin mRNA expression was associated with a substantial increase in $[Ca^{2+}]_i$ (Fig. 1). In resting cardiomyopathic myocytes bathed in 1 mM $CaCl_2$, $[Ca^{2+}]_i$ was 254.3 ± 21.0 nM (n = 6), which was significantly higher than the mean level of 123.8 ± 11.2 nM (n = 6) observed in control cells. The superfusion with 60 mM KCl induced a rapid and sustained increase in $[Ca^{2+}]_i$ in both groups of myocytes (Fig. 1). Moreover, in the presence of KCl, both the absolute $[Ca^{2+}]_i$ value and the increment in $[Ca^{2+}]_i$ over the basal value were significantly higher in cardiomyopathic cells (511.0 ± 36.5 and 255.2 ± 12.4 nM, respectively; n = 6) than in normal myocytes (298.0 ± 16.1 and 176.0 ± 19.8 nM, respectively; n = 6). Verapamil (2 μM) rapidly restored basal $[Ca^{2+}]_i$ in both groups of cells (Fig. 1). Fig. 1 shows that prodynorphin mRNA expression paralleled the changes in $[Ca^{2+}]_i$ observed both in control and cardiomyopathic myocytes following the treatments described above. A significant increase in prodynorphin mRNA levels was observed following the exposure of control cells to KCl. In particular, while a treatment with 60 mM KCl for 1 h was ineffective (not shown), the exposure of myocytes to 60 mM KCl for 4 h increased prodynorphin mRNA from 2.9 ± 0.28 pg/μg total RNA (basal value, mean ± S.E., n = 6) to 10.8 ± 1.0 pg/μg total RNA (mean ± S.E., n = 6). A 4-h exposure of cardiomyopathic myocytes to 60 mM KCl significantly increased prodynorphin mRNA above the basal levels and the levels observed in KCl-treated control cells (Fig. 1). The stimulatory effect of KCl was prevented by the incubation of control or cardiomyopathic cells in the presence of 2 μM verapamil (Fig. 1). In resting cardiomyopathic myocytes that have been treated for 4 h with 10 μM BAPTA/AM, an agent acting as an intracellular Ca^{2+} chelator (21, 22), the levels of prodynorphin mRNA were significantly lower than those observed in these cells under basal conditions, but were still significantly higher than the mRNA levels detected in untreated control cells (Fig. 2). Buffering of

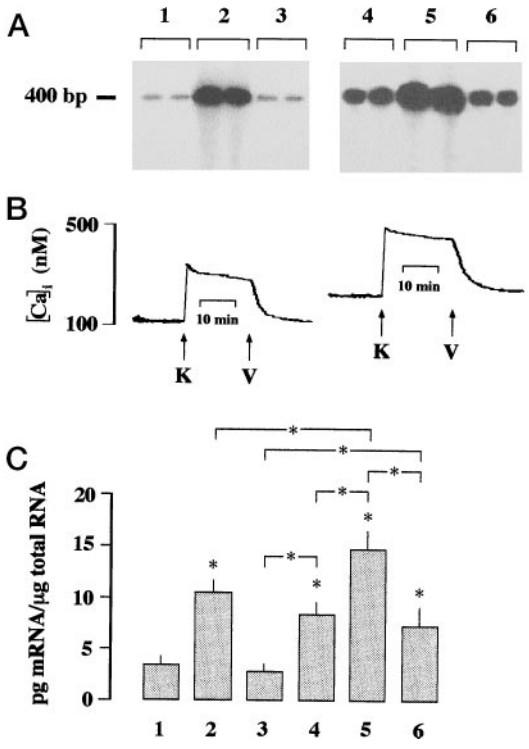

FIG. 1. **Effect of KCl on prodynorphin mRNA expression and $[Ca^{2+}]_i$ in isolated normal or cardiomyopathic myocytes treated in the absence or presence of verapamil.** The myocardial cells were isolated from 60-day-old control (F1B) or cardiomyopathic (BIO 14.6) hamsters. *Panel A* shows representative 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. *Lane 1*, untreated control cells; *lanes 2* and *3*, treatment of control myocytes with 60 mM KCl for 4 h in the absence or presence of 2 μM verapamil, respectively; *lane 4*, untreated cardiomyopathic myocytes; *lanes 5* and *6*, treatment of cardiomyopathic myocytes with 60 mM KCl for 4 h in the absence or presence of 2 μM verapamil, respectively. *Panel B* shows representative tracings of the changes in $[Ca^{2+}]_i$ induced at rest in a single control (*left*) or cardiomyopathic (*right*) myocyte by cell treatment with 60 mM KCl (*K*), in the absence or presence of 2 μM verapamil (*V*). In these experiments the myocardial cells were superfused with a buffer containing (mM): 116.4 NaCl, 5.4 KCl, 1.6 $MgSO_4$, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, 5.6 D-glucose, 1.0 $CaCl_2$. This medium was continuously gassed with 95% O_2, 5% CO_2 (pH 7.36 ± 0.05). Averaged values of prodynorphin mRNA levels are reported in *panel C*. 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).

$[Ca^{2+}]_i$ during the treatment with BAPTA/AM was confirmed by the observation that this compound abolished the electrically driven (1 Hz) cytosolic Ca^{2+} transients in isolated cells superfused with 1 mM $CaCl_2$ (Fig. 2). Fig. 2 shows that no significant difference in prodynorphin mRNA levels was observed among normal and cardiomyopathic myocytes when the cardiomyopathic cells were incubated with BAPTA/AM in the presence of chelerythrine (5 μM) or calphostin C (1 μM), two selective PKC inhibitors (23–26).

We next investigated whether the effects produced on prodynorphin mRNA expression by the observed changes in $[Ca^{2+}]_i$ may result from differences in the transcriptional status of the myocardial nucleus. By using an *in vitro* run-off transcription assay, we found that the exposure of control myocytes to 60 mM KCl for 4 h resulted in a marked increase in the nuclear transcription of the prodynorphin gene that was completely suppressed by cell incubation in the presence of 2 μM verapamil (Fig. 3). The incubation of cardiomyopathic cells

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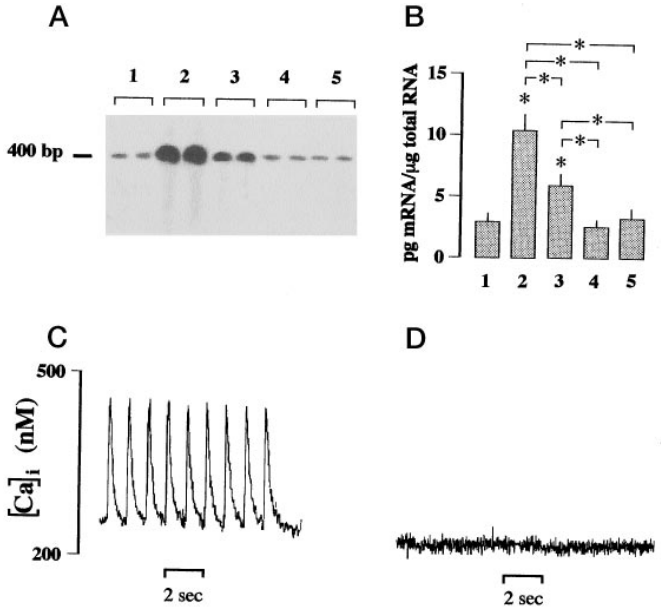

FIG. 2. **Prodynorphin mRNA expression in cardiomyopathic myocytes treated with the intracellular Ca^{2+} chelator BAPTA/AM in the absence or presence of PKC inhibitors.** Cardiac myocytes were isolated from 60-day-old control or cardiomyopathic hamsters. *Panel A* shows representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA. Autoradiographic exposure was performed as described in Fig. 1. *Lane 1*, untreated control myocytes; *lane 2*, untreated cardiomyopathic cells; *lane 3*, treatment of cardiomyopathic myocytes with 10 μM BAPTA/AM for 4 h; *lanes 4* and *5*, treatment of cardiomyopathic cells with 10 μM BAPTA/AM for 4 h in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively. Mean values ± S.E. (n = 6) of prodynorphin mRNA levels are reported in *panel B*; *, significantly different from the control value; ⌐*¬, significant difference between two groups (one-way analysis of variance, Newman Keul's test). *Panel C* shows a typical record of cytosolic Ca^{2+} transients elicited in a single cardiomyopathic cell by field stimulation at 1 Hz via two platinum electrodes placed in the bathing fluid (Grass stimulator, model SD9, Grass Instrument Co., Quincy, MA). In the same cell, the electrically driven cytosolic Ca^{2+} transients were completely abolished following a 30-min treatment in the presence of 10 μM BAPTA/AM (*panel D*).

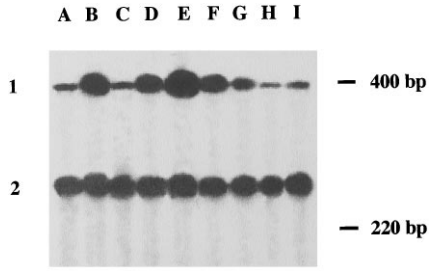

FIG. 3. **Analysis of prodynorphin gene transcription in isolated myocardial nuclei.** Myocardial nuclei were isolated from myocytes of both control or cardiomyopathic hamsters of 60 days, and the nuclear run-off assay was performed as described in a companion study (44). Autoradiograms are representative of six separate experiments. *Lane 1*, transcription of the prodynorphin gene; *lane 2*, cyclophilin mRNA. Autoradiographic exposure was for 2 days on Kodak X-Omat film with an intensifying screen. The *bars* on the right indicate 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* and *C*, nuclei were isolated from control cells that have been exposed to 60 mM KCl for 4 h in the absence or presence of 2 μM verapamil, respectively; *D*, nuclei were isolated from untreated cardiomyopathic cells; *E* and *F*, nuclei were isolated from cardiomyopathic cells treated for 4 h with 60 mM KCl in the absence or presence of 2 μM verapamil, respectively; *G*, nuclei were isolated from cardiomyopathic myocytes that have been treated for 4 h with 10 μM BAPTA/AM; *H* and *I*, nuclei were isolated from cardiomyopathic cells exposed for 4 h to 10 μM BAPTA/AM in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively.

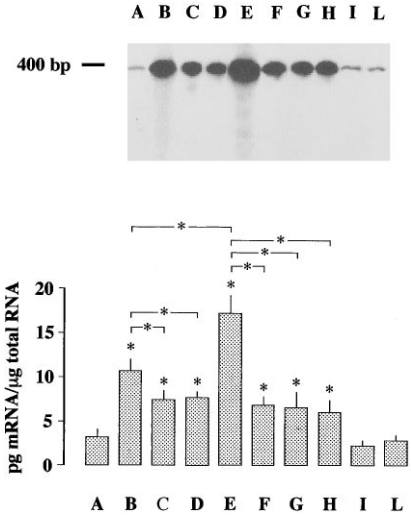

FIG. 4. **Additive effects of 1,2-dioctanoyl-*sn*-glycerol and KCl on prodynorphin mRNA expression in normal myocytes.** Cells were isolated from 60-day-old control hamsters. The *upper panel* shows representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA. Autoradiographic exposure was performed as described in Fig. 1. *A*, untreated cells; *B*, 60 mM KCl for 4 h; *C* and *D*, 4 h of exposure to 60 mM KCl in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively; *E*, 4 h of exposure to 60 mM KCl in the presence of 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol; *F*, 4 h of treatment with 60 mM KCl and 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol in the presence of 2 μM verapamil; *G* and *H*, 4 h of treatment with 60 mM KCl and 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol in the presence of 5 μM chelerythrine or 1 μM calphostin C, respectively; *I* and *L*, 4 h of treatment with 60 mM KCl and 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol in the presence of 2 μM verapamil plus 5 μM chelerythrine or plus 1 μM calphostin C, respectively. Mean values ± S.E. (n = 6) of prodynorphin mRNA levels are reported in the lower panel. *, significantly different from the control value; ⌐*¬, significant difference between two groups (one-way analysis of variance, Newman Keul's test).

in the presence of 60 mM KCl enhanced the rate of gene transcription above that observed in nuclei that have been isolated from KCl-treated control myocytes. Such a stimulatory effect of KCl was prevented by the incubation of cardiomyopathic cells in the presence of verapamil (Fig. 3). The incubation of cardiomyopathic myocytes with BAPTA/AM resulted in a significant decrease in the transcription rate of the prodynorphin gene, that, however, was superimposable to that observed in nuclei isolated from control myocytes only when the cardiomyopathic cells were exposed to the intracellular Ca^{2+} chelator in the presence of chelerythrine or calphostin C (Fig. 3).

Fig. 4 shows that the increase in prodynorphin mRNA levels produced by KCl in control myocytes was attenuated by cell exposure to 5 μM chelerythrine or 1 μM calphostin C. Furthermore, a 4-h treatment of control myocytes with KCl in the presence of the PKC activator 1,2-dioctanoyl-*sn*-glycerol (27) produced additive effects on the expression of prodynorphin mRNA (Fig. 4). Under these experimental conditions, verapamil, chelerythrine, or calphostin C, administered alone, were unable to reduce mRNA levels to the values observed in untreated control cells. The treatment with a combination of KCl and the diglyceride failed to affect prodynorphin mRNA expression only when the control myocytes were exposed to the Ca^{2+} antagonist in the presence of 5 μM chelerythrine or 1 μM calphostin C (Fig. 4).

In a companion study (44), PKC-α (80 kDa), PKC-δ (78 kDa), PKC-ϵ (97 kDa), and PKC-ζ (75 kDa) were detected in total cellular extracts from hamster ventricular myocytes. In this study, we observed that the treatment of control myocytes for

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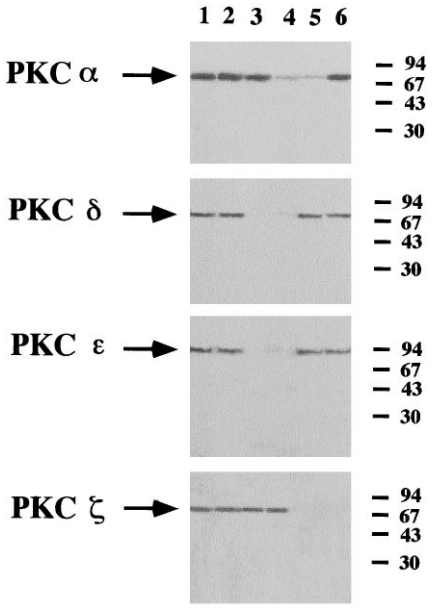

FIG. 5. **Effect of KCl treatment on the subcellular distribution of PKC isozymes in normal myocytes.** Total cell lysates, cytosolic and nuclear fractions were prepared from myocytes that have been isolated from 60-day-old control hamsters and then incubated for 30 min in the absence or presence of 60 mM KCl. Equal amounts of protein (20 μg) from each sample were subjected to 8% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting as described in a companion study (44). 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, cytosolic, or nuclear fractions isolated from untreated cells; *lanes 2*, *4*, and *6* correspond, respectively, to total cell lysates, cytosolic, or nuclear fractions isolated from KCl-treated myocytes.

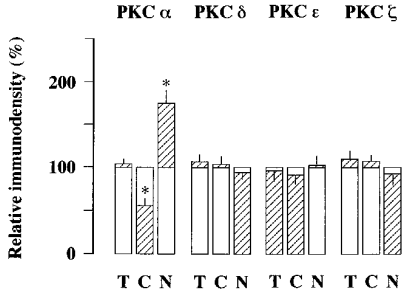

FIG. 6. **Quantitative analysis of the subcellular distribution of PKC isozymes in normal myocytes treated in the absence or presence of KCl.** Data are expressed as percentage changes in the intensity of autoradiographic bands of total extracts (*T*), cytosolic (*C*), or nuclear (*N*) fractions from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

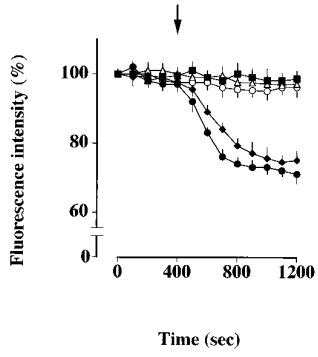

FIG. 7. **Effect of KCl treatment on nuclear PKC activity from normal hamster myocytes.** Cells were dissociated from the heart of 60-day-old control (F1B) hamsters. Nuclear PKC activity was measured in the presence of the acrylodan-labeled MARCKS peptide, according to the method described in a companion study (44). 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 phosphatidylserine, 0.1 μg 1,2-dioctanoyl-*sn*-glycerol, and 75 nM acrylodan-labeled MARCKS peptide. Peptide phosphorylation was started by the addition of 10 μg of nuclear protein (*arrow*) and was followed at 37 °C. As the acrylodan-peptide becomes phosphorylated, it undergoes a time-dependent decrease in its fluorescence at 480 nm. ♦, nuclei were isolated from untreated cells; ●, nuclei were isolated from myocytes exposed for 30 min to 60 mM KCl; △ and ○, nuclei isolated from KCl-treated cells were preincubated for 30 min with 5 μM chelerythrine or 1 μM calphostin C, respectively, 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 800 s, ● was significantly different from ♦; no significant difference was observed between △ or ○ and ■ (one-way analysis of variance, Newman Keul's test).

30 min in the presence of 60 mM KCl increased the amount of PKC-α in the nuclear fraction (Fig. 5, Fig. 6). PKC-α was weakly expressed in the nucleus of untreated cells (Fig. 5) and its increase in the nucleus of KCl-treated myocytes was associated with a decline in isozyme expression in the cytosolic fraction (Figs. 5 and 6). Myocyte exposure to KCl failed to affect the expression of PKC-δ, PKC-ϵ, or PKC-ζ (Figs. 5 and 6). The phosphorylation of the acrylodan-labeled myristoylated alanine-rich PKC substrate (MARCKS) peptide, a high affinity fluorescent substrate for PKC (28–31), occurred at a higher rate in the presence of nuclei isolated from control myocytes exposed for 30 min to 60 mM KCl than in the presence of nuclei isolated from untreated control cells (Fig. 7). No significant change in acrylodan-peptide fluorescence was observed in the presence of nuclei isolated from KCl-treated control cells and subsequently exposed to chelerythrine (5 μM) or calphostin C (1 μM) 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).

In the companion study (44), we have shown that the expression of immunoreactive dynorphin B (ir-dyn B) was significantly increased both in acetic acid extracts and in the incubation medium from cardiomyopathic myocytes. In this study, the incubation for 4 h of control myocytes with 60 mM KCl resulted in an increase in ir-dyn B in cells and the medium that was completely suppressed by cell treatment in the presence of verapamil (Fig. 8). A 4-h exposure of control myocytes to KCl in the presence of 1,2-dioctanoyl-*sn*-glycerol additively increased the amount of ir-dyn B both in the cells and in the incubation media (Fig. 8). This treatment failed to affect dyn B expression in control cells that have been incubated with verapamil in the presence of 5 μM chelerythrine (Fig. 8) or 1 μM calphostin C (not shown). The exposure of cardiomyopathic myocytes to BAPTA/AM significantly reduced ir-dyn B both in the cells and in the medium. No significant difference in dyn B expression was observed between the cardiomyopathic and control cells when the cardiomyopathic myocytes were incubated with BAPTA/AM in the presence of 5 μM chelerythrine (Fig. 8) or 1 μM calphostin C (not shown).

DISCUSSION

In the present study, we found that $[Ca^{2+}]_i$ not only started at a substantially higher level in resting cardiomyopathic myocytes compared with control cells but was increased by a significantly higher value in cardiomyopathic than in normal cells in response to KCl treatment. These results are in agreement

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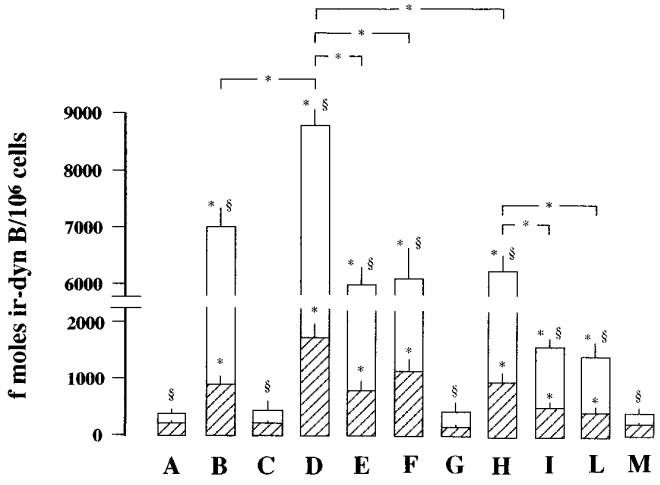

FIG. 8. **Effect of KCl, BAPTA/AM and PKC inhibitors on the expression of ir-dyn B in myocardial cells and in their incubation media.** Cardiac myocytes were isolated from 60-day-old control or cardiomyopathic hamsters. *Hatched bars*, ir-dyn B in myocytes; *white bars*, ir-dyn B in the medium. *A*, untreated control myocytes; *B*, control myocytes exposed to 60 mM KCl for 4 h; *C*, control myocytes treated with 60 mM KCl for 4 h in the presence of 2 μM verapamil; *D*, control cells exposed for 4 h to 60 mM KCl in the presence of 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol; *E*, control cells treated for 4 h with 60 mM KCl and 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol, in the presence of 2 μM verapamil; *F*, control cells exposed for 4 h to 60 mM KCl and 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol, in the presence of 5 μM chelerythrine; *G*, control cells treated for 4 h with 60 mM KCl and 0.2 μg/ml 1,2-dioctanoyl-*sn*-glycerol, in the presence of 2 μM verapamil plus 5 μM chelerythrine; *H*, untreated cardiomyopathic myocytes; *I*, 4 h of exposure of cardiomyopathic cells to 10 μM BAPTA/AM; *L*, 4 h of treatment of cardiomyopathic cells with 5 μM chelerythrine; *M*, cardiomyopathic myocytes treated with BAPTA/AM for 4 h in the presence of 5 μM chelerythrine. 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).

with the observations of Sen *et al.* (11, 12), showing that diastolic $[Ca^{2+}]_i$ was higher in cardiomyopathic myocytes isolated from BIO 14.6 hamsters than in control cells and that $^{45}Ca^{2+}$ content increased more in cardiomyopathic than in control cells following the exposure to effectors that elevate transarcolemmal Ca^{2+} flux. The current experimental data raise at least three possibilities: (i) there is increased Ca^{2+} flux at the level of sarcolemmal Ca^{2+} channels, (ii) there are abnormalities of Ca^{2+} sequestration in the sarcoplasmic reticulum (SR), and (iii) the ability of the cardiomyopathic cell to extrude Ca^{2+} in response to Ca^{2+} loading conditions is abnormal. There is increasing experimental evidence indicating that the above mechanisms may occur in cardiomyopathic myocytes and that altered $[Ca^{2+}]_i$ regulation may represent a prominent feature in the primary hereditary cardiomyopathy of the Syrian hamster. A simultaneous reduction of the sarcolemmal and sarcoplasmic reticulum Ca^{2+} ATPase activities and gene expression has been shown in myocardial cells from BIO 14.6 cardiomyopathic hamsters (32), suggesting that a reduced capacity of these calcium pumps would result in alterations in intracellular Ca^{2+} homeostasis and in myocardial contractility. Moreover, Finkel *et al.* (33, 34) reported an increase in the number of ryanodine-binding sites in BIO 14.6 hamster hearts and hypothesized the presence of a defect in the ryanodine-sensitive sarcoplasmic reticulum calcium release channel, which may contribute to an increased $[Ca^{2+}]_i$. A marked increase in the number of the high affinity 1,4-dihydropyridine receptors and related voltage-sensitive Ca^{2+} channels has also been observed in cardiac membranes from cardiomyopathic hamsters (8, 35). Evidence has been recently provided that T-type Ca^{2+} channels are abnormal in isolated cardiomyopathic myocytes (36), suggesting that increased Ca^{2+} entry via T-type Ca^{2+} current may play an important role in eliciting the intracellular Ca^{2+} overload and the abnormalities in excitation-contraction coupling observed in the Syrian hamster model of cardiomyopathy.

We have previously shown that prodynorphin mRNA levels and prodynorphin gene transcription were markedly increased in cardiomyopathic myocytes isolated from BIO 14.6 hamsters as compared with myocardial cells obtained from normal hamster hearts. In the present study, a number of experimental results suggest that abnormal intracellular Ca^{2+} loading and altered $[Ca^{2+}]_i$ regulation may contribute to the overexpression of the prodynorphin gene in the cardiomyopathic myocyte. First, the increase in prodynorphin mRNA levels observed under basal conditions in cardiomyopathic cells was associated with an increase in resting $[Ca^{2+}]_i$ and was significantly attenuated when cardiomyopathic myocytes were exposed to the intracellular Ca^{2+} chelator BAPTA/AM. Second, both $[Ca^{2+}]_i$ and prodynorphin mRNA levels were increased in response to KCl-mediated depolarization in control as well as in cardiomyopathic myocytes. Thirdly, in both groups of KCl-treated myocytes, the presence of the Ca^{2+} channel blocker verapamil restored basal $[Ca^{2+}]_i$ and prodynorphin mRNA values. Fourth, the higher $[Ca^{2+}]_i$ observed in cardiomyopathic myocytes compared with control cells following KCl treatment was associated with prodynorphin mRNA levels that were higher than those observed in KCl-treated control cells. The possibility that intracellular Ca^{2+} overload may have increased prodynorphin mRNA expression by acting at the transcriptional level is supported by the results in nuclear run-off experiments, showing that: (i) KCl treatment increased the rate of prodynorphin gene transcription in both control and cardiomyopathic myocytes, (ii) myocyte exposure to verapamil abolished the transcriptional effect of KCl, and (iii) the intracellular Ca^{2+} chelator BAPTA/AM significantly inhibited the increase in prodynorphin gene transcription observed in cardiomyopathic cells.

The observation that both the intracellular and secreted ir-dyn B were increased by KCl treatment in control myocytes and were markedly lowered by the exposure of cardiomyopathic cells to BAPTA/AM indicates that the regulatory effect of $[Ca^{2+}]_i$ on prodynorphin gene expression was associated with changes in prodynorphin mRNA translation into a biologically active end product of the gene and in the subsequent release of the opioid peptide from the myocardial cell. However, BAPTA/AM failed to completely abolish the increase in prodynorphin gene transcription as well as the increase in prodynorphin mRNA and dyn B expression as observed in cardiomyopathic cells, while effective in chelating intracellular Ca^{2+}. These results suggest that other signaling mechanism(s) may also have a role in regulating the expression of the prodynorphin gene through the cardiomyopathy.

As previously shown, PKC activation may contribute to increase prodynorphin gene expression in cardiomyopathic cells. A number of interrelated observations in the present study suggest that intracellular Ca^{2+} overload and PKC activation may represent the two major signaling mechanisms involved in the overexpression of the prodynorphin gene during the cardiomyopathic process. First, the treatment of control myocytes with KCl in the presence of the PKC activator 1,2-dioctanoyl-

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sn-glycerol additively increased both prodynorphin mRNA levels and dyn B expression. Second, neither verapamil nor chelerythrine, nor calphostin C, were able to completely abolish these additive effects. Third, only the association of a Ca^{2+} channel blocker with a selective PKC inhibitor led to a total suppression of the additive responses elicited by KCl and 1,2-dioctanoyl-*sn*-glycerol. The fourth observation is that a combined treatment of cardiomyopathic myocytes with an intracellular Ca^{2+} chelator and a PKC inhibitor completely abolished the increase in prodynorphin gene expression observed in these cells. These findings also suggest that PKC activation and intracellular Ca^{2+} overload might have enhanced prodynorphin gene expression by acting, at least in part, through independent pathways. In this regard, it has been shown that the stimulation of proenkephalin gene expression by Ca^{2+}/calmodulin pathways may occur independently of PKC activation (37) and that intracellular Ca^{2+} loading may affect the expression of a number of opioid and non opioid genes by acting through signaling pathways and/or transcription factors that are partially independent of those involved in protein kinase C- or A-mediated events (38, 39). On the other hand, the finding that myocyte treatment in the presence of PKC inhibitors slightly blunted the KCl-induced increase in prodynorphin mRNA levels seems to indicate that the stimulatory effect elicited on prodynorphin gene expression by a raise in $[Ca^{2+}]_i$ may not be entirely independent of PKC activation. Such a hypothesis appears to be confirmed by the observation that the treatment of control myocytes in the presence of KCl induced the translocation of PKC-α to the nucleus and increased nuclear PKC activity. These results are in agreement with previous studies showing that KCl depolarization stimulated PKC activity and translocation in adult rat ventricular cardiac myocytes (40). Failure of KCl treatment to affect the expression of PKC-δ, -ϵ, or -ζ is also in agreement with the observation that, differently from PKC-α, these isozymes lack the Ca^{2+}-binding C2 domain and may be therefore considered as Ca^{2+}-independent PKC isotypes (41).

The implications of the current experimental results are still difficult to be determined. However, the data presented here may suggest that the alterations in intracellular Ca^{2+} homeostasis described in cardiomyopathic myocytes, by increasing both prodynorphin gene expression and the release of a biologically active opioid peptide, may activate an autocrine pathway eliciting myocardial responses to the interaction of the secreted peptide with κ opioid receptors at the cell surface. In this regard, opioid agonists have been found to deplete of Ca^{2+} an intracellular storage site (16, 19) and to decrease Ca^{2+} current (I_{Ca}) of L-type Ca^{2+} channels in myocardial cells (42). Although these effects produce a decrease in contractility (19, 42), they may also be viewed as a mechanism counteracting the detrimental consequences of intracellular Ca^{2+} overload and proving beneficial during the progression of the cardiomyopathy. Moreover, as we discussed in a companion study (44), the ability of κ opioid receptor stimulation to increase cytosolic pH and therefore to increase the myofilament responsiveness to Ca^{2+} (43) may limit the decrease in twitch amplitude resulting from opioid-mediated effects on Ca^{2+} homeostasis.

The analysis of the adaptive responses of cardiomyopathic myocytes to manipulations of the expression of the prodynorphin gene might be useful to understand the role of this opioid gene in the cardiomyopathic process and is the subject of further investigations.

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