Preproenkephalin mRNA expression in developing rat heart and in cultured ventricular cardiac muscle cells

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Heart muscle tissue has previously been reported to have the highest content of preproenkephalin (ppEnk) mRNA of any tissue in the adult rat. We have determined that it is present in the ventricular cardiac muscle cells of the heart and is developmentally regulated. The expression of ppEnk mRNA was observed to be low throughout the first 2 weeks of postnatal development and decreases substantially during week 3. Expression was again low by week 4, but by adulthood (approx. 3 months), it reached a maximum. ppEnk mRNA was actively expressed in primary cardiac muscle cell cultures prepared from both neonatal and adult rats. Its steady-state content in cell cultures was observed to be increased by cyclic AMP and 3-isobutyl-1-methylxanthine. The phorbol ester phorbol 12-myristate 13-acetate elicited a transient effect (i.e. an increase was observed at 4 h and a return to control values by 24 h). We speculate that enkephalin may play a multi-functional role in the differentiation of neonatal cardiac muscle cells and in the terminally differentiated adult heart cell. We demonstrate that the primary culture systems employed in this study will be useful models with which to explore both transcriptional and translational regulation of ppEnk mRNA in the heart.

INTRODUCTION

Early cardiac muscle cell differentiation is an intricate process involving simultaneous cell proliferation and cytodifferentiation. It was shown previously that, during week 3 of postnatal development, cardiac muscle cells of the rat cease DNA replication and cell division, and subsequent growth of the muscle tissue is due exclusively to cell enlargement [1–4]. Throughout this developmental period, significant morphological and biochemical alterations occur within the myocytes. Expression of cell-proliferation genes is inactivated, whereas that of muscle-specific differentiation genes is activated. Besides alterations in gene expression, several other events also occur. These include functional adrenergic innervation of the tissue and development-dependent increases in the concentrations of noradrenaline and cyclic AMP [5].

Since Hughes *et al.* discovered [Met⁵]- and [Leu⁵]-enkephalin in 1975 [6], much experimental work has been carried out on the function and mechanism of action of these small opioid peptides. Preproenkephalin (ppEnk) mRNA, which codes for these peptides, has been shown to have a wide range of tissue distribution. Very recently Howells *et al.* [7] reported that ppEnk mRNA is present in rat ventricular heart tissue. Although the peptide contents were reported to be very low, this tissue was found to contain the highest amount of ppEnk mRNA of any tissue in the rat.

The studies reported here were undertaken: (i) to determine if ppEnk mRNA is present in the muscle cells of the heart; (ii) to determine the extent of ppEnk mRNA expression in developing ventricular cardiac muscle (VCM) tissue; and (iii) to study the regulation of ppEnk mRNA expression in purified VCM cell cultures.

MATERIALS AND METHODS

Isolation of VCM tissue during development

Timed-pregnant Sprague–Dawley rats were obtained from Holtzman (Madison, WI, U.S.A.) on day 14 of gestation. On day 18 of gestation, two animals were killed by a lethal injection of sodium pentobarbital, and the hearts were rapidly removed from the fetuses. The atria and large vessels were carefully trimmed away, and the ventricular tissue was washed in phosphate-buffered saline [11] and flash-frozen in liquid N₂. Neonatal rats were raised in litters of ten. Two litters were used for both fetal and neonatal time points. The rats were killed by decapitation for the first four neonatal time points and by cervical dislocation for the last 15 time points. The ventricular tissue was collected and flash-frozen as described above.

Isolation and culture of neonatal and adult VCM cells

Neonatal-rat VCM cells were isolated by a modification of the technique of Kasten [8]. Briefly, VCM tissue was removed aseptically from 3-day-old neonatal rats, minced, and then digested with 0.125% trypsin in Hanks balanced salt solution overnight at 4 °C. Neonatal VCM cells were then recovered by repeated digestions of the tissue in 10 ml of 0.1% collagenase in phosphate-buffered saline at 37 °C in a shaking water bath. The cells were gently pelleted by centrifugation at 2000 rev./min for 3 min, and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum. All samples were pooled, and the cells were differentially plated [9] on Corning T75 flasks for 2 h to enrich for myocytes and decrease contamination by non-muscle

Abbreviations used: VCM, ventricular cardiac muscle; ppEnk, preproenkephalin; DBcAMP, N⁶,2'-O-dibutyryl cyclic AMP; BrcAMP, 8'-bromo cyclic AMP; PMA, phorbol 12-myristate 13-acetate; IBMX, 3-isobutyl-1-methylxanthine.

cells. VCM cells were then either dissolved in 4 M-guanidinium solution (4 M-guanidinium isothiocyanate, 5 mM-sodium citrate, pH 7.0, 0.1 M- β -mercaptoethanol, 0.5% sodium lauryl sarcosinate) for RNA isolation, or cultured in T75 flasks in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml). The culture media were changed at the end of 24 h, and 24 h later cells were used for the outlined experiments.

Adult VCM cells were isolated from female Sprague-Dawley rats (200–250 g) exactly as previously described [10,11]. Freshly isolated cells were either dissolved in 4 M-guanidinium for total RNA isolation or plated on collagen-coated T75 flasks and cultured exactly as described by Claycomb & Lanson [10]. Non-muscle-cell contamination was eliminated by addition of 10 μ M-cytosine 1- β -D-arabinoside for 7 days [11]. On day 7 of culture the cells were grown in cytosine arabinoside-free medium and fed every other day. On day 12 of culture the cells were used for the described experiments.

Five flasks of neonatal cells and three flasks of adult cells were used for each RNA isolation point. After exposure to the various agents for the indicated time period, each flask was washed once with 3 ml of phosphate-buffered saline and allowed to drain briefly. The cells were solubilized for RNA isolation by the addition of 4 M-guanidinium solution to the flasks.

RNA isolation and gel electrophoresis

Total RNA was isolated by the guanidinium isothiocyanate/CsCl method essentially as described by Chirgwin *et al.* [12] and Maniatis *et al.* [13]. The frozen tissue was removed from -70°C and immediately placed in the guanidinium solution. While thawing, the tissue was homogenized with a Brinkman Polytron. Then 1 g of CsCl was added per 2.5 ml of guanidinium homogenate, and the mixture was layered over a 1.2 ml cushion of 5.7 M-CsCl/0.1 M-EDTA, pH 7.5, in Beckman SW50.1 centrifuge tubes and centrifuged at 110000 *g* for 15 h at 20°C . After careful removal of the supernatant, the insides of the tubes were wiped dry and the RNA pellet was resuspended in RNA buffer (10 mM-Tris, pH 7.4, 5 mM-EDTA, 1% SDS). RNA was extracted with an equal volume of chloroform [containing 4% (v/v) 3-methylbutan-1-ol]/butan-1-ol (4:1, v/v). The aqueous phase was recovered, and the organic phase was re-extracted with an equal volume of RNA buffer. The two aqueous extracts were pooled and precipitated with 0.1 vol. of 3 M-sodium acetate and 2.2 vol. of 95% (v/v) ethanol. The purity and quantity of the isolated RNA were determined by reading the A_{260} and A_{280} . The samples were stored at -70°C .

RNA samples were electrophoresed on a denaturing 1.2%-agarose/2.2 M-formaldehyde gel, stained with ethidium bromide, transferred to Zeta-Probe nylon membrane (Bio-Rad), and baked under vacuum for 2 h at 80°C . The Zeta-Probe membrane was photographed on a transilluminator to assess the transfer and integrity of the RNA before hybridization. The membranes were then prehybridized in a solution consisting of 50% (v/v) formamide, 1 mM-EDTA, 7% SDS, 0.25 M- NaH_2PO_4 , pH 7.0, 0.25 M-NaCl and 0.1 mg of single-stranded herring testes DNA/ml for 12 h at 42°C . Hybridizations were carried out at 42°C for 48 h. The membranes were washed three times at 62°C in solution I (1 mM-EDTA, 40 mM- NaH_2PO_4 , pH 7.0, and 5% SDS) and three times

at 62°C in solution II (1 mM-EDTA, 40 mM- NaH_2PO_4 , pH 7.0, and 1% SDS) as described by Church & Gilbert [14]. Autoradiography was performed with Kodak X-Omat RP film and DuPont Lightning-Plus intensifying screens at -70°C for 1–7 days.

Plasmids used for hybridization analysis

The following DNA clones were used for probes: pRPE2, a 938-base-pair rat ppEnk cDNA, was given by Steve Sabol [15]; pF0108A, a human histone-H4 gene clone, was given by Gary Stein [16]; and TKcDNA, a 1200-base-pair mouse thymidine kinase cDNA, was given by Reinhold Hofbauer [17]. All the above inserts (except for TK) were cut out of the plasmid vector by using restriction endonucleases and purified by electrophoresis on a 1.0%-agarose gel. The insert-containing band was excised from the gel, electroeluted from agarose, and purified on a Nacs Prepac column (BRL). The DNA was nick-translated by using [α - ^{32}P]dATP (New England Nuclear, sp. radioactivity > 400 Ci/mmol) to a specific radioactivity of $(1-2) \times 10^8$ c.p.m./ μ g.

Densitometry

Densitometry was performed by using Biomed Instruments (Fullerton, CA, U.S.A.) scanner software. Changes (fold) in ppEnk mRNA amounts were calculated by normalizing the enkephalin signal against a constitutively expressed mRNA (mRNA coding for the ribosomal protein L7). The resulting values were then used to determine relative changes (fold) between the various developmental time points.

RESULTS

In 1986 Howells *et al.* [7] reported that ppEnk mRNA was expressed in rat heart muscle tissue in very high amounts. We asked whether ppEnk mRNA was being expressed in the cardiac muscle cells or in the non-muscle cells of the heart. Cardiac muscle tissue of the adult rat is very heterogeneous as to cell type. Adult muscle cells are binucleated and comprise 75–80% of the heart's mass, yet are only 20–25% of the total cell population [18,19]. We thought it possible that the ppEnk mRNA observed in the intact heart could be due to neurons associated with this tissue. Total RNA was isolated from purified populations of freshly isolated 3-day-old neonatal (Fig. 1, lane 1) and adult (Fig. 1, lane 2) VCM cells, and from intact tissue of these same-aged rats (Fig. 1, lanes 3 and 4). The expression of ppEnk mRNA was observed to be equivalent in the cells and the tissue, establishing that the muscle cells were the source of this mRNA. One band that was detected by autoradiography coincided with an mRNA of approx. 1500 bases. This has previously been reported to be the size of ppEnk mRNA in rat heart tissue, by both cDNA- and oligonucleotide-directed hybridization analysis [7]. This experiment also indicated to us that ppEnk mRNA expression was developmentally regulated. We expanded the study to include 20 time points to clarify the pattern. The integrity and quantity of the RNA in each lane was evaluated by the ethidium bromide staining of the RNA transferred to the nylon membrane (Fig. 2). ppEnk mRNA expression was low from 18 days fetal age until day 10 of postnatal development. During week 3 after birth (days 14–21) there was a 5-fold decrease in the

Fig. 1. Northern-blot analysis of ppEnk mRNA expression in neonatal- and adult-rat freshly isolated VCM cells and whole tissue

Total RNA was isolated and electrophoresed through a denaturing formaldehyde/agarose gel and transferred by capillary action to the nylon membrane Zeta-Probe. The size-fractionated RNAs were hybridized with a ^{32}P -labelled preproenkephalin-cDNA insert and autoradiographed (exposure 2 days). Lanes: total RNA (15 μg /lane) from (1) freshly isolated neonatal ventricular cardiac myocytes, (2) freshly isolated adult ventricular cardiac myocytes, (3) neonatal ventricular cardiac tissue and (4) adult ventricular cardiac tissue. The size of the transcripts were determined relative to 18 S and 28 S RNA, which are assumed to be 1.87 and 4.72 kb (kilobases) respectively.

hybridization intensity, and it remained at this value until day 21 of development. During week 4, approx. 21 days after birth, there was a 5-fold increase in ppEnk mRNA hybridization intensity, and by day 50 *post partum* there was another 3-fold increase. This hybridization intensity reaches a maximum in the adult rat (90 days), approx. 50-fold higher than the 4-day-neonatal time point.

We have isolated RNA from each time point in the developing rat four times, and have consistently seen a bimodal developmental pattern for the expression of ppEnk mRNA. Using histone H4 and TK as probes, we have re-affirmed that cell proliferation and DNA replication essentially cease in the rat during week 3 of postnatal development [1–4] (Fig. 2). The low extent of expression of histone H4 after day 16 is due to an mRNA subspecies of this histone, which is independent of DNA synthesis [20].

We used cardiac-muscle cell-culture systems to study the regulation of ppEnk mRNA. We used several agents on the neonatal VCM cultures: (1) phorbol 12-myristate

13-acetate (PMA); (2) both 8'-bromo (Br) and dibutyryl (DB) cyclic AMP; and (3) 3-isobutyl-1-methylxanthine (IBMX). We chose these agents to determine if the second-messenger system of cyclic AMP-dependent protein kinase or the protein kinase C pathways could influence the pattern of expression of ppEnk mRNA. Endogenous stores of cyclic AMP have previously been shown to increase during late development of cardiac tissue. Therefore we wished to determine if the amount of ppEnk mRNA could be influenced by this agent in a primary culture. Each agent was added to the culture for 24 h, and then total cellular RNA was isolated and analysed. We observed that both DBcAMP and BrcAMP increased the enkephalin mRNA in the neonatal VCM cells (Fig. 3, and results not shown).

Comb *et al.* previously reported [21] that PMA induces the expression of a proenkephalin-chloramphenicol acetyltransferase fusion gene when transiently expressed in CV-1 cells in culture. They also noted that PMA induction of this fusion gene could be augmented if PMA was added along with the cyclic-AMP-dependent phosphodiesterase inhibitor IBMX. We repeated this experiment with our primary heart cell cultures. Neonatal VCM cultures were exposed to IBMX (0.1 mM), IBMX (1 mM), PMA (100 ng/ml), IBMX (0.1 mM) + PMA (100 ng/ml), and IBMX (1 mM) + PMA (100 ng/ml). One set of PMA-containing flasks was treated for 4 h, and all the other flasks were treated for 24 h. A transient increase in ppEnk mRNA by PMA was observed. At 4 h PMA elicited a response, whereas at 24 h a decrease in ppEnk mRNA amounts below control was observed (Fig. 3a, lanes 4 and 5). IBMX (1 mM) alone increased the steady-state amounts of ppEnk mRNA (Fig. 3a, lane 2). However, at a lower concentration (0.1 mM), IBMX had little effect (Fig. 3a, lane 7). When IBMX (0.1 mM) was added with PMA, a synergistic effect was seen (Fig. 3a, lane 8). Other experiments (Fig. 3b) show that both BrcAMP and PMA also stimulate the expression of ppEnk mRNA in terminally differentiated adult VCM cultures. PMA also was observed to have a transient effect in these adult myocyte cultures.

DISCUSSION

The work presented here demonstrates three findings: (i) ppEnk mRNA is present within the ventricular muscle cells of the rat heart; (ii) ppEnk mRNA expression is developmentally regulated; and (iii) ppEnk mRNA is expressed in VCM cultures, and its steady-state amount can be altered by cyclic AMP, IBMX and PMA. It is important to note that different processes may account for the change in the steady-state amount of ppEnk mRNA. These include changes in mRNA transcription and/or stability.

Howells *et al.* [7] have shown that adult rat heart contains greater amounts of ppEnk mRNA than does any other tissue in the body. However, the enkephalin peptide content in the heart is only 3% of that in brain [7]. It is possible that VCM, being devoid of secretory granules [22], does not have the ability to store the enkephalin peptide, but may store its mRNA for translation at the appropriate time. This could explain the high content of ppEnk mRNA and the lack of enkephalin-containing peptide in the heart muscle cell. Several other processes could also explain these observations: (i) ppEnk mRNA may be translated

Fig. 2. Composite Northern-blot analysis of the developmental expression of mRNA for preproenkephalin (ppEnk), histone H4 (H4) and thymidine kinase (TK)

The RNA was isolated from VCM tissue of 20 developmental time points. Total RNA (12 μ g/lane) was electrophoresed on a denaturing formaldehyde/agarose gel and transferred to nylon membranes. The ethidium bromide staining pattern of the transferred RNA is shown in the upper part of the composite. The size-fractionated RNAs were hybridized with the indicated ^{32} P-labelled cDNA inserts and autoradiographed (exposed for 1 day, except for thymidine kinase, which required 5 days).

Fig. 3. Northern-blot analysis of (a) neonatal and (b) adult VCM cell cultures

Cells were isolated and cultured as described in the text for 24 h before the following additions. (a) Neonatal: lane 1, nothing (control); lane 2, 1 mM-IBMX; lane 3, 1 mM-BrcAMP; lane 4, PMA (100 ng/ml) (4 h culture period); lane 5, PMA (100 ng/ml); lane 6, 1 mM-IBMX + PMA (100 ng/ml); lane 7, 0.1 mM-IBMX; lane 8, 0.1 mM-IBMX + PMA (100 ng/ml). (b) Adult: lane 1, nothing (control); lane 2, BrcAMP; lane 3, PMA (100 ng/ml) (4 h culture period); lane 4, PMA (100 ng/ml). The cells were cultured for an additional 24 h (except where indicated for 4 h) in the presence of each of the above agents. Total RNA was isolated, analysed (15 μ g/lane), and autoradiographed.

constitutively and immediately degraded or secreted from the cardiac myocyte; (ii) ppEnk mRNA may be stored as mRNA and translated into protein only when needed; or (iii) ppEnk mRNA is not translated at all, and its high content in the heart is a biological artifact.

The observed developmental expression pattern of heart ppEnk mRNA is interesting. It has previously been shown that intracellular contents of cyclic AMP and nor-adrenaline, and adenylate cyclase activity, increase progressively during late fetal and early neonatal

development, indicating functional adrenergic innervation of this tissue [2–5,23–27]. We have shown that ppEnk mRNA expression in neonatal VCM cells is very responsive to cyclic AMP. Therefore, functional innervation and/or developmentally regulated hormones that modulate cyclic AMP concentrations could control the biosynthesis of specific mRNAs such as that for enkephalin, in a developmental, differentiation-specific, manner.

It has been shown in several tissues that endogenous opioids act on opiate receptors in such a way as to inhibit cell proliferation and promote cell differentiation [28,29]. The simultaneous decline in ppEnk mRNA and the switch from hyperplastic growth to hypertrophic growth during week 3 of development [3,5] leads us to two inferences: (i) enkephalin may act to inhibit cell proliferation and promote cell differentiation in cardiac tissue in an autocrine or paracrine fashion, or (ii) its decline could be the result of the reorganization/reprogramming of the cellular genome when hyperplastic growth switches to hypertrophic growth.

The substantial increase in ppEnk mRNA from day 30 to adulthood may indicate an additional function for this peptide in heart muscle. Several studies have attempted to correlate the effect of enkephalin on cardiovascular function in the adult animal. No clear-cut result has yet emerged. Completely opposing effects in laboratory animals are observed by different investigators (i.e. bradycardia or tachycardia, and hypotension or hypertension), depending on its manner of administration [30,31]. It seems quite plausible that enkephalin may be produced by the adult VCM tissue to influence the physiological performance of the heart. Although it is evident that cardiovascular function can be altered by endogenous opioids [28–31], the question still remains: does the heart influence its own function by producing these opioids? We believe that the cardiac muscle cell cultures used in the present study will be a useful model system with which to answer this question.

The transient stimulation effect on ppEnk mRNA amounts by PMA would seem to be very similar to the transient induction of *c-fos* in other systems [32–34]. The biological effects of PMA are thought to be mediated by protein kinase C [35,36]. It would appear that the synergistic effect observed when the VCM cells are cultured with both PMA and IBMX could be explained by a prolonged increase in intracellular cyclic AMP. This result could indicate that the two major second-messenger systems, protein kinase C and cyclic AMP-dependent protein kinase, may somehow be interacting [35–38] to influence the expression of ppEnk mRNA in rat heart.

In conclusion, enkephalin could play several roles in cardiac tissue. During early development it may promote cellular differentiation and the switch from hyperplastic to hypertrophic growth. During adulthood it may be acting as a neurotransmitter to influence cardiovascular function and/or in pain attenuation after myocardial injury, i.e. silent heart attack.

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