Weil, Joachim, Thomas Eschenhagen, Gerrit Fleige, Clemens Mittmann, Ellen Orthey, and Hasso Scholz. Localization of preproenkephalin mRNA in rat heart: selective gene expression in left ventricular myocardium. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H378–H384, 1998.—The enkephalins are derived from a common precursor protein known as preproenkephalin (ppENK). Enkephalins appear to be one of the endogenous ligands for the opiate receptors. In the rat the ventricular myocardium contains more ppENK mRNA than any other tissue. To gain further insight into the role of cardiac enkephalins, the regional and developmental distribution of ppENK mRNA was studied by Northern blotting and in situ hybridization. In the early postnatal period, ppENK mRNA is low in atrial and ventricular myocardium. With maturation, ppENK expression increases threefold in left and right ventricular tissue, but not in the atria or cardiac conductive system. Interestingly, ppENK mRNA levels are four times higher in the left than in the right chamber. Thus, to our knowledge, ppENK is the only gene exhibiting marked differences in expression between the adult right and left ventricles. Given the left-side preference of ppENK expression, the possibility is raised that the left ventricle is an endocrine organ that supplies the body with enkephalins.

MATERIALS AND METHODS

Processing of tissue. Wistar rats of different ages (3 days and 4, 8, and 14 wk) were used for the experiments. Neonatal rats were of either sex; all other animals were male. Animals were killed by decapitation. Hearts were rapidly removed, washed free of blood in ice-cold 0.9% NaCl, and weighed. For in situ hybridization, some hearts were filled with Tissue Tek (Miles, Elkhart, IN) and frozen in n-hexane precooled on dry ice. The tissue was mounted on microtome specimen holders with the use of Tissue Tek and stored at −80°C until further processing. For RNA preparation the excised hearts were dissected into atria, left and right ventricles, and septum and frozen in liquid nitrogen.

Preparation of RNA and Northern blot analysis. Total RNA was extracted with the commercially available kit RNAzol (Biotecx Laboratories, Houston, TX) according to the manufacturer’s instructions. Briefly, 100–150 mg of frozen atrial or ventricular myocardium or 3 × 10^6 isolated cardiomyocytes or noncardiomyocytes were transferred to 600 µl of RNAzol solution and homogenized with a Polytron (Kinematica, Littau, Switzerland), extracted with phenol-chloroform, and precipitated with isopropanol, and precipitated RNA was washed with ethanol (75%). RNA was solubilized in sterile
and pyrogen-free water. The concentration was determined photometrically at 260 nm. RNA was stored at −80°C. RNA blotting, cDNA labeling, hybridization, and quantification were performed essentially as described previously (34). Total RNA (5–10 µg) from atrial or ventricular myocardium was separated by electrophoresis on 1% agarose-formaldehyde gels and transferred to nylon membranes (Schleicher and Schuell). Plasmid with the cDNA insert of the rat ppENK gene was a kind gift from Dr. M. Boluyt (National Institutes of Health, Bethesda, MD). An SmaI/SacI fragment (935 bp) was used for Northern blot hybridization. To correct measurements for minor loading differences, all membranes were rehybridized with a32P-labeled cDNA coding for the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Blots were washed at a final stringency of 0.2× standard saline citrate-0.1% SDS at 65°C, exposed on imaging plates (model BAS-IP MP 2040 P, Fuji) for 24 h or X-ray films for 3 days, and scanned by a phosphoimager (model BAS 2000, Fuji). Hybridization signals were quantified using TINA 2.0 (Raytest) and Zerodescan (CSP).

In situ hybridization. A BamHI/EcoRI cDNA fragment (944 bp) containing the full-length insert of ppENK was subcloned into pBluescript SK (Stratagene, La Jolla, CA). Transformation in Escherichia coli and plasmid preparation were performed by standard molecular biology methods (28). Sense and antisense cRNA for ppENK were transcribed in vitro and radiolabeled with35S-UTP as previously described (7). cRNA probes were digested to shorter fragments (100–200 nucleotides) by mild alkaline hydrolysis. Cryosections of whole hearts were used for experiments. Prehybridization procedures consisted of 4% paraformaldehyde fixation followed by protein kinase K (20 µg/ml Tris-EDTA buffer; Boehringer Mannheim) digestion for 7 min. Sections were postfixed with 4% paraformaldehyde, washed twice with PBS and dehydrated in a graded series of alcohol washes and xylene.

Table 1. Biometric data and ratio of normalized ppENK mRNA levels of left to right ventricular myocardium in rats of different age

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Body Weight, g</th>
<th>Heart Weight</th>
<th>ppENK LV/RV Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>114.0 ± 6.0</td>
<td>496 ± 31</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>8</td>
<td>325.3 ± 1.5</td>
<td>1,013 ± 35</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>518.3 ± 10.1</td>
<td>1,250 ± 67</td>
<td>4.0 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3/group. Total heart weight is given as wet weight in absolute amount and relative to body weight. ppENK, preproenkephalin; LV, left ventricular myocardium; RV, right ventricular myocardium.
and once with 0.9% NaCl, and immediately used for hybridization. Sections were prehybridized with hybridization solution [50% formamide, 10% dextran sulfate, 10 mmol/l dithiothreitol, 5× Denhardt’s solution (Ficoll, polyvinylpyrrolidone, and BSA, 1 mg/ml each), 0.9 mmol/l NaCl, 60 mmol/l Na₂HPO₄, 6 mmol/l EDTA, 0.5% SDS, and 200 µg/ml tRNA from yeast] for 1–2 h at 50°C in a humid chamber (50% formamide). Denatured cRNA probes (5 min, 65°C) were added to fresh hybridization solution at a concentration of 14 × 10⁶ dpm/ml. Hybridization was performed in 50 µl/section for 12–24 h at 50°C. Sections were washed as described previously (7) at a final stringency of 0.2× standard saline citrate at 65°C by means of RNase digestion (10 U RNase I and 10 µg/ml RNase A; Boehringer Mannheim). Sections were dehydrated in an ethanol series (40–100%), air dried, dipped in Kodak NTB-2 photo emulsion, and exposed for 5–7 days. Emulsion-coated slides were developed in Kodak D-19 (16 g/l) and fixed in Kodak Unifix (150 g/l). Photomicrography was performed with a Zeiss Axioplan photomicroscope.

Histological localization of the cardiac conductive system. To localize the cardiac conductive system in rat heart, every 10th section was processed according to a method described by El-Badawi and Schenk (6). This method allows the histochemical demonstration of acetylcholinesterases that are concentrated in the cardiac conductive system (4). Briefly, sections were fixed in PBS-buffered 4% formaldehyde (Merck, Darmstadt, Germany), washed twice with distilled water, and then stained for 1–2 h at 37°C in 0.5 mg/ml acetylthiocholiniodide, 38 mmol/l sodium acetate, 2 mmol/l acetic acid, 4.25 mmol/l sodium citrate, 10 mmol/l copper sulfate, 0.5 mmol/l potassium ferricyanide, and 0.08 mmol/l tetraisopropyl pyrophosphoramide (Sigma Chemical). A brownish color developed in positive areas. The reaction was stopped by washing with distilled water for 1 min, and sections were counterstained with Gill’s hematoxylin (Sigma Chemical).

Statistics. Values are arithmetic means ± SE. Statistical significance was estimated using Student’s t-test for unpaired observations. P < 0.05 was considered to be significant.

RESULTS

Northern blot analysis. Hybridization of Northern blots with a labeled ppENK cDNA was used to control the specificity of the hybridization signal. In accordance with former results (14), the ppENK cDNA probe detected a single band at ~1.5 kb in Northern blots of total RNA from various tissues of the rat. As shown in Fig. 1, ppENK mRNA levels in myocardial tissue exceeded levels found in other tissues. To study the regional distribution of ppENK mRNA expression in the heart, we performed Northern blots with total RNA extracted from atrial, septal, and ventricular myocardium of 8-wk-old (young adults) Wistar rats. As illustrated in Fig. 2A, ppENK mRNA was mainly expressed in the left ventricle and the interventricular septum, was approximately threefold lower in right ventricular tissue, and was hardly detectable in the atria. Quantitative analysis revealed a >10-fold higher expression of ppENK mRNA in the septum than in the atria (Fig. 2B). Because ppENK gene expression is known to be developmentally regulated (31), Northern blots from left and right ventricular myocardium of different-aged rats were performed. As shown in Fig. 3, ppENK mRNA
expression increased in both ventricles roughly in parallel with aging. The intensity of the signal was 3.2- and 3.3-fold higher in the right and left ventricular myocardium ($P < 0.05$), respectively, in adolescent (4-wk-old) than in young adult (8-wk-old) rats. However, further aging was not accompanied by a significant increase in ppENK mRNA levels. The biometric data of the rats are given in Table 1. Interestingly, the left-to-right ventricular ratio of ppENK mRNA levels remained unchanged during all ages investigated (Table 1). As demonstrated in Fig. 4, there was a strong relationship ($r = 0.93$) between the expression of ppENK mRNA in the left ventricle and absolute heart weight.

To look for the expression pattern of ppENK in more detail, we performed in situ hybridization on serial cryosections from rat hearts of different ages.

Specificity of the ppENK probe. Cryosections of rat brain (cerebellar cortex) were used to control the specificity of $^{35}$S-labeled cRNA in sense and antisense orientation. Hybridization with cRNA in sense orientation revealed no signal (Fig. 5B), whereas the antisense probe was markedly concentrated in a subpopulation of cells that were scattered throughout all lobes of the cerebellar cortex (Fig. 5A). These cells have been labeled by others using in situ hybridization (29) and immunohistochemistry (30) and are thought to be Golgi cells.

Regional distribution of ppENK expression in myocardial tissue. The distribution of ppENK mRNA was studied in sections of hearts from neonatal (1- to 3-day-old, $n = 3$), adolescent (4-wk-old, $n = 3$), and adult (14-wk-old, $n = 3$) Wistar rats. In neonatal hearts the hybridization signal for ppENK mRNA was distributed uniformly throughout the heart, including atria and ventricles (Fig. 6A). In general, the signal intensity was weak and only slightly above the nonspecific binding of the sense probe (Fig. 6, D–F). In contrast, in the heart of adolescent rats the ppENK expression was much stronger in the ventricle than in the atria (Fig. 6B). There was no specific signal in the atria. As shown by Northern blots, ppENK mRNA levels were considerably higher in septal and left ventricular myocardium than in the right ventricle (Fig. 6B). This difference was even more pronounced in the heart of older rats. As shown in Figs. 6C and 7A, ppENK was mainly localized in the working myocardium of the interventricular septum and, to a lesser degree, in the free left ventricular wall. It appeared that ppENK is mainly expressed in the subendocardial layers of the septum (Figs. 6C and 7A). The right ventricle and both atria showed only nonspecific binding. As shown in Fig. 7A, the accumulation of ppENK mRNA in the interventricular septum has a clusterlike pattern. Because stimulation of opioid receptors is known to induce alterations in heart rate, the expression of ppENK was also investigated in parts of the cardiac conductive system. To localize the cardiac conductive system, acetylcholinesterase was histochemically demonstrated in adjacent sections. As shown in Fig. 8A, there was no enrichment of ppENK mRNA in the cardiac conductive system of the ventricle. In contrast, signal intensity was clearly lower than in working myocardium of the interventricular septum.

**DISCUSSION**

The present study investigated the regional and developmental distribution of ppENK mRNA in rat heart. The main findings were as follows. 1) The expression level of ppENK in atrial and ventricular myocardium is low in the early postnatal period. 2) With
maturation, ppENK mRNA markedly increases in the working myocardium of the left ventricle and accumulates in the subendocardial layer of the interventricular septum. Thus, to our knowledge, ppENK is the only gene exhibiting marked differences in expression between the adult right and left ventricle and therefore could serve as a molecular marker. Given the left-side preference of ppENK expression and the size of the left ventricle, the possibility exists that the left chamber is an endocrine organ that supplies the body with enkephalins.

At the first glimpse, our finding is in line with earlier reports showing that ppENK is developmentally regulated. These experiments demonstrated low ppENK mRNA levels in rat ventricular myocardium throughout the first weeks of life with a subsequent increase during adulthood, reaching a maximum after 3–4 mo (31). A more recent study showed a further increase in ppENK mRNA and opioid peptide levels in myocardial tissue from Wistar rats during advanced aging (2). This age-associated pattern of ppENK expression in the myocardium can also be seen in other rat strains, such as Fischer 344 rats, which are frequently used as an aging model (3).

In this respect, regulation of ppENK mRNA expression appears to show a similar pattern known from other cardiac genes, e.g., atrial natriuretic peptide (ANP) or the inositol 1,4,5-trisphosphate receptor (10, 36).

However, in the light of the present experiments, it appears that aging per se, which would affect all chambers of the heart, is not the sole trigger for upregulation of ppENK in the left ventricle. Instead, high levels of ppENK mRNA in the left chamber strongly suggest that hemodynamic load, a major difference between the left ventricle and the remaining heart, is responsible for this phenomenon. At birth there is a transition characterized by a rapid decline in right ventricular afterload and an increase in left ventricular load that proceeds during the course of maturation. It has been postulated that these changes are responsible for the isoform switch of contractile proteins seen in the postnatal period (25). Furthermore, it is known that enhanced wall stress, as seen under pathophysiological conditions, e.g., increased end-systolic ventricular pressure, can upregulate transforming growth factor-β, vascular endothelial growth factor, and ANP or downregulate sarco(plasmic) Ca²⁺-ATPase and phospholamban mRNA expression in the myocardium (17, 23, 24, 36). However, to our knowledge, studies on chamber-specific differences in expression of most of these proteins are lacking. Under physiological conditions, where load is highest in the left ventricle, differences in gene expression between the left and right ventricle are not known. Even more ANP, although known to be induced by pressure or volume overload, is expressed in atrial tissue at much higher levels than in the ventricles. This suggests that, in the normal heart, factors other than load play a dominant role in the biosynthesis of this peptide. Therefore, the selective expression of ppENK in the left ventricle is unique.

Fig. 7. ppENK mRNA distribution in rat heart. Dark-field photomicrographs of RV chamber of adjacent transverse sections through a 14-wk-old rat heart hybridized with 35S-labeled ppENK cRNA in antisense (A) and sense (B) orientation are shown under identical conditions. PM, right papillary muscle. Compared with RV myocardium, ppENK mRNA levels are markedly enriched in subendocardial layers of interventricular septum.

Are there factors other than load that are different in the heart that might explain the selective expression of ppENK in the left ventricle? Steady-state ppENK mRNA in neonatal cardiomyocytes can be increased by cAMP, suggesting that differences in sympathetic stimulation could account for the distinct expression of ppENK (31). However, because sympathetic innervation and outflow are known to be higher in atrial than in ventricular myocardium and higher in right than in left ventricular tissue, this explanation is highly unlikely (9, 21).

A second parameter known to be different in left and right ventricles is the amount of interstitial fibrosis that increases with aging almost selectively in the left heart (1, 12). This would imply higher levels of ppENK in fibroblasts than in cardiomyocytes. Preliminary data on ppENK expression in cultured cardiomyocytes and
noncardiomyocytes revealed no difference in expression levels (unpublished data). However, the patchy appearance of ppENK accumulation in the septa of older rats cannot be exclusively due to differences in load and points to an additional role of fibrosis.

What may be the physiological or pathophysiological relevance of these findings? Investigations regarding the role of enkephalins on cardiovascular function yielded opposing results (e.g., positive or negative inotropic effects) depending on the model used (18, 33). Recently, Pepe and co-workers (27) showed that Leu-enkephalin inhibits the β1-adrenoceptor-induced positive inotropic effect and intracellular cAMP formation in isolated rat hearts. Thus enkephalins may serve as an intracardiac negative modulator of β-adrenergic stimulation comparable to adenosine and ACh. This negative-feedback mechanism is expected to prevent metabolic demand, to exceed supply of substrate, and to protect the heart from calcium overload. Such a role is compatible with the data showing that ppENK mRNA levels are significantly elevated in myocardial tissue from cardiomyopathic hamsters and spontaneously hypertensive rats (5, 26). Furthermore, endogenous opioids have been implicated in the regulation of blood pressure and heart rate. Depending on the model used, endogenous opioids showed pressor/tachycardiac or depressor/bradycardiac responses (8). During the preparation of this manuscript, Hao and Rabkin (11) showed in the Dahl salt-dependent rat model that intravenous administration of enkephalin-derived peptides produced an immediate, short-term decrease in heart rate and a marked increase in blood pressure. Also, ppENK mRNA levels were found to be higher in the left than in the right ventricle and much less in the atria than in the ventricles, thus confirming the difference seen in our study.

In addition, it has been shown in several tissues that the endogenous opioids inhibit cell proliferation and promote cell differentiation (37, 38). In newborn rats, in vivo administration of [Met5]enkephalin to neonatal rats depresses DNA synthesis of myocardial cells (22). Thus it is tempting to speculate that the myocardial production of endogenous opioid peptides during the course of maturation of the developing heart acts as a negative regulator of cell proliferation and growth. However, indirect effects of [Met5]enkephalin on, e.g., blood pressure or heart rate may also account for the inhibition of myocardial DNA synthesis. Therefore, direct effects of enkephalins on isolated cardiomyocytes or noncardiomyocytes have to be elucidated.

The antiadrenergic and possible antihypertropic properties as well as the systemic effects of enkephalins derived from ppENK would suggest an autocrine/paracrine mode of action. Given the left-side preference of ppENK expression, as shown in this study, the possibility is raised that the left ventricle serves as a sensor of hemodynamic load as well as an endocrine organ that supplies the body with enkephalins under conditions of increased wall stress.

This work is part of the doctoral thesis of G. Fleige at the University of Hamburg and has been presented at the Annual Meeting of the Deutsche Gesellschaft für Kardiologie-Herz-und Kreislaufforschung, Mannheim, Germany, 1997, and published in abstract form (Z. Kardiol. 86, Suppl. 2: 463, 1997).

Address for reprint requests: J. Weil, Abteilung Allgemeine Pharmakologie, Universitäts-Krankenhaus Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany.

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