Prevalence and Turnover of Peptidylglycine \( \alpha \)-amidating Monooxygenase mRNA in Atrial Cardiomyocytes

Jean-Yves Maltese*, Ana Maria Oyarce and Betty A. Eipper

Department of Neuroscience, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore MD 21205, USA

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J.-Y. Maltese, A. M. Oyarce and B. A. Eipper. Prevalence and Turnover of Peptidylglycine \( \alpha \)-amidating Monooxygenase mRNA in Atrial Cardiomyocytes. Journal of Molecular and Cellular Cardiology (1996) 28, 155–163. Peptidylglycine \( \alpha \)-amidating monooxygenase (PAM), the enzyme responsible for the \( \alpha \)-amidation of neuroendocrine peptides, is more prevalent in the atrium of the heart than in pituitary or brain. RNase protection assays indicate that PAM transcripts account for approximately 0.5% of the mRNA in the neonatal atrium and 0.06% of the mRNA in the neonatal ventricle. In primary atrial cardiomyocyte cultures PAM mRNA turns over slowly, with a half-life of approximately 20 h. Levels of PAM mRNA in primary atrial cardiomyocytes are increased to 165% of control upon treatment with dexamethasone and decreased to 63% of control upon treatment with thyroid hormone.

Introduction

Atrial myocytes contain large dense core granules whose morphology closely resembles that of the large dense core granules found in neurons and endocrine cells (DeBold et al., 1991; Rosenzweig and Seidman, 1991). While the large dense core granules of neurons and endocrine cells contain mature peptide products derived from endogenous prohormones, the granules in atrial myocytes contain primarily unprocessed prohormone and endoproteolytic cleavage occurs at or near the time of secretion (Glomoski et al., 1990; Rosenzweig and Seidman, 1991; Greenwald et al., 1992; Sei et al., 1992). Prohormone convertases 1 and 2, the subtilisin-like endoproteases thought to play a key role in prohormone cleavage in neurons and endocrine cells, are not expressed at high levels in the atrium (Bloomquist et al., 1991; Zheng et al., 1994).

In contrast, levels of the peptide amidating enzyme, peptidylglycine \( \alpha \)-amidating monooxygenase (PAM), are higher in atrium than in any other tissue examined (Braas et al., 1989; Maltese and Eipper, 1992; Lew and Smith, 1993). Although endoproteolytically processed forms of PAM are stored in the large dense core granules of neurons and endocrine cells, uncleaved integral membrane PAM proteins predominate in atrium and ventricle (Eipper et al., 1992; Maltese and Eipper, 1993; Oyarce and Eipper, 1993). As for pro-ANP, endoproteolytic cleavage of PAM occurs at or near the time of secretion (Glomoski et al., 1990; Rosenzweig and Seidman, 1991; Maltese and Eipper, 1993).
Although pro-ANP is not processed into \( \alpha \)-amidated product peptides, atrial granules contain precursors to enkephalin, thyrotropin releasing hormone (TRH), neuropeptide Y, adrenomedullin and dynorphin, each of which can be processed to yield \( \alpha \)-amidated product peptides (Howells et al., 1986; Springhorn and Claycomb, 1989; Low et al., 1990; Marek and Mains, 1990; Carnell et al., 1992; Sakata et al., 1993; Ichiki et al., 1994; Ventura et al., 1994). Metabolic labeling studies demonstrated that PAM accounts for 0.3 to 0.5% of the total protein synthesis in primary cultures of neonatal atrial cardiomyocytes (Maltese and Eipper, 1993). The expression of PAM in the heart shows developmental regulation, with striking changes in atrial and ventricular levels of PAM in the perinatal period (Ouaik et al., 1989). PAM expression in cultures of atrial cardiomyocytes is responsive to glucocorticoids and cAMP (Thiele et al., 1989).

In some tissues levels of PAM transcript are rapidly modulated. For example, in hippocampal dentate granule neurons, levels of PAM transcript peak within 1 to 4 h following a single electroconvulsive treatment and return to baseline within 24 h (Bhat et al., 1993). The forms of PAM transcript present in the atrium and ventricle change rapidly near the time of birth (Ouaik et al., 1989). These rapid alterations in the level or form of PAM mRNA suggest the occurrence of rapid changes in gene transcription and/or mRNA stability. The mechanisms underlying these changes in mRNA level have not yet been investigated; the promoter for rat PAM is GC-rich and lacks a TATA motif (Hand et al., 1994). In this series of studies we used an RNase protection assay to determine the prevalence of PAM mRNA in neonatal atrium and ventricle, determine the half-life of PAM mRNA in primary cultures of atrial cardiomyocytes and examine the effect of thyroid hormone and glucocorticoid treatment on expression of PAM mRNA in these cells.

Materials and Methods

Preparation of cardiocyte cell cultures

Atrial tissue taken from 40 neonatal rats (Sprague-Dawley) on the day of birth (postnatal day 1) was dissociated using trypsin and collagenase (DeBold et al., 1991). Cells (2 \( \times \) 10\(^5\) cells/cm\(^2\)) were plated on fibronectin coated dishes (4 \( \mu \)g fibronectin/cm\(^2\)) in DMEM-F12 supplemented with 10% fetal bovine serum. Proliferation of fibroblasts was limited by treating cultures with 10 \( \mu \)M cytosine-\( \beta \)-D-arabinofuranoside for 24 h. On the second day of culture, cells were placed into complete serum-free medium [DMEM-F12, 10 \( \mu \)g/ml insulin, 5 \( \mu \)g/ml transferrin, 1 \( \times \) 10\(^{-6}\)M Na\( \text{SeO}_3\), 1 mg/ml bovine serum albumin (fatty acid-free), 1 \( \times \) 10\(^{-9}\)M triiodothyronine, 1 \( \mu \)M dexamethasone (American Reagent), 15 mM HEPES, pH 7.4] containing 2 \( \mu \)M cytosine-\( \beta \)-D-arabinofuranoside. Cells were subsequently maintained in this serum-free medium lacking cytosine-\( \beta \)-D-arabinofuranoside. For treatment of atrial cardiomyocytes with triiodothyronine or dexamethasone, cultures were fed with serum-free medium lacking both hormones on day 6 of culture. After 48 h in complete serum-free medium lacking triiodothyronine and dexamethasone, cultures were fed with serum-free medium containing various concentrations of triiodothyronine or dexamethasone; in general, cultures were analysed 48 h after hormone treatment. Unless indicated otherwise, chemicals for cell culture were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Northern blot analysis

Total RNA was extracted, fractionated on 1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes as described (Ouaik et al., 1990). The SDS-PIPES buffer system (Bloomquist et al., 1991) was used with prehybridization for 30 min at 65°C and hybridization overnight at 65°C with 1 to 2 \( \times \) 10\(^6\) cpm/ml cDNA probe (labeled with [\( ^{32}P\)]-dCTP by random priming) (Prime-It kit; Stratagene). Filters were washed twice with 5% SDS/1 \( \times \) SSC for 15 min at 65°C and exposed to X-OMAT film (Kodak) for 1 to 4 h. For detection of all forms of PAM mRNA, the 1.3 kb Pst1/BamH1 fragment of rPAM-1 was used (bp 356-1682) (Ouaik et al., 1990).

Levels of the mRNA for ribosomal protein S26 are relatively invariant amongst tissues (Vincent et al., 1993); therefore PAM signals were normalized to the amount of S26 mRNA on each blot. A cDNA probe for rat S26 cDNA was generated using oligonucleotide primers (25-mers) to amplify a fragment from cDNA prepared from adult rat atrial RNA; the 354 bp fragment (nt 37 to nt 390) was subcloned into pBluescript (SK minus) and detected by autoradiography. The prevalence of PAM mRNA in neonatal atrial and ventricular levels of PAM in the perinatal period (Ouaik et al., 1989). These rapid alterations in the level or form of PAM mRNA suggest the occurrence of rapid changes in gene transcription and/or mRNA stability. The mechanisms underlying these changes in mRNA level have not yet been investigated; the promoter for rat PAM is GC-rich and lacks a TATA motif (Hand et al., 1994). In this series of studies we used an RNase protection assay to determine the prevalence of PAM mRNA in neonatal atrium and ventricle, determine the half-life of PAM mRNA in primary cultures of atrial cardiomyocytes and examine the effect of thyroid hormone and glucocorticoid treatment on expression of PAM mRNA in these cells.
Determination of mRNA half-life

Several of the methods described by Harrold et al., 1991) were used to determine the half-life of PAM mRNA in primary cultures of neonatal atrial cardiomyocytes. 5,6-Dichloro-1-β-ribofuranosyl benzimidazole (DRB), an inhibitor of RNA polymerase II, was solubilized in serum-free medium (350 µM) by agitation at 37°C for 3 h. The effect of 70 µM DRB on synthesis of poly(A +) RNA in these primary cultures was verified in preliminary experiments. After 7 days in culture, cells were exposed to serum-free medium containing 70 µM DRB (Harrold et al., 1991) for periods of time ranging from 4 to 48 h. After 24 h of exposure to DRB, cellular morphology remained normal; 48 h of exposure to DRB resulted in vacuolized, unhealthy looking cells, precluding the use of even higher concentrations of DRB (Mes-sina, 1989; Marshall et al., 1991). Equal amounts of total RNA were obtained from control cultures and from cultures treated with DRB for 24 h. Total RNA prepared from DRB-treated cultures was subjected to Northern blot analysis. The 1.3 kb PstI/ BamH1 fragment of rPAM-1 (bp 356-1682) was used to detect all forms of PAM mRNA. Den-sitometric analyses were performed as described above. In three experiments with actinomycin D (5 µg/ml) (Harrold et al., 1991; Zaidi and Malter, 1994), half-life estimates of 8 to 12 h were obtained; however, cells appeared vacuolized and unhealthy within 20 h, making it impossible to use this drug to estimate the half-life of a long-lived mRNA such as PAM.

Results

Abundance of PAM mRNA

Northern blot analysis and biosynthetic labeling experiments both indicated that PAM was a major product in the atrium of neonatal rats and in cultured atrial myocytes (Maltese and Eipper, 1993; Braas et al., 1989). The PAM gene is subject to alternative splicing and PAM-1 and PAM-2 mRNAs are the major forms found in the atrium (Fig. 1). In order to quantify more precisely the total amount of PAM mRNA present and to compare PAM expression to ANP expression, we developed an RNase protection assay with a probe capable of measuring all forms of PAM mRNA (Fig. 1). In two independent experiments, 1 µg RNA prepared from postnatal
Abundance of PAM mRNA. (a) The structures of the PAM mRNAs most prevalent in rat atrium are shown along with the location of the two RNase protection probes and the cDNA probe used in these studies. (b) Total RNA extracted from postnatal day 2 or adult rat atrium (5 μg) and ventricle (10 μg) or the indicated amount of PAM or S26 sense RNA was hybridized with approximately 2 x 10^5 cpm [32P]-labeled Ant3 PAM probe and 3 x 10^5 cpm [32P]-labeled S26 probe as described. After overnight hybridization, samples were digested with a mixture of RNase A and RNase T1, precipitated and electrophoresed through 5% acrylamide gels containing 8 M urea. Size standards are shown to the left. The sense RNA standard includes PAM and S26: the PAM probe protects a fragment of 385 bases while the S26 probe protects a fragment of 354 bases; the intact PAM mRNA is approximately 4000 bases long.

day 2 rat atrium contained 129 ± 39 pg PAM mRNA; the same amount of postnatal day 2 ventricular RNA contained 17.1 ± 6.1 pg PAM mRNA. If we estimate the poly(A +) content of atrial and ventricular RNA at 2 to 5% of the total RNA (Harrold et al., 1991), PAM transcripts account for 0.26 to 0.65% of atrial mRNA and 0.034 to 0.085% of ventricular mRNA. Assuming that each atrial cardiomyocyte contains approximately 40 pg total RNA (Maltese and Eipper, 1992), each would contain about 2400 molecules of PAM mRNA. Adult atrium contained 8.5-fold more PAM mRNA than adult ventricle (118 ± 53 v 13.8 ± 7.6 pg PAM mRNA/μg total RNA); when normalized to levels of S26 mRNA, PAM mRNA levels in adult atrium were approximately double those in postnatal day 2 atrium.

Half-life of PAM mRNA

Although levels of PAM mRNA have been shown to vary considerably in response to various stimuli, the contributions of transcription rate and mRNA stability have not yet been investigated. We used two different methods to measure the turnover of PAM mRNA in primary atrial cardiomyocytes (Harrold et al., 1991). We utilized 5,6-dichloro-1-β-ribofuranosyl benzimidazole, an adenosine analog which inhibits transcription by RNA polymerase II within an hour after its application to cell cultures. Atrial cultures were exposed to 5,6-dichloro-1-β-ribofuranosyl benzimidazole (70 μM) for varying periods of time and total RNA was then subjected to Northern blot analysis (Fig. 2a). Data obtained in four independent experiments (normalized to total RNA) were compiled to yield an estimate of the half-life of PAM transcripts.

(b)
and 12 h; the PAM message was clearly not turning over rapidly.

Regulation of PAM mRNA levels

In the heart, as in the pituitary and the hypothalamus, levels of PAM mRNA are subject to hormonal regulation (Thiele et al., 1989; Ouafik et al., 1990; Grino et al., 1990). Glucocorticoids and thyroid hormone are major hormonal regulators of cardiac tissue and we previously observed a stimulatory effect of dexamethasone, a synthetic glucocorticoid, on PAM expression in atrial cardiomyocytes using Northern blot analysis (Thiele et al., 1989). Glucocorticoids are well known for their hypertrophic effects on cardiac cells and thyroid hormones have profound effects on heart rate and myocardial contractility (Shanker et al., 1987; Rohrer and Dillman, 1988; Argentin et al., 1991). Primary atrial cardiomyocytes kept in culture for 6 days were maintained in the absence of either hormone for 48 h and then treated with either dexamethasone or thyroid hormone for 48 h. RNAs from control and treated cultures were examined following Northern blot analysis or RNase protection. As shown in Figure 3, treatment with 100 nM or 1 μM dexamethasone resulted in an increase in levels of PAM mRNA. Treatment with 10 nM or 100 nM triiodothyronine reduced levels of PAM mRNA. Similar results were obtained using the RNase protection assay (Fig. 4); triiodothyronine treatment reduced levels of PAM mRNA to 66 ± 9% of control (P < 0.005) while treatment with dexamethasone increased levels of PAM mRNA to 170 ± 20% of control (P < 0.005).

Discussion

High level expression of PAM is observed in the atria of several species including rat, bovine and ovine (Eipper et al., 1988; Braas et al., 1989; Maltese and Eipper, 1992; Lew and Smith, 1993), suggesting that the enzyme serves an important function in this tissue. Although several precursors to α-amidated peptides are expressed in the atrium of the heart, the predominant endogenous peptides, ANP and brain natriuretic peptide, are not α-amidated and potentially α-amidated products are found at much lower levels. Estimates of PAM prevalence based on metabolic labeling and mRNA prevalence agree remarkably well; approximately 0.5% of the mRNA and protein synthesis in the atrium of the neonatal rat is devoted to PAM. In comparison, proANP mRNA accounts for 1 to 3% of the total mRNA in the atrium (Rosenzweig and Seidman, 1991). The high levels of PAM found in this tissue in the absence of correspondingly high levels of substrate suggest that PAM might serve a different function in the atrium.

Although expression of PAM in primary atrial cardiomyocytes can be manipulated by treatment with T₃ or dexamethasone, the system is not a rapidly responsive one. The half-life of newly synthesized PAM-2 protein was previously shown to be approximately 8 h in primary cultures of neonatal rat atrium and these studies demonstrate that the half-life of PAM transcripts is approximately 20 h. Half-lives of different mRNAs can vary from 20 min to over 24 h (Sachs, 1993). Levels of PAM mRNA in vivo are subject to fairly rapid regulation during development (Ouafik et al., 1989) and in response to electroconvulsive shock (Bhat et al., 1993). Between embryonic day 21 and postnatal day 1, total levels of PAM mRNA in the rat atrium drop three-fold and the splicing pattern is completely altered (Ouafik et al., 1989). The factors responsible for the rapid turnover of PAM transcripts in vivo do not appear to be functional in these primary cultures.

Thyroid hormones produce a general increase in total RNA and mRNA levels in the rat heart and have a selective effect on expression of a few mRNA species including phospholamban, dihydropyridine-sensitive slow Ca²⁺ channels and sarcoplasmic Ca²⁺ ATPase (Shanker et al., 1987; Rohrer et al., 1991; Seppet et al., 1993; Kimura et al., 1994). Thyroid hormone-stimulated products in the heart are generally energy consuming enzymes; contraction velocity is increased due to a shift in the myosin heavy chain isoform expressed and changes in diastolic relaxation are attributed to alterations in the speed of myocytic Ca²⁺ sequestration (Rohrer and Dillman, 1988; Rohrer et al., 1991). Thyroid hormone treatment of cultured atrial myocytes approximately doubles levels of ANP mRNA and ANP secretion (Gardner et al., 1987; Matsubara et al., 1987).

Addition of thyroid hormone to primary cultures of neonatal atrial myocytes produces a small decrease in levels of PAM mRNA. PAM mRNA levels in the adult rat anterior pituitary decline more dramatically as thyroid hormone levels rise from hypothyroid to euthyroid; in contrast, PAM expression in adult male rat atrium was unaffected by thyroid hormone levels (Ouafik et al., 1990). The rat PAM promoter is a GC-rich, TATA-less promoter lacking any consensus thyroid hormone response elements in the 2000 nucleotides
Regulation of PAM mRNA levels by dexamethasone and triiodothyronine. After growth in the absence of dexamethasone or triiodothyronine for 2 days, triplicate cultures of atrial cardiomyocytes were treated with the indicated dose of hormone for 48 h. Total RNA (2 μg) was fractionated on denaturing gels, transferred to nylon membranes and hybridized simultaneously with cDNA probes for PAM (1.3 kb probe) and the ribosomal protein S26. (a) A representative Northern blot. (b) Data from three independent experiments like the one shown in panel (a) have been compiled. Differences between samples and controls were significant at the $P < 0.05$ level using the Student's $t$-test.

preceding the transcriptional start site (Hand et al., 1991; Greenwald et al., 1992; Sei et al., 1992). PAM mRNA levels are increased in parallel with pro-ANP mRNA levels in response to dexamethasone. Like pro-ANP, some of the endogenous precursors to α-amidated peptides identified in the atrium might undergo rapid cleavage and α-amidation coupled to secretion to yield peptides that play an autocrine or paracrine role in the effect of thyroid hormone or glucocorticoids on atrial growth. The PAM promoter lacks a consensus glucocorticoid response element (Hand et al., 1994); however, it contains a putative AP1 site, suggesting a mechanism through which the glucocorticoid receptor could regulate transcription of the PAM gene (Yang-Yen et al., 1990; Vig et al., 1994).

Current studies on the determinants of mRNA stability suggest that mRNAs are inherently stable; the rapid turnover observed for certain transcripts...
Figure 4  Analysis of regulation using RNase protection assay. Triplicate cultures of atrial cardiomyocytes were treated as described in Figure 3. (a) Total RNA (5 μg) was analysed by RNase protection using the rPHMs riboprobe (2.5 × 10^6 cpm/sample) and the S26 riboprobe (8 × 10^5 cpm/sample). Protected fragments were analysed on a 3% acrylamide gel containing 8 M urea. (b) Normalized data were averaged; differences from control were significant at the P<0.005 level.

requires the presence of specific signals (Sachs, 1993; Cleveland and Yen, 1989). Two elements in the 3'-untranslated region of PAM transcripts may play a role in turnover: two AUUUA motifs, which serve as destabilizing elements in transcripts encoding several lymphokines, cytokines and immediate early genes (Cleveland and Yen, 1989; Sachs, 1993), are present; an 86 nucleotide segment at the 3'-end of PAM transcripts is the most highly conserved part of the nucleotide sequence (Stoffers et al., 1989). The lack of rapid turnover of PAM in atrial cardiomyocytes maintained in serum-free culture despite what appears to be rapid turnover in vivo suggests that additional factors or tissue-specific interactions are required to activate this process.

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References


