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TISSUE-SPECIFIC CHANGES IN DISTRIBUTION OF PAM ACTIVITY, mRNA LEVELS, AND PROTEIN FORMS

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The high levels of peptidylglycine α-amidating monoxygenase (PAM, EC 1.14.17.3) found in adult rat atrium led us to examine PAM expression in rat atrium and ventricle from embryonic day 14 through adulthood. Immunocytochemical studies using antisera to PAM identified cardiocytes as the major site of PAM expression in atrium and ventricle throughout development. Levels of PAM mRNA and PAM activity exhibited distinctly different developmental profiles in atrium and ventricle. Ventricle PAM mRNA and PAM activity were highest from embryonic days 14 through 18, declined at the time of birth, rose slightly during the first postnatal week, and declined toward adult levels. Atrial PAM mRNA and PAM activity were low at embryonic day 14, rose to a peak immediately before birth, declined at the time of birth, and then rose after birth. Levels of atrial PAM mRNA and PAM activity were not directly correlated at all developmental stages. Two major forms of PAM mRNA (4.2 ± 0.1 and 3.8 ± 0.1 kilobase(s)) were identified in atrium and ventricle throughout development. The prevalence of the two forms varied with developmental stage, with atrium and ventricle containing similar forms at each stage. Western blots of atrial and ventricular membranes revealed the existence of a developmental stage-specific distribution of PAM protein among forms ranging in mass from 125 to 94 kDa. In both atrium and ventricle PAM activity was primarily soluble from embryonic days 14 through 16 and primarily particulate after birth. The role of PAM in the heart is not yet clear, but the presence of tissue-specific and developmentally regulated alterations in PAM mRNA, PAM protein, and PAM activity suggests that this peptide processing enzyme plays a key role in the heart.

Small bioactive peptides are derived from larger precursor proteins following a series of post-translational cleavage and modification steps (1–3). For many of these peptides, full biological activity is dependent on α-amidation of the carboxyl-terminal amino acid. Peptidylglycine α-amidating monoxygenase (PAM; EC 1.14.17.3), a copper-, molecular oxygen-, and ascorbate-dependent enzyme, produces α-amidated product peptides from a variety of glycine-extended peptide substrates and has been identified in many tissues (4–6). cDNAs encoding PAM have been cloned and sequenced from bovine intermediate pituitary and frog skin libraries (7–9). The bovine cDNA encodes a 108,207-dalton protein containing an amino-terminal signal sequence followed by a putative propeptide (Fig. 1). The catalytic domain comprises the amino-terminal third of the molecule and is followed by an intragranular domain, a hydrophobic putative membrane spanning domain, and a carboxyl-terminal, 85-amino acid putative cytoplasmic tail. Endoproteolytic cleavage at a subset of the 10 pairs of basic amino acid residues found in the bovine precursor protein may generate the soluble forms of PAM purified from the bovine neurointermediate lobe (PAM-A, 54,000 daltons; PAM-B, 38,000 daltons). The distribution of PAM activity among soluble and membrane fractions is tissue-specific (10), with over one-half of the PAM activity in the rat anterior pituitary being membrane-associated.

With the discovery of atrial natriuretic factor, the endocrine role of the heart is now recognized (11–13). Recently, high levels of membrane-associated PAM activity and PAM mRNA were identified in adult rat and bovine heart atrium (14); more than 70% of total atrial PAM activity is membrane-bound, and the specific activity is 5– to 20-fold greater than corresponding pituitary fractions. Although the peptide products known to be derived from pro-atrial natriuretic factor are not α-amidated and substrates for atrial PAM activity have not yet been identified, the unexpectedly high levels of PAM activity and PAM mRNA in adult atrial tissue led us to begin to study the regulation and function of PAM in cardiac tissue. As one means of understanding the role of PAM in the heart, we decided to study its ontogenesis in rat atrium and ventricle. Atrial and ventricular PAM expression from embryonic day 14 through adulthood was studied by measuring soluble and membrane-associated PAM activity, assessing the levels and forms of PAM mRNA by Northern analysis, determining the forms of PAM protein by Western analysis, and examining its cellular localization using immunocytochemical techniques. Like myosin and troponin in cardiac tissue (15, 16), PAM was found to exhibit striking changes during development.

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∥ The abbreviations used are: PAM, peptidylglycine α-amidating monoxygenase; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate; kb, kilobase(s).
MATERIALS AND METHODS

Preparation of Tissue Extracts—Timed pregnant rats were purchased from Holtzman Laboratory (Madison, WI); the day on which the dam was postnatal day 1. Homogenates of atrial and ventricular tissue (apical half) from embryonic, postnatal, and adult (90 days) rats were separated into soluble and particulate fractions as described (10). Briefly, the tissues were homogenized in 20 mM NaTes, pH 7.4, 10 mM mannitol, containing 0.3 mg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 16 μg/ml benzamidine using a ground glass homogenizer at 4°C. The homogenates were frozen and thawed three times and centrifuged for 5 min at 1,000 × g to sediment nuclei and cell debris. The homogenates were then centrifuged for 60 min at 100,000 × g. The supernatants were saved and the pellets were washed once by resuspension in the same buffer and recentrifugation. The supernatants from the two high speed centrifugations were pooled and used to measure soluble PAM activity. The pellets were resuspended in the same buffer containing 1% Triton X-100 as previously described (10); following centrifugation for 60 min at 100,000 × g the supernatants were used to measure solubilized, membrane-associated PAM activity. All samples were stored at −70°C until time of assay. Protein concentrations were determined using the biuret method (Pierce Chemical Co.) and bovine serum albumin as standard.

Amidation Assays—Amidation assays were performed in duplicate essentially as described (17). Unless otherwise indicated, assay tubes contained 20,000-25,000 cpm of mono-[14C]-D-Tyr-Val-Gly, 0.4 μM D-Tyr-Val-Gly, 400 μM ascorbate, 0.1 mM CuSO₄, catalase (100 μg/ml), and 0.16-2.4 μg of protein in 120 mM NaTes buffer, pH 8.5. Reaction velocities were generally expressed as picomoles of product formed per μg protein/h. The variation between duplicate samples was less than 5%. The reaction velocities reported are initial velocities using a concentration of substrate at least 10-fold below the Km of the enzyme for peptide substrate. In general, no more than 10% of the substrate was converted to product in the assay. For assays carried out in the presence of Triton X-100, the final assay mixture contained less than 0.02% Triton X-100.

RNA Preparation and Analysis—Total RNA was prepared from the atria and ventricles of rats of differing ages using the acid guanidinium isothiocyanate/phenol/chloroform procedure (18). The RNA was denatured and then electrophoresed on 1% agarose gels and the concentration of RNA was measured spectrophotometrically at 260 nm. The RNA was then hybridized to a nick-translated double-stranded cDNA probe and the hybridization was allowed to proceed at 52°C for 2-3 h. Unhybridized probe was removed by washing the nitrocellulose filters at 60°C in 0.1× sodium chloride, 0.1% sodium dodecyl sulfate and 0.1% sodium pyrophosphate followed by washing in 0.1× sodium chloride, 0.1% sodium dodecyl sulfate. The filter was then air-dried and exposed to film. Radioactivity was quantitated using a KODAK X-Omat film with a Kodak Medical Imaging System 1000. The results were analyzed using a KODAK Scientific Image Analyzer. A KODAK X-Omat film with a Kodak Medical Imaging System 1000 was used to determine the amount of cRNA present in the samples.

Preparation of Synthetic Peptides—bPAM-(561-579) and bPAM-(945-961) were prepared by the solid-phase procedure (19, 20). Using the Applied Biosystems Model 430A synthesizer with methylbenzhydrylamine resin as solid support, tert-butoxycarbonyl-protected amino acids were purchased from Applied Biosystems and from Bachem Fine Chemicals (Torrance, CA). Peptides were cleaved from the solid support by means of anhydrous hydrogen fluoride containing 10% anisole in a Kiel-F distillation apparatus. The peptides were purified by gel filtration on Sephadex G-25 eluted with 1.0 M acetic acid, followed by ion exchange chromatography on DEAE-cellulose (Whatman DE-52) eluted with a linear gradient from 0.1 to 2.0 M ammonium bicarbonate, pH 8.4. bPAM-(561-579) eluted at the point of the acid gradient, while bPAM-(945-961) eluted at a concentration close to 2.0 M ammonium bicarbonate. Homogeneity of the peptides was confirmed by amino acid analysis on the Beckman Model 6300 instrument after 6 N HCl hydrolysis (24 h, 110°C in vacuo) and by sequence and partial amino acid analysis using the Beckman System 880 sequencer. Synthesis and purification of synthetic bPAM-(561-579) and bPAM-(945-961) was carried out using the pH-stat method (21) and the purified material was analyzed for purity by amino acid analysis and by high pressure liquid chromatography (HPLC).

Preparation of Antisera—An immunogen, purified bovine neurointermediate pituitary PAM-A and -B (rabbit Ab365, Ab-bP-(A+B)) was affinity purified and used as described (7). bPAM-(561-579) and bPAM-(945-961) were linked to keyhole limpet hemocyanin (0.5 mg of peptide/mg hemocyanin) with glutaraldehyde (21) or 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (Bio-Rad) (22), and each conjugate was to be used to immunize two female New Zealand White rabbits (50 or 100 μg of peptide/primary injection; 50 μg of peptide/booster). bPAM-(288-310) was linked to soybean trypsin inhibitor (0.5 mg of peptide/mg soybean trypsin inhibitor) and bovine serum albumin (1 mg of peptide/ml resin) and affinity purification was carried out using the pH-stat method (21) and the purified material was analyzed for purity by amino acid analysis and by HPLC.

Preparation of bPAM Fusion Proteins—A fusion protein produced in Escherichia coli CAG456 cells infected with λPAM-1 (7), one antisera of each type (Ab69, Ab-bP-(561-579); Ab74, Ab-bP-(945-961); and Ab59, Ab-bP-(288-310)) was selected for affinity purification. Synthetic bPAM-(561-579) and bPAM-(945-961) were linked to activated CH-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) (1 mg of peptide/ml resin) and affinity purification was carried out using glycine-HCl as the eluent for Ab-BP-(945-961) and 3.0 M KSCN or 4.5 M MgCl₂ as the eluents for the antibody. Western blot analysis showed that the antibody recognized the 48 KDa protein in the CAG456 cell lysate and was adequate sensitivity for use on Western blots.

Immunocytochemistry—Fetal, postnatal, and adult rat cardiac tissue was obtained from Sprague-Dawley rats (Sprague-Dawley, CA). The tissues were frozen and sections were stained for PAM as described previously (14). Briefly, cardiac tissues were fixed by immersion in 4% paraformaldehyde for 2 h, washed in 0.15 M sodium...
phosphate buffer, pH 7.5, containing 0.32 M sucrose, and processed were then incubated with peroxidase substrates diaminobenzidine at a dilution of 1:1000 in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1% gelatin for 48 h at 4°C. Incubations in a 1:400 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) were for 80 min, and incubations in a 1:200 dilution of avidin-biotin-peroxidase complex were for 60 min. The sections before (14) to verify the staining patterns. Staining with a working dilution of Ab-bP-(561-579) or Ab-bP-(945-961) was completely absorbed upon preincubation with 1 μg/ml of the appropriate synthetic peptide.

RESULTS

PAM Activity in the Heart during Development—The expression in PAM activity in atrial and ventricular tissue was found to be developmentally regulated. High levels of membrane-associated PAM activity were first detectable in the rat atrium at embryonic day 16 (Fig. 2A). The specific activity increased 2-fold by embryonic day 18, reaching levels higher than those in the adult atrium; the specific activity then consistently declined around the time of birth. After birth the specific activity of membrane-associated atrial PAM rose until around postnatal day 5, reaching levels equal to or greater than those observed late in embryonic development; the specific activity then gradually decreased 3-fold to reach adult levels after postnatal day 21. The specific activity of soluble PAM in atrium was approximately 10-fold lower than membrane-associated PAM for all of the age groups examined. The developmental pattern for soluble atrial PAM activity was also complex, with a decline around the time of birth followed by maintained high levels until postnatal day 21 (Fig. 2B).

Membrane-associated PAM activity appeared in the developing ventricle before it appeared in the atrium (Fig. 3). The specific activity of membrane-associated PAM was higher in the ventricle at embryonic day 14 than in corresponding fractions from the adult anterior pituitary (10); in contrast, at embryonic day 14, atrial membrane-associated PAM activity was very low (compare Figs. 2A and 3A). The specific activity of membrane-associated ventricular PAM decreased substantially by the time of birth, consistently rose slightly in the first postnatal week, and then declined slowly to the low levels observed in adult ventricle following postnatal day 7. After embryonic day 18, the specific activity of PAM in particulate fractions from the ventricles was at least 10-fold lower than in corresponding atrial fractions. The specific activity of soluble ventricular PAM declined steadily from embryonic day 14 to adult (Fig. 3B).

For both atrium and ventricle, soluble forms of PAM activity predominated from embryonic day 14 to embryonic day 16; at embryonic day 14 less than 20% of the PAM activity in atrium or ventricle was particulate (Fig. 4A). From embryonic day 14 through postnatal day 3, the percentage of
the amount of the rPAM mRNAs. Two forms of PAM mRNA were present in varying proportions at earlier ages, and the forms of PAM mRNA clearly underwent developmental regulation (Fig. 5A). The data for PAM mRNA have been normalized to the overall specific activity of PAM can be calculated as a function of age (Fig. 4B). Total atrial PAM specific activity exhibited a markedly different developmental profile, rising rapidly before birth, increased transiently after birth until postnatal day 7 and then declined to the lower levels seen in adult ventricle. As in the atrium, the forms of PAM mRNA in the ventricle underwent developmental regulation (Fig. 5A, lower). The total amount of PAM mRNA present in the ventricles at each age exhibited a time course distinctly different from the time course for PAM mRNA in the atrium (Fig. 5B). Thus, PAM gene expression in atrium and ventricle exhibited tissue specificity along with a complex developmental profile.

Although the absolute amounts of PAM mRNA in atrium and ventricle varied in an independent fashion, the switching among different forms of rPAM mRNA during development occurred coordinately in atrial and ventricular tissues (Fig. 5A). In the atrium, PAM mRNA was first detectable at embryonic day 14, rising to a peak at embryonic day 20. The levels of atrial PAM mRNA then declined dramatically, remaining low through the first postnatal week and then gradually increasing to achieve adult levels following postnatal day 21.

High levels of PAM mRNA also appeared in the ventricles during embryonic development (Fig. 5A, lower). The highest levels of ventricular PAM mRNA were seen at embryonic days 14–16 and approached the levels seen in adult atrium (Fig. 5B). Levels of ventricular PAM mRNA diminished rapidly before birth, increased transiently after birth until postnatal day 7 and then declined to the lower levels seen in adult ventricle. As in the atrium, the forms of PAM mRNA in the ventricle underwent developmental regulation (Fig. 5A, lower). The total amount of PAM mRNA present in the ventricles at each age exhibited a time course distinctly different from the time course for PAM mRNA in the atrium (Fig. 5B). Thus, PAM gene expression in atrium and ventricle exhibited tissue specificity along with a complex developmental profile.

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Fig. 5. Northern blot analysis of PAM mRNA during development. A, an aliquot of total RNA (5 μg) from atria or ventricles of animals of the ages indicated was fractionated as described under "Materials and Methods"; the dye band was allowed to migrate 22 cm in order to allow adequate resolution of the various forms of rPAM mRNA. The blots were hybridized with the 0.7-kb bPAM cDNA probe and exposed to x-ray film for 18 h (atrium) or 48 h (ventricle). Similar analyses carried out on an independent set of animals demonstrated the same shifts in prevalence of 3.8- and 4.2-kb forms of PAM mRNA as a function of developmental stage. Blots were subsequently stripped and reprobed with a cDNA probe corresponding to 18 S rRNA to permit correction for the amount of sample actually transferred to Nytran. B, for densitometric analysis of PAM mRNA levels during development, the amount of PAM mRNA was normalized to the amount of 18 S rRNA. Blots containing both adult rat atrial (■) and ventricular (▴) RNA allowed comparison of PAM mRNA levels in the two tissues. Data shown are expressed as a ratio to the adult atrial PAM mRNA level. Results from an independent analysis of the same samples agreed within 13%; data from a second complete developmental time course expressed as the -fold change from adult atrial PAM mRNA levels but not normalized to 18 S rRNA agreed with the data shown within 15%.

Although significant amounts of two forms of rPAM mRNA were present in older animals (postnatal day 21 to adult). It is not yet clear whether the PAM mRNAs expressed in atrium and ventricle throughout the various developmental stages are identical to the forms present in the adult.

Immunocytochemical Localization of PAM during Heart Development—To determine whether PAM is expressed in cardiac myocytes or in other supporting cell types during development, atrium and ventricle from animals of different ages were prepared for light microscopic immunocytochemistry (Fig. 6). Immunocytochemical studies with affinity purified Ab-bP-(945–961), directed against the putative cytoplasmic tail of bPAM, demonstrated a specific staining reaction in both atrial and ventricular cardiocytes (Fig. 6, A–H). A dark immunocytochemical stain was observed in atrial cardiocytes with Ab-bP-(945–961) for animals of all ages examined (Fig. 6, A, C, E, and G). The pattern observed was similar to that previously seen for PAM in adult bovine atrium (14). The staining in the ventricle was most intense from embryonic day 14 to postnatal day 3 and declined to barely detectable levels in adult ventricle (Fig. 6, B, D, F, and H). The immunocytochemical staining profile obtained with Ab-bP-(945–961) in the ventricle paralleled ventricular PAM expression as measured by PAM activity and mRNA levels.

Using affinity purified Ab-bP-(561–579), directed against the intragranular domain of PAM, weak staining appeared in isolated atrial cells at embryonic day 14 (data not shown); by embryonic day 20, a dark punctuate perinuclear staining pattern was observed in most of the atrial myocytes (Fig. 6, I). A similar pattern of atrial staining with Ab-bP-(561–579) persisted in each of the age groups examined and in adult tissue (data not shown). In contrast, no specific staining was observed when ventricular tissue was stained with Ab-bP-(561–579) (Fig. 6, J). The difference in the ability of Ab-bP-(561–579) and Ab-bP-(945–961) to visualize ventricular PAM may relate to the different specificity of the antisera. Tissue-specific post-translational processing or subcellular routing of the PAM precursor could result in the differential retention of the intragranular and cytoplasmic domains of the PAM precursor in atrial and ventricular cardiocytes.

Forms of PAM Protein in the Heart during Development—Since the forms of PAM mRNA expressed in the heart undergo developmental regulation (Fig. 5A), changes in the forms of PAM protein present were sought. Western blots utilizing antibodies specific for the catalytic and intragranular
domains of PAM were used to determine the apparent molecular weight of the cross-reactive protein. For atrial membrane fractions from animals of each age were fractionated as described under "Materials and Methods." Each of the samples shown in A contained 45 pmol/h PAM activity (lane E18, 8.2 μg of protein; lane P3, 7.12 μg of protein; lane P14, 15.6 μg of protein; lane A, adult, 22 μg of protein) and were visualized with Ab-bP-(A+B) at a 1:1000 dilution. Each of the samples shown in B contained 30 pmol/h PAM activity and were visualized with Ab-bP-(561–579).

As shown for E18 (E18 (con)), staining for all samples was blocked by addition of 10 μg/ml bPAM-(561–579). C, aliquots of ventricular membranes and soluble fractions from animals of the indicated age were fractionated as in B. The aliquot of soluble ventricular PAM (E14 (sol)) contained 4 pmol/h PAM activity and 45 μg of protein and was visualized with Ab-bP-(288–310) at a dilution of 1:1000. Samples of ventricular membrane PAM each contained 30 μg of protein and variable amounts of PAM activity (lane E18, 16.4 pmol/h; lane P3, 6.8 pmol/h; lane P14, 4.35 pmol/h; lane A, adult, 1.4 pmol/h) and were visualized with Ab-bP-(561–579) at a 1:1000 dilution.


discussion

The high levels of PAM activity observed in the adult rat atrium (14) were exceeded during atrial development. A peak of atrial PAM activity immediately preceded birth and a second broader peak followed the decline in specific activity that occurred at birth (Fig. 4B). Levels of PAM mRNA in the atrium also peaked immediately before birth and declined at the time of birth, but failed to rise in parallel with levels of PAM activity during the postnatal period (Fig. 5B). While levels of PAM mRNA rose substantially between postnatal day 7 and adulthood, PAM specific activity declined 2-fold. This dissociation between levels of PAM mRNA and enzyme activity may reflect variation in the forms of PAM present, altered translational efficiency for PAM mRNA, or variable rates of secretion of PAM from the tissue. At each developmental stage examined, immunocytochemical studies indicated that atrial myocytes were the major cellular source of PAM.

Although levels of PAM mRNA and activity in the adult rat ventricle were substantially lower than in the adult atrium, levels of ventricular PAM exceeded levels of atrial PAM early in development. Levels of ventricular PAM mRNA and enzymatic activity were maximal from embryonic days 14 through 18 and declined at birth; a moderate increase in ventricular PAM mRNA and activity occurred in the first postnatal week followed by a decline to adult levels. The dissociation of PAM mRNA and activity observed in the atrium during development was not observed in the ventricle. Immunocytochemical studies again indicated that cardiocytes were the major cellular source of ventricular PAM at all ages examined. Although embryonic and early postnatal ventricular cardiocytes could be visualized with Ab-bP-(945–961), they could not be visualized with Ab-bP-(561–579). The reasons for this discrepancy are not yet clear and may reflect the presence of different forms of mRNA or the use of different subcellular processing schemes.

During development of the endocrine pancreas, levels of PAM activity and thyrotropin releasing hormone, an α-amidated peptide whose production requires PAM activity, rise transiently before declining to the low levels observed in the adult (27). It is tempting to speculate that the transient expression of substrates for PAM parallels enzyme expression in the atrium and ventricle.

Two major forms of PAM mRNA, 4.2 and 3.8 kb, were observed in varying proportions throughout development (Fig. 5A). Distinctive shifts in the pattern of PAM mRNAs present occurred before birth, at the time of birth, during the first postnatal week, and before adulthood. Throughout this complex developmental course, the forms of PAM mRNA in atrium and ventricle changed in a coordinated fashion. In both atrium and ventricle, PAM activity shifted from primarily soluble at embryonic days 14 and 16 to primarily particulate after embryonic day 20. Although the level of PAM expression in atrium and ventricle varied independently, shifts in forms of PAM mRNA and distribution of activity between soluble and particulate fractions occurred in a coordinate fashion. Particulate fractions contained forms of PAM protein ranging in mass from 94 to 125 kDa; similar forms were seen in atrium and ventricle. The prevalence of the
various forms of particulate PAM varied during development, but it is not yet possible to relate individual proteins to individual mRNAs. For example, while the 3.8- and 4.2-kb forms of PAM mRNA predominated at P3 and P14, nearly identical protein patterns were observed for P3 and P14 on Western blots.

Developmental studies on the expression of several contractile proteins in the heart have demonstrated state-specific expression of individual members of gene families as well as stage-specific splicing of mRNA transcripts. In chicken and rat cardiac muscle the large troponin isoform predominates during embryogenesis, while the smaller form occurs exclusively in the adult (16, 28). During heart and skeletal muscle differentiation, both the light and heavy chains of myosin undergo stage-specific isoform switching (15, 29); these changes are subject to hormonal regulation (30, 31). The major forms of PAM mRNA in the adult atrium appear to result from alternate splicing (32). Determining the forms of PAM mRNA and protein present throughout cardiac development, the factors regulating their tissue-specific and developmentally regulated expression, and their role the cardiac function remain projects for the future.

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REFERENCES