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Dynorphin Gene Expression and Release in the Myocardial Cell*

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The expression of the prodynorphin gene was investigated in adult cultured rat ventricular cardiac myocytes by using a sensitive solution hybridization RNase protection assay for the quantitative analysis of prodynorphin mRNA. Myocyte culture in high KCl resulted, after 4 h, in a marked increase in cellular prodynorphin mRNA, while a KCl treatment for 6, 12, or 24 h progressively down-regulated the levels of prodynorphin mRNA below the control value. Immunoreactive dynorphin B, a biologically active end product of the precursor, was found to be present in the culture medium in significantly higher amounts than in the cardiac myocytes. The levels of this biologically active K opioid receptor agonist significantly increased after 4 h of KCl treatment and were markedly reduced following a 24-h exposure of the cardiac myocytes to KCl. These KCl-induced effects were all abolished by cell incubation in the presence of the calcium channel blocker verapamil. In single cardiac myocytes, acute stimulation of K opioid receptors with dynorphin B or with the selective agonist U-50,488H increased the level of cytosolic calcium. This effect was abolished by the specific K opioid receptor antagonist (Mr-1452) and was not affected by the removal of calcium from the bathing medium. These results suggest that an opioid gene may influence the myocardial function in an autocrine or paracrine fashion.

Opioid peptides have long been known to control the cardiac function through reflex mechanisms at the level of the central or peripheral nervous system (1, 2). However, opioid receptors have been identified in rat ventricular sarcolemma (3), indicating that opioid peptides may also elicit direct effects in the myocardial cell. We have recently shown that cardiac myocytes respond to the stimulation of K opioid receptors with a sustained increase in the formation of Inositol (1,4,5)P_3 and inositol (1,3,4)P_4 (4) and with a protein kinase C-dependent enhancement of the Na^+/H^+ antiporter activity (5). These effects modified both calcium and pH cytosolic homeostasis and were associated to remarkable changes in the contractility of the myocardial cell (5, 6). Furthermore, prodynorphin-derived peptides have been reported to be present in the atria and in the ventricles of guinea pig and rat hearts (7, 8) as well as in human atria (8). However, these studies were performed in multicellular preparations and could not discriminate on the cell type accounting for the opioid synthesis. We have recently provided evidence that the prodynorphin mRNA is synthesized in rat ventricular cardiac myocytes in amounts comparable to those observed in the whole ventricle (9). This suggests that the myocardial cell may represent an important source for prodynorphin-derived peptides in the ventricular tissue.

In the present study, we investigated the effects of KCl-induced cell calcium loading on the expression of prodynorphin gene as well as on the release of immunoreactive dynorphin B (ir-dyn B),[1] a biologically active end product which acts as a selective K agonist (10), in cultured ventricular myocytes from adult rats. We also described, in single cardiac myocytes, the effects elicited on the cytosolic calcium level by the acute stimulation of K opioid receptors. The possibility that an opioid gene may play an autocrine or paracrine role in the regulation of the myocardial function is discussed.

MATERIALS AND METHODS

Isolation of Cardiac Myocytes—Hearts from 2–3-month-old male WISTAR rats were retrogradely perfused through the aorta in the presence of a bicarbonate buffer (pH 7.4) containing low calcium and collagenase (11). Following the isolation procedure, the adult cultured cardiac myocytes were subjected to a short-term serum-free primary culture, according to the method described by Piper *et al.* (12). For peptide and mRNA studies, the cardiac myocytes were cultured, under 5% CO_2 atmosphere at 37 °C, in Petri dishes (60-mm Falcon dishes, Becton Dickinson) at a density of 2×10^5 cells/dish in 3 ml of M-199 medium (with Earls salts), containing 0.2% bovine serum albumin, 10^{-8} M insulin, 2.5×10^{-4} M penicillin G, 2.5×10^{-4} M streptomycin, and 10^{-5} M cytosine arabinofuranoside. One day after plating, cells were treated with 60 mM KCl, in the absence or presence of 2 μM verapamil.

RNA Extraction and Determination of Prodynorphin mRNA—Total RNA was isolated from the adult cultured cardiac myocytes by using the method of Chomczynsky and Sacchi (13). The levels of prodynorphin mRNA were determined, as previously described (9), by using a sensitive solution hybridization RNase protection assay (14, 15).

Identification of Dynorphin B-like Material—Ir-dyn B was measured by a radioimmunoassay procedure, by using the antiserum "13 S" raised against dynorphin B and capable of recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dynorphin B in their sequence (16, 17). Acetic acid extracts from cardiac myocytes or pooled samples from the culture medium were processed by reverse-phase high performance liquid chromatography (rp-HPLC), followed by the radioimmunoassay analysis of the collected fractions, according to a previously described method (18). Immunoreactivity was attributed to authentic dynorphin B by comparison to the elution position of a synthetic standard.

Estimation of Cytosolic Calcium Level in Single Myocardial Cells—Dissociated cardiac myocytes, bathed in HEPES-buffered medium, were loaded at 23 °C with the ester derivative (AM form) of the Ca^{2+} probe indo-1 (19), dissolved in dimethyl sulfoxide and mixed with fetal calf serum and a dispersing agent (Pluronic F-12, BASF Wyandotte, Wyandotte, MI), according to a previously described procedure (20).

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[1] The abbreviations used are: ir-dyn B, immunoreactive dynorphin B; rp-HPLC, reverse-phase high performance liquid chromatography.

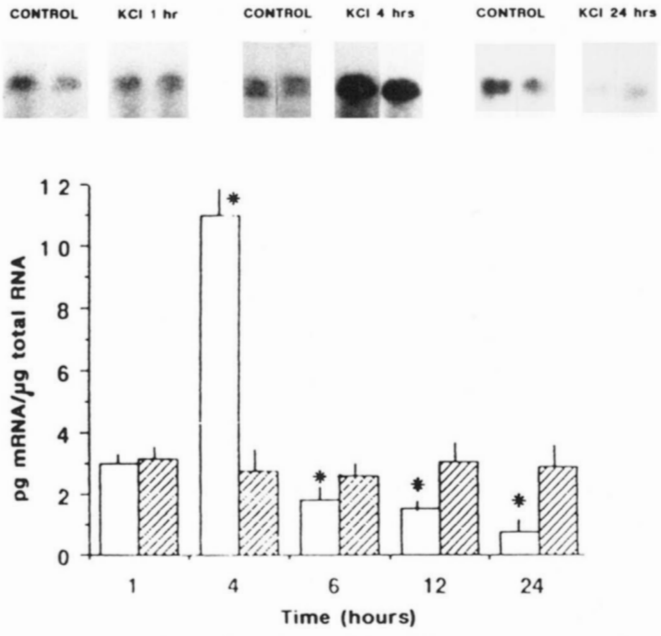

FIG. 1. **Regulation of prodynorphin mRNA expression by KCl in adult cultured rat ventricular myocytes.** The *upper panel* shows representative autoradiograms of the ribonuclease protection analysis of myocardial prodynorphin mRNA in the absence (*CONTROL*) or presence of 60 mM KCl. Exposure was for 2 days on Kodak X-Omat film with an intensifying screen. The *lower panel* reports the time course of the effect of KCl on the expression of prodynorphin mRNA in cultured myocytes. ▨, control; □, KCl. The data are expressed as mean value ± S.E. (n = 6). *, significantly different from the control value.

Cytosolic calcium was monitored in single, indo-1-loaded, adult rat ventricular cardiac myocytes by using a videomicroscopic technique described in detail elsewhere (6, 21). Briefly, indo-1 fluorescence from an individual cardiac myocyte on the stage of an inverted microscope was excited by epi-illumination with 10-μs flashes of 350 ± 5 nm of light. Paired photomultipliers collected indo-1 emission by simultaneously measuring spectral windows of 391–434 and 457–507 nm selected by bandpass interference filters. The ratio of indo-1 emission at the two wavelengths was calculated as an index of cytosolic [Ca^{2+}], by using a pair of fast integrator sample-and-hold circuits under the control of a VAX 11/730 computer.

Acute Stimulation of Myocardial K Opioid Receptors—Dynorphin B or the selective K opioid receptor agonist U-50,488H were rapidly "puffed" from a micropipette positioned directly above a single resting myocyte (the concentrations of dynorphin B or U-50,488H in the pipette were 10 or 100 μM, respectively). Pressure pulses of 20 p.s.i. and 2 s in duration were applied to the pipette with a picospritzer II (General Valve Corp., Fairfield, N.J.).

Data Analysis—The statistical analysis of the data was performed by using the unpaired Student's t-test, assuming a p value less than 0.05 as the limit of significance.

RESULTS AND DISCUSSION

RNA was extracted from adult cultured rat ventricular cardiac myocytes and then subjected to a solution hybridization RNase protection analysis, using a synthetic ^{32}P-antisense probe for detection. The quantity of prodynorphin mRNA derived under basal conditions from the myocardial cells was 3.2 ± 0.4 pg/μg total RNA (mean ± S.E., n = 6) (Fig. 1). The expression of prodynorphin mRNA was found to be modulated by myocyte incubation in the presence of 60 mM KCl. A 4-h exposure to KCl increased the level of prodynorphin mRNA up to 11.0 ± 0.86 pg/μg total RNA (mean ± S.E., n = 6), while incubation of the myocytes with KCl for 6, 12, or 24 h progressively down-regulated prodynorphin mRNA levels below the control value (Fig. 1). On the contrary, exposure of the myocardial cells to KCl for 1 h did not affect significantly the level of prodynorphin mRNA (Fig. 1). The fact that the incubation with KCl did not evoke an increase in the activity of creatine phosphokinase in the medium and did not affect the amount of total RNA extracted from the myocytes (data not shown) indicates that these cells were not damaged throughout the treatment. In the presence of the calcium channel blocker verapamil (2 μM), the levels of prodynorphin mRNA following cell incubation with 60 mM KCl for 4 or 24 h were 3.35 ± 0.50 pg/μg total RNA (mean ± S.E., n = 6) and 2.94 ± 0.33 pg/μg total RNA (mean ± S.E., n = 6), respectively, and resulted not significantly different from the control value. Therefore, the KCl-induced effects, which may reflect changes in mRNA transcription and/or stability, appear to be mediated by an increase in the intracellular calcium level. In this regard, sustained membrane depolarization with KCl and cell calcium loading have been shown to modulate opioid gene expression in neuronal cells (22–25) and the expression of G proteins and protooncogenes in both neuronal and myocardial cells (26, 27).

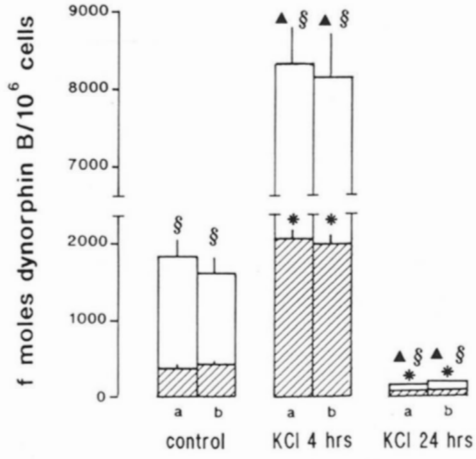

FIG. 2. **Regulation of ir-dyn B levels by KCl.** Adult cultured rat ventricular myocytes were treated for 4 or 24 h with 60 mM KCl. ▨, ir-dyn B in cultured cardiomyocytes; □, ir-dyn B in the medium. Each single value in the medium was calculated in a final volume of 15 ml, corresponding to the volume of pooled samples of the culture medium from 10^6 cells. Data were obtained in the absence (a) or presence (b) of a peptidase inhibitor mixture containing 20 μM bestatin, 0.3 μM thiorphan, 1 mM leucyl-L-leucine, 3 μM poly-L-lysine, 15 μM captopril, 30 μM 1–10-phenanthroline, 6 μM 1,4-dithiothreitol. The data are expressed as mean values ± S.E. (n = 6). *, significantly different from its own control value; ▲, significantly different from its own control value; §, □ significantly different from ▨.

We evaluated the presence of dynorphin B-like material both in the myocytes and in the culture medium as an index of prodynorphin mRNA translation into a biologically active end product. We found that the medium from cardiac myocytes cultured under basal conditions contained a molecular form with the same retention time as authentic dynorphin B; furthermore, rp-HPLC analysis of the medium from myocytes cultured for 4 h in the presence of 60 mM KCl revealed a significant increase in the level of ir-dyn B (Fig. 2). On the contrary, cell culture with KCl for 24 h resulted in a marked decrease in the amount of the ir-dyn B detected in the medium (Fig. 2). The incubation of the cardiomyocytes with a peptidase inhibitor mixture did not affect significantly the levels of ir-dyn B in the medium, both in the absence or presence of KCl (Fig. 2). This indicates the relative stability of the opioid peptide in this culture model, an issue which is further supported by the fact that incubation of the myocytes in the presence of ^{125}I-dynorphin B for 4 or 24 h did not affect the elution position of this radiolabeled standard throughout the rp-HPLC (data not shown). Consistent amounts of ir-dyn B were also found in acetic acid extracts from cardiac myocytes (Fig. 2). Cell treatment with 60 mM KCl for 4 h elicited a marked increase in the myocardial level of ir-dyn B, which was conversely reduced to

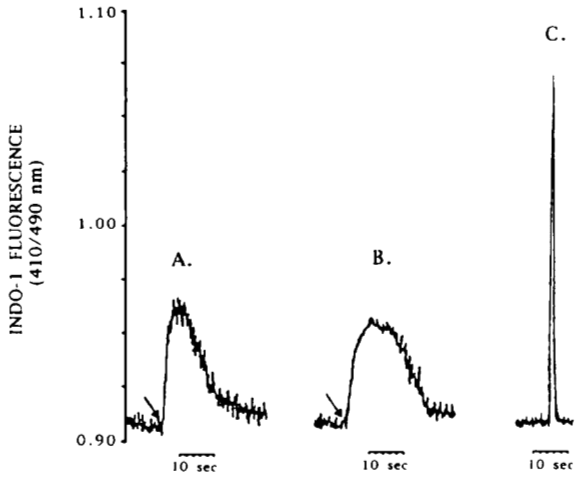

FIG. 3. **Effect of K opioid receptor stimulation on cytosolic calcium level in single rat ventricular cardiac myocytes.** The figure shows representative tracings of the changes in cytosolic Ca^{2+} that occur when the selective K opioid receptor agonist U-50,488H (*A*) or dynorphin B (*B*) are rapidly released (*arrow*) on a single resting rat cardiac myocyte superfused with a buffer of the following composition (mM): NaCl, 137; $MgSO_4$, 1.2; KCl, 5; HEPES, 20; D-glucose, 15 (pH 7.4). Extracellular Ca^{2+} was 1.0 mM. *C* shows, for comparison, an electrically driven cytosolic Ca^{2+} transient which was elicited in the same cell by field stimulation via two platinum electrodes placed in the bathing fluid (Grass stimulator, model SD9, Grass Instrument Co., Quincy, MA).

less than 20% of the control value following a 24-h exposure to KCl (Fig. 2). However, the cellular levels of ir-dyn B were significantly lower than those detected in the medium. This suggests that, in the ventricular myocardial cell, which lacks secretory granules (28), the prodynorphin-derived peptides may not be targeted for storage but may be constitutively released shortly after synthesis.

Acute release of dynorphin B or of the selective K opioid receptor agonist U-50,488H (29) over single cardiac myocytes (see "Materials and Methods") similarly increased the intracellular calcium level (Fig. 3). The opioid effect was abolished by myocyte superfusion in the presence of 1 μM Mr-1452, a specific receptor antagonist (30), and was still observed in the presence of 0 Ca^{2+} and EGTA (0.1 mM) in the bathing medium (data not shown), indicating that the opioid action was specific in nature and that calcium was released from an intracellular storage site. It may be of interest that these effects were obtained following a direct local exposure to the opioid agonists, as it might occur when biologically active dynorphin-related peptides are released from the nerve endings supplying the heart muscle (31, 32) or from the cardiac myocyte itself.

These results and the finding that a biologically active prodynorphin-derived peptide is synthesized and released in cardiac myocytes suggest that the myocardial expression of the prodynorphin gene might be involved in the regulation of the cardiovascular function through an autocrine or paracrine mechanism.

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