Developmental regulation of peptidylglycine alpha-amidating monooxygenase (PAM) in rat heart atrium and ventricle. Tissue-specific changes in distribution of PAM activity, mRNA levels, and protein forms.

L Ouafik, V May, H Keutmann, B Eipper

To cite this version:

L Ouafik, V May, H Keutmann, B Eipper. Developmental regulation of peptidylglycine alpha-amidating monooxygenase (PAM) in rat heart atrium and ventricle. Tissue-specific changes in distribution of PAM activity, mRNA levels, and protein forms.. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 1989, 264, pp.5839-45. <hal-01802518>
Developmental Regulation of Peptidylglycine α-Amidating Monoxygenase (PAM) in Rat Heart Atrium and Ventricle

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

(Received for publication, September 23, 1988)

L'Houcine Ouafik§, Victor May†, Henry T. Keutmann‡, and Betty A. Eipper‡

From the ‡Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and the §Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

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. 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 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 carboxy-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 putative 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.

* This work was supported by Grants DK-32949 from the National Institutes of Health and Grants DA-00266 and DA-00098 from the National Institute on Drug Abuse. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Neuroscience, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

§ To whom correspondence should be addressed: Dept. of Neuroscience, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

1 The abbreviations used are: PAM, peptidylglycine α-amidating monoxygenase; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino(ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate; kb, kilobase(s).

5839
MATERIALS AND METHODS

Preparation of Tissue Extracts—Timed pregnant rats were purchased from Harlan Laboratories (Madison, WI); the day pregnant 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, 15 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 x g to sediment nuclei and cell debris. The homogenates were then centrifuged for 60 min at 100,000 x 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 x 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 otherwise noted, assay tubes contained 20,000-25,000 cpm of [14C]-Tyr-Val-Gly, 400 µM ascorbate, 8 µM 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% 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 bovine serum albumin), 20 mM sodium phosphate, pH 6.5,0.1% SDS, 5%. The reaction velocities reported are initial velocities using a concentration 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 (25) and a synthesized oligonucleotide (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/mg. 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-Butyloxycarbonyl-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 fluoride containing 10% anisole in a K2F 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 (25) and a synthesized oligonucleotide (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/mg. For each separate experiment, this ratio in adult atrial tissue was scaled to 1.0.
phosphate buffer, pH 7.5, containing 0.32 M sucrose, and processed for paraffin embedding. The sections (8 μm) were then deparaffinized, 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 HzO2 (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 ab-sorbed 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
Developmental Regulation of Heart PAM

The amount of rPAM mRNA. Two forms of PAM mRNA were present in varying proportions at earlier ages, and the forms of PAM mRNA clearly underwent developmental regulation (Fig. 3). The total amount of PAM mRNA present in each sample was quantitated by densitization of the autoradiograms; the data for PAM mRNA have been normalized to the amount of 18S rRNA in each sample (Fig. 3A). 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.

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. The total amount of PAM mRNA present in each sample was quantitated by densitization of the autoradiograms; the data for PAM mRNA have been normalized to the amount of 18S rRNA in each sample (Fig. 3). 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.

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. The total amount of PAM mRNA present in each sample was quantitated by densitization of the autoradiograms; the data for PAM mRNA have been normalized to the amount of 18S rRNA in each sample (Fig. 3). 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.

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. 5A). 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.
Developmental Regulation of Heart PAM

were subsequently stripped and reprobed with a cDNA probe corre-

Animal demonstrated the same shifts in prevalence of 3.8- and 4.2-

Development-To determine whether PAM is expressed in car-

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 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. 7A). A similar pattern was observed with Ab-bP-(561-579) (Fig. 7B) and Ab-bP-(288-310) (data not shown). Staining was completely blocked by inclusion of the appropriate synthetic peptide (Fig. 7B, 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. 7C). 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. 7C, lane E18(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. 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.

Acknowledgments—We wish to thank Dr. John Burnier for synthesizing the bPAM-soybean trypsin inhibitor conjugate and Dr. Barbara Sollner-Webb for providing the Xenopus ribosomal RNA plasmid. We also thank Dick Mains, Karen Braas, Doris Stoffers, and Cris Green for their insight, encouragement, and thoughts during the completion of these studies.

REFERENCES