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Developmental Regulation of Peptidylglycine α-Amidating Monooxygenase (PAM) in Rat Heart Atrium and Ventricle

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

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The high levels of peptidylglycine α-amidating monooxygenase (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. Ventricular 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 monooxygenase (PAM[1]; 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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[1] The abbreviations used are: PAM, peptidylglycine α-amidating monooxygenase; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}ethanesulfonic 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 Laboratories (Madison, WI); the day of birth is postnatal day 1. Homogenates of atrial and ventricular tissue (apical half) from embryonic, postnatal, and adult (90 day) 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 bicinchoninic acid protein assay reagent (Pierce Chemical Co.) and bovine serum albumin as standard.

Amidation Assays—Amidation assays were performed in duplicate essentially as described (17). Unless indicated otherwise, assay tubes contained 20,000–25,000 cpm of mono-^{125}I-D-Tyr-Val-Gly, 0.4 μM D-Tyr-Val-Gly, 400 μM ascorbate, 8 μM $CuSO_4$, catalase (100 μg/ml), and 0.16–2.4 μg of protein in 120 mM NaTES buffer, pH 8.5. Reaction velocities are 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 K_m 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% detergent (10).

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 final RNA pellets were resuspended in diethyl pyrocarbonate-treated water, and the concentration of RNA was measured spectrophotometrically by absorbance at 260 and 280 nm. At embryonic day 14, atria from 22 pups were pooled and yielded about 25 μg of total RNA. By embryonic day 20, atria from 18 pups were used to produce 34 μg of total RNA. At postnatal day 3, atria from four pups were used to produce 20 μg of total RNA. Equivalent amounts of ventricular tissue from animals of each age were extracted.

RNA was denatured and then electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0, and transferred to Nytran (Schleicher & Schuell) by capillary action in 20 × SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0). Additional 5-μg aliquots of RNA were fractionated and visualized with acridine orange to monitor the quality and amount of the RNA. The Nytran filters were baked in a vacuum oven at 80 °C for 2 h. The baked filters were prehybridized at 42 °C for 6–8 h in 50% deionized formamide, 5 × SSC, 4 × Denhart's solution (50 × Denhart's solution is 1% Ficoll-400, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 20 mM sodium phosphate, pH 6.5, 0.1% SDS, 0.1 mg/ml sonicated carrier herring sperm DNA. The 0.7-kb *Eco*RI fragment of bovine PAM cDNA (782–1503 base pairs) was used as probe (7). DNA fragments (100 ng) recovered from agarose gels were radiolabeled by nick translation (Du Pont-New England Nuclear) with 100 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Corp.) to a specific activity of 0.5–1.2 × 10^9 cpm/μg DNA. Nick-translated probe was heat-denatured and added to the pre-hybridization solution (10^6 cpm/ml), and hybridization was allowed to proceed at 42 °C for 36–44 h. Filters were washed in 2 × SSC, 0.1% SDS at room temperature for 2 × 30 min and in 0.2 × SSC, 0.1% SDS at 52 °C for 2 × 30 min. Filters were exposed to x-ray film at −70 °C with an intensifying screen. Molecular weights were estimated by comparison to an RNA ladder (Bethesda Research Laboratories) fractionated on a parallel lane and stained with acridine orange or blotted and probed with nick-translated wild type λDNA. In order to correct for the actual amount of RNA applied to each lane, blots were stripped and hybridized to cDNA probes derived from 18 S and 28 S frog rRNA (the 3.8- and 4.2-kb *Nco*I fragments derived from pX1r101a, kindly provided by Dr. Barbara Sollner-Webb, Johns Hopkins Medical School).

For quantitation, autoradiograms were densitized using a LOATS RAS-1000 image analysis system (Amersham Corp.). Known amounts of a nick-translated double-stranded cDNA probe were applied to a nitrocellulose membrane using a slot blot apparatus (Schleicher & Schuell), and densitization of this autoradiogram provided a standard curve for converting integrated optical density into disintegrations/min. The amount of PAM mRNA (disintegrations/min) in each sample was then normalized to the amount of rRNA (disintegrations/min) in that sample, giving a ratio of PAM mRNA/rRNA. For each separate experiment, this ratio in adult atrial tissue was scaled to 1.0.

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 Kel-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 outset of the 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 preview analysis using the Beckman System 890 sequencer. bPAM-(288–310) was synthesized and purified by Dr. John Burnier (Genentech Inc.) by standard solid-phase technique (19).

Preparation of Antisera—Antiserum to purified bovine neurointermediate pituitary PAM-A and -B (rabbit Ab36, 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-dimethylaminopropyl)carbodiimide HCl (Bio-Rad) (22), and each conjugate was used to immunize two female New Zealand White rabbits (50 or 100 μg of peptide/primary injection; 50 μg of peptide/boost). bPAM-(288–310) was linked to soybean trypsin inhibitor (0.5 mg of peptide/mg soybean trypsin inhibitor) via its terminal cysteine residues using the heterobifunctional cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester by Dr. John Burnier (23). Three female New Zealand White rabbits were each immunized with conjugate containing 300 μg of peptide and boosted with conjugate containing 75 μg of peptide. Based on the ability to bind ^{125}I-labeled peptide and/or the ability to stain the β-galactosidase/bPAM fusion protein produced in *Escherichia coli* CAG456 cells infected with λPAM-1 (7), one antiserum 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 $MgCl_2$ as the eluents for Ab-bP-(561–579) as described (24). bPAM-(288–310) was linked to Affi-Gel 15 (2 mg of peptide/ml gel), and affinity purification was carried out using glycine-HCl as the eluent. A diagrammatic representation of the regions of bPAM to which the different antisera were raised is shown in Fig. 1.

Western Blot Analysis—Samples of soluble or particulate fractions were separated on slab gels containing 10% acrylamide and 0.25% *N,N'*-methylene-*bis*-acrylamide using the buffer system of Laemmli (25). Aliquots of crude membrane fractions were boiled into SDS for separation on slab gels or solubilized with Triton X-100 to permit determination of the amount of PAM activity present. Proteins were electrophoretically transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol (26) and visualized with Ponceau S (Sigma). Molecular weights were estimated by comparison with standard proteins (Rainbow markers; Amersham Corp.). Nitrocellulose strips were immunostained using a variety of primary rabbit antisera and alkaline phosphatase conjugated to affinity purified goat anti-rabbit immunoglobin (Bio-Rad Immuno-Blot assay kit) as described (14). Antisera to bPAM-(945–961) failed to detect the bovine λPAM-1 fusion protein or bovine atrial PAM with adequate sensitivity for use on Western blots.

Immunocytochemistry—Fetal, postnatal, and adult rat cardiac tissues were prepared and immunocytochemically 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

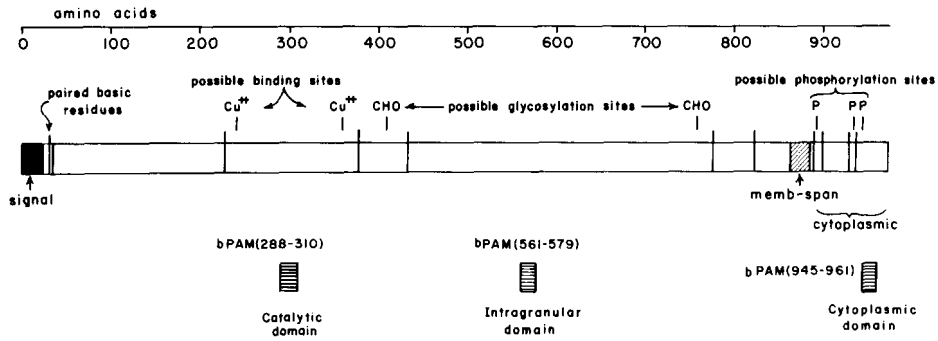

FIG. 1. **Structure of the PAM precursor.** The structure of the bPAM precursor deduced from a cDNA cloned from a bovine intermediate pituitary cDNA library is shown (7). The structure of rat atrial PAM is highly homologous (32). Rabbit antisera were generated against synthetic peptides corresponding to the regions of the bPAM precursor shown. Ab-bP-(288–310) recognizes a sequence in the catalytic domain, Ab-bP-(561–579) recognizes a sequence in what is referred to as the intragranular domain, and Ab-bP-(945–961) recognizes a sequence in a region expected to form a cytoplasmic tail. Ab36[Ab-bP-(A+B)] was produced against purified PAM A + B (7). All of the antisera used were generated to bPAM and were selected for their ability to cross-react with rPAM.

phosphate buffer, pH 7.5, containing 0.32 M sucrose, and processed for paraffin embedding. The sections (8 μm) were then deparaffinzed, rehydrated, and stained. Primary affinity purified antisera were used 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 60 min, and incubations in a 1:200 dilution of avidin-biotin-peroxidase complex were for 60 min. The sections were then incubated with peroxidase substrates diaminobenzidine (0.3 mg/ml) and H_2O_2 (0.03%) in 0.05 M Tris-HCl, pH 7.6, for 15 min. Appropriate method and specificity controls were performed as 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. 2*A*). 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. 2*B*).

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. 2*A* and 3*A*). 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

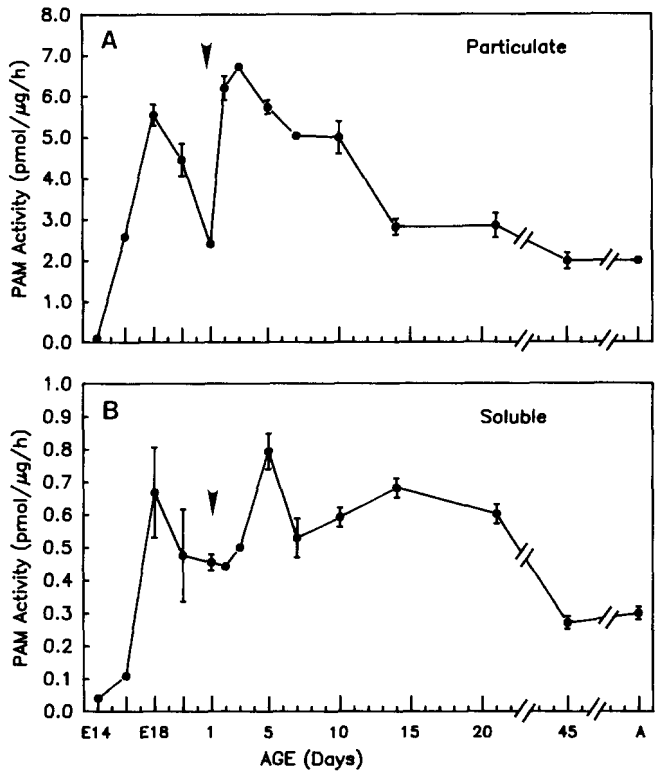

FIG. 2. **Ontogenesis of PAM activity in rat atrium.** Membrane-associated (*A*) and soluble (*B*) PAM activity was measured as described under "Materials and Methods." Data represent the average specific activity of duplicate determinations from two entirely separate experiments ± range. Where absent, the error is within the symbol. The *arrowheads* indicate day of birth. Note the 8-fold expanded scale for the *y* axis in *B*.

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. 3*B*).

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. 4*A*). From embryonic day 14 through postnatal day 3, the percentage of the

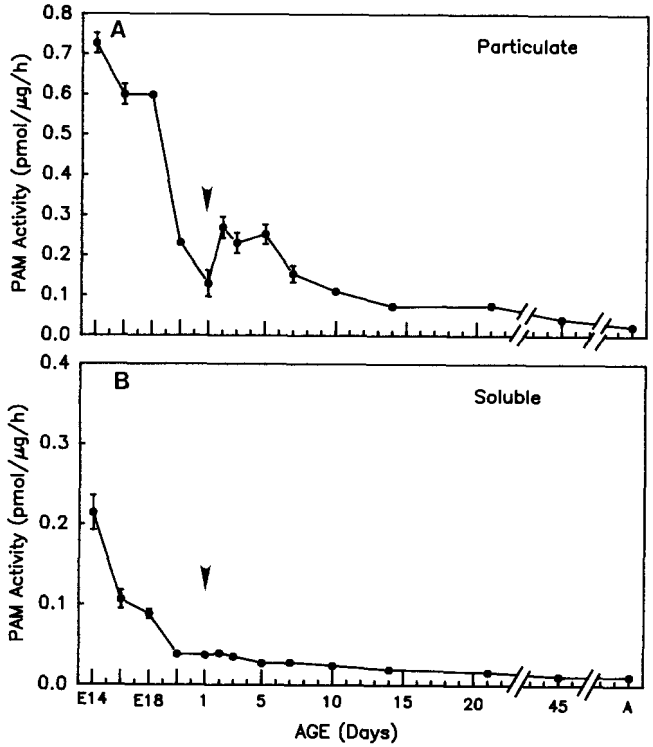

FIG. 3. **Ontogenesis of PAM activity in rat ventricle.** Membrane-associated (*A*) and soluble (*B*) PAM activity was measured as described under "Materials and Methods." Data represent the average specific activity of duplicate determinations from two entirely separate experiments ± range. The *arrowheads* indicate day of birth. Note the 2-fold expanded scale for the *y* axis in *B*.

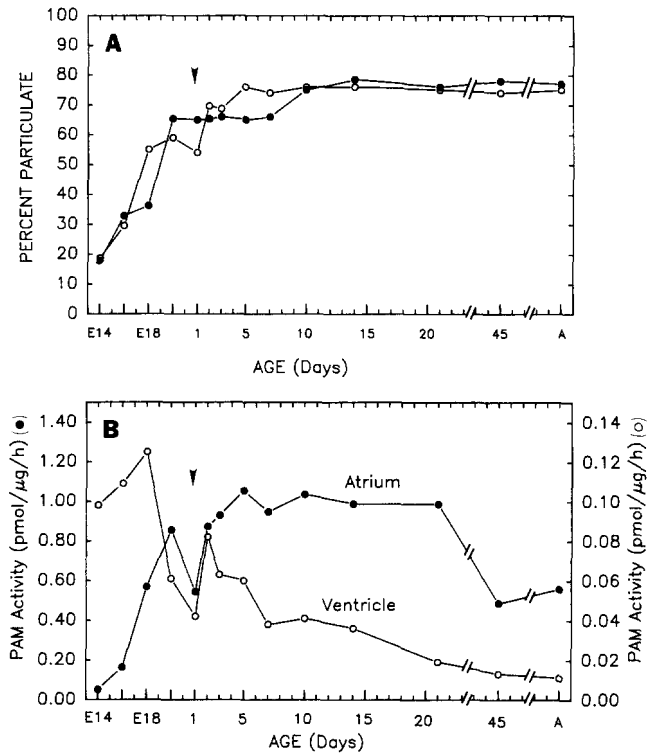

FIG. 4. **Changes in heart PAM activity during development.** *A*, total soluble and membrane-associated PAM activity were determined for each sample, and the percentage of the total PAM activity accounted for by membrane-associated activity was calculated. *B*, total PAM specific activity is plotted; data from two independent experiments were averaged and agreed within ± 10%. *Arrowheads*, date of birth.

total tissue PAM activity that was membrane-associated increased in parallel in atrium and ventricle, so that particulate PAM became the dominant form (70% of total tissue PAM activity), as observed previously in adult atrial and ventricular tissues.

By taking into account the amount of soluble and particulate protein and the specific activity of PAM in both fractions, the overall specific activity of PAM can be calculated as a function of age (Fig. 4*B*). Total atrial PAM specific activity rose rapidly from embryonic day 14 until the time of birth; following a transient decline, it reached a plateau that was maintained through postnatal day 21 and then declined 2-fold to reach the adult level. Total ventricular PAM specific activity exhibited a markedly different developmental profile, with the highest levels occurring from embryonic days 14 through 18; a rapid decline around the time of birth was followed by a rise in the first postnatal week and a decline to adult levels.

Forms of PAM mRNA in the Heart during Development—Total RNA was isolated from the atria and ventricles of rats ranging in age from embryonic day 14 through adult and PAM mRNA was examined by Northern blot analysis (Fig. 5*A*). Equal amounts of total RNA from animals of each age were fractionated, and a bPAM cDNA probe was used to visualize the rPAM mRNAs. Two forms of PAM mRNA (4.2 ± 0.1 and 3.8 ± 0.1 kb) similar in size to the two major forms of PAM mRNA identified previously in adult rat atrium and ventricle were present in varying proportions at earlier ages, and the forms of PAM mRNA clearly underwent developmental regulation (Fig. 5*A*). The total amount of PAM mRNA present at each age was quantitated by densitization of the autoradiograms; the data for PAM mRNA have been normalized to the amount of 18 S rRNA in each sample (Fig. 5*B*). 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. 5*A*, *lower*). The highest levels of ventricular PAM mRNA were seen at embryonic days 14–16 and approached the levels seen in adult atrium (Fig. 5*B*). 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. 5*A*, *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. 5*B*). 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 through development occurred coordinately in atrial and ventricular tissues (Fig. 5*A*). In the atrium and ventricle, a 3.8-kb form of PAM mRNA predominated at embryonic day 14. Similar amounts of a 4.2- and a 3.8-kb form were observed from embryonic days 18 through 20, and the larger form of rPAM mRNA predominated by embryonic day 21. From birth until postnatal day 3, a smaller form of rPAM mRNA was again predominant. There was a gradual switch to a predominance of a larger form of rPAM mRNA from postnatal days 5 through 14

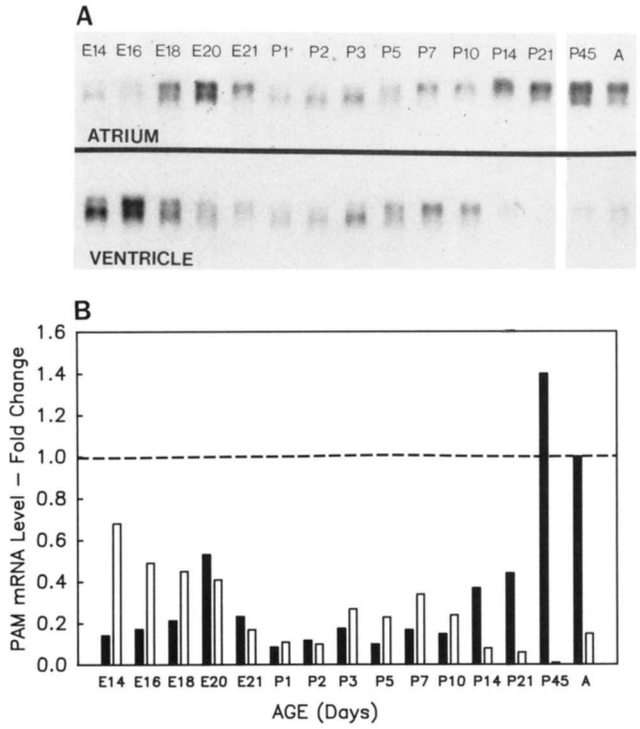

FIG. 5. **Northern blot analysis of PAM mRNA during development.** *A*, an aliquot of total RNA (5 μg) from atria or ventricles of animals of the ages indicated was fractionated as described under "Materials and Methods"; the dye band was allowed to migrate 22 cm in order to allow adequate resolution 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 prevalance 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%.

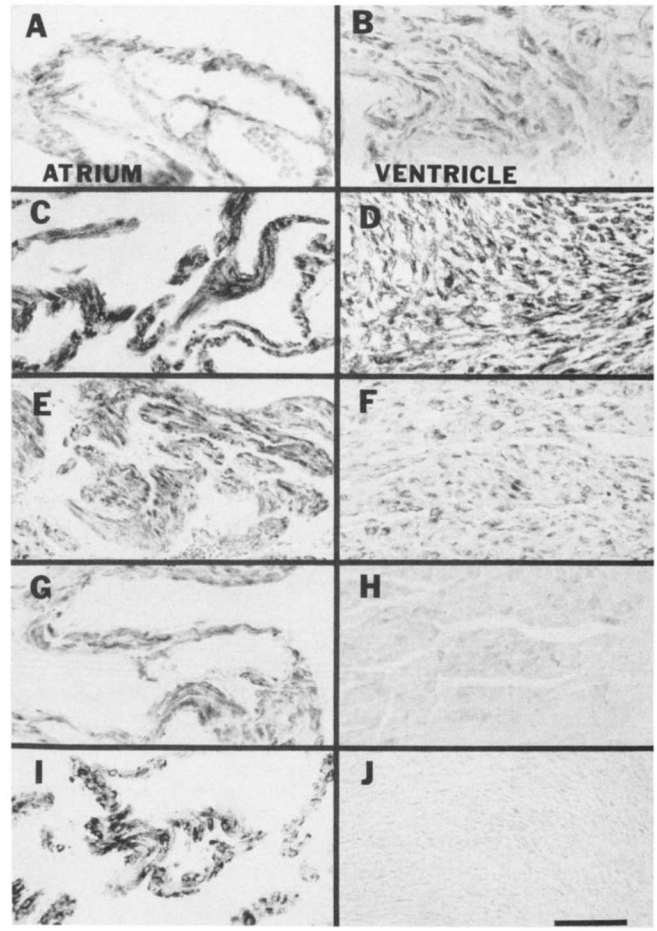

FIG. 6. **Immunocytochemical staining of heart tissues for PAM.** *A–H*, fetal, neonatal, and adult heart tissues were immunocytochemically stained for PAM using Ab-bP-(945–961) for the cytoplasmic domain. Atrial tissues are shown on the *left*, ventricular tissue on the *right*. Age groups are as follows: embryonic day 14 (*A, B*); embryonic day 20 (*C, D*); postnatal day 3 (*E, F*); adult (*G, H*). *I* and *J*, staining using Ab-bP-(561–579) on embryonic day 20 atrium (*I*) and ventricle (*J*) are shown for comparison. *Scale bar*, 50 μm.

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. 5*A*), changes in the forms of PAM protein present were sought. Western blots utilizing antibodies specific for the catalytic and intragranular

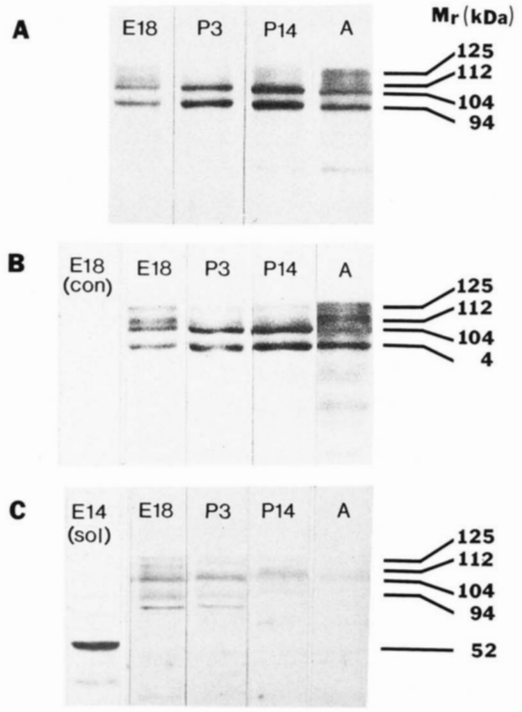

FIG. 7. **Western blot analysis of PAM.** *A* and *B*, aliquots of atrial membranes from animals of the indicated ages were fractionated as described under "Materials and Methods." Each of the samples shown in *A* contained 45 pmol/h PAM activity (*lane E18*, 8.2 μg of protein; *lane P3*, 7.12 μg of 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.

domains of PAM were used to determine the apparent molecular weight of the cross-reactive protein. For atrial membrane fractions from different developmental stages, equal amounts of PAM activity were applied to the gel (Fig. 7, *A* and *B*). Antiserum to purified bovine PAM-A and -B detected a complex cluster of proteins ranging in mass from 94 to 125 kDa (Fig. 7*A*). A similar pattern was observed with Ab-bP-(561–579) (Fig. 7*B*) and Ab-bP-(288–310) (data not shown). Staining was completely blocked by inclusion of the appropriate synthetic peptide (Fig. 7*B*, *lane E18(con)*). Although the relative proportion of material contained in each band varied from embryonic day 18 through adulthood, cross-reactive proteins of similar size (125 ± 2, 112 ± 6, 104 ± 4, and 94 ± 5 kDa) were present throughout development.

The lower specific activity of PAM in ventricular membrane fractions compared to atrial membrane fractions made it impossible to load equivalent amounts of PAM activity for all of the developmental stages. Therefore, a constant amount of ventricular membrane protein from animals of each age was subjected to Western analysis (Fig. 7*C*). A complex pattern of bands ranging in size from 94 to 125 kDa was again observed with Ab-bP-(561–579). Staining of the 94–125-kDa bands was blocked by addition of the appropriate synthetic peptide; staining of the sharp band below the 94-kDa band on embryonic day 18 and on postnatal day 3 was not blocked and was considered to be nonspecific. At embryonic day 14 the ventricles contained a significant amount of soluble PAM activity. Western blot analysis of samples containing soluble ventricular PAM activity revealed a major cross-reactive protein of 52 ± 2 kDa using Ab-bP-(288–310) (Fig. 7*C*, *lane E14(sol)*).

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. 4*B*). 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. 5*B*). 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. 5*A*). 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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