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Differential Regulation of Peptide α-Amidation by Dexamethasone and Disulfiram

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ABSTRACT

α-Amidation is essential for the function of many peptides in intercellular communication. This C-terminal modification is mediated in a two-step process by the hydroxylase and lyase activities of the bifunctional enzyme, peptidylglycine α-amidating monooxygenase (PAM). The first step, catalyzed by peptidylglycine-α-hydroxylating monooxygenase (PHM; EC 1.14.17.3), is rate limiting in the process, and therefore subject to regulation. Dexamethasone and disulfiram (tetraethylthiuram disulfide; Antabuse) were used as in vivo treatments to study the regulation of PHM expression and activity in cardiac atrium. Our findings show that both dexamethasone and disulfiram treatment increase the activity of PHM in atrial tissue but that they do so by distinctly different mechanisms. Dexamethasone elevated tissue levels of PAM mRNA and protein concurrently, suggesting that glucocorticoids regulate PAM expression at the level of gene transcription. In contrast, disulfiram treatment, which depletes stores of α-amidated peptides, increased the specific activity of PHM without affecting the level of PAM expression. The catalytic efficiency of PHM was enhanced by raising the V_{max} of the enzyme. Importantly, this increase in V_{max} was retained through purification to homogeneity, indicating that either a covalent modification or a stable conformational change had occurred in the protein. These novel findings demonstrate that the rate-limiting enzyme in the bioactivation of peptide messengers is differentially regulated by transcriptional and post-transcriptional mechanisms in vivo. It is proposed that regulation of PHM's expression and catalytic efficiency serve as coordinated physiologic mechanisms for maintaining appropriate levels of α-amidating activity under changing conditions in vivo.

Neural and endocrine peptides perform a diverse and indispensable array of functions in intercellular communication. More than half of these peptides require α-amidation for receptor recognition and signal transduction. α-Amidation is a terminal modification in peptide biosynthesis and can itself be rate limiting in the overall production of α-amidated peptides. This essential post-translational modification is catalyzed by peptidylglycine α-amidating monooxygenase (PAM; E.C. 1.14.17.3), a bifunctional enzyme localized in the *trans*-Golgi network and secretory granules (Bradbury and Smyth, 1991; Eipper et al., 1992; Eipper et al., 1993). PAM is encoded by a single gene and constitutes the only known mechanism for peptide α-amidation in vivo. The peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase activities of PAM sequentially catalyze α-amidation in a two-step process. In this sequence, PHM is rate limiting and requires molecular oxygen, ascorbate and copper for activity.

The regulation of α-amidation is complex and includes mechanisms that control the expression and processing of PAM mRNA and protein. Hormonal control of PAM expression is evident in the anterior pituitary and atrium, where levels of PAM mRNA are increased by hypothyroidism (Ouafik et al., 1990) or treatment with glucocorticoid (Thiele et al., 1989). Alternative splicing proceeds in a tissue-specific manner to generate at least seven different forms of the enzyme (Ouafik et al., 1990, 1992; Katopodis and May, 1990; Eipper et al., 1992a, 1993; Suzuki et al., 1993). This structural diversity is increased further by endoproteolytic processing (Eipper et al., 1992b). Together, alternative splicing and proteolytic cleavage determine whether PAM proteins will be membrane-bound or soluble and whether the two catalytic domains will be linked or separated. The complex patterns of PAM expression are tightly controlled in a tissue-specific and developmental manner. Differential processing is also important for directing intracellular routing (Milgram et al., 1992, 1993; Eipper et al., 1993) and can influence the kinetic properties of the different molecular forms of PAM (Husten et al., 1993).

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ABBREVIATIONS: PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; Tris, tris(hydroxymethyl)-aminomethane; HIC, hydrophobic interaction chromatography; MQ, Mono-Q; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride.

There is evidence that another mechanism exists for the regulation of peptide α-amidation (Mueller et al., 1993). Reduced Cu^{2+} in vivo, induced by administration of disulfiram (tetraethylthiuram disulfide; Antabuse), causes a dramatic decrease in tissue concentrations of α-amidated peptides. In response, PHM protein is modified such that its activity is actually increased when the enzyme is assayed at optimal Cu^{2+} concentrations in vitro. Kinetic experiments performed on unpurified tissue extracts showed that this increase in activity is attributable to an elevation in the enzyme's maximal velocity (V_{max}) with no change in K_M. Importantly, expression levels of PHM are not altered by disulfiram treatment. The reduced peptide α-amidation and increased V_{max} of PHM persist for more than 2 weeks following cessation of treatment and cannot be explained by the continued presence of disulfiram, which is metabolized rapidly (Faiman, 1987). Rather, the time course for changes in α-amidation and the altered functional characteristics of PHM protein indicate that the enzyme is modified as a compensatory response to the inhibition of α-amidation. Thus, the observed modulation of enzyme activity is thought to arise from a physiologic mechanism that normally regulates the activity of PHM.

This project was designed to directly compare the effects of in vivo dexamethasone and disulfiram administration on the regulation of PAM expression and PHM activity. Our working hypothesis has been that the disulfiram-induced elevation in PHM's V_{max} arises from covalent post-transcriptional modification of the enzyme, which can occur independently from changes in PAM expression. To test this hypothesis, we used disulfiram as a means to alter the enzymatic activity of PHM, in conjunction with dexamethasone to enhance PAM expression. The present findings demonstrate that disulfiram's effect on PHM's V_{max} persists through purification to homogeneity, and can occur either independently or in conjunction with increased production of PAM protein. These novel findings provide compelling evidence that PHM activity is regulated by covalent modification, and establish a foundation for efforts designed to define the structural nature of the modification involved.

Materials and Methods

Animal Treatments and Tissue Collection. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA or Taconic Farms, Germantown, NY) weighing 250 to 300 g were housed under a 12-h light/dark cycle and received food and water ad libitum. Disulfiram (Sigma Chemical Co., St. Louis, MO) and dexamethasone (Phoenix Pharmaceutical, Inc., St. Joseph, MO) were prepared in 0.9% saline containing 0.5% Tween 80 and administered daily (6–8 days) by s.c. injection (disulfiram, 300–400 mg/kg; dexamethasone, 1–5 mg/kg; n = 30–60 animals per group). Control animals received injections of vehicle only. Animals were sacrificed (CO_2 asphyxiation) 24 h after the last injection. Dissected atria were rinsed in ice-cold Dulbecco's PBS and stored at −70°C.

Preparation of Soluble PHM from Atrial Membranes. Frozen atria were minced and homogenized in 10 vol (gm/ml) of 20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesolfonic acid, sodium salt, pH 7.0, containing 0.25 M sucrose, 0.6 M KCl, and protease inhibitors (1 mM EDTA, 0.3 mg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml lima bean trypsin inhibitor, 10 μg/ml bacitracin, and 10 μg/ml leupeptin) using a Polytron (Brinkman Instruments, Westbury, NY) at power setting 3.5 for 10 to 15 s followed by Potter-Elvehjem glass/Teflon homogenization (five up and down strokes). The homogenate was centrifuged at 6500 rpm in a Sorval RC-5 centrifuge using a SS34 rotor ($4900g_{av}$) for 15 min at 4°C. The resulting pellet was rehomogenized (glass/Teflon) in 5 to 10 ml homogenization buffer and centrifuged as above. The supernatants were pooled and centrifuged at $233{,}000g_{av}$ for 60 min at 4°C. The resulting membrane pellet (microsomal fraction) was resuspended by glass/Teflon homogenization in 10 to 20 ml of 20 mM Tris-HCl, pH 8.0, containing protease inhibitors and subjected to three freeze-thaw cycles. The membranes were brought to 1 M NaCl, dispersed by glass/Teflon homogenization and repelleted by ultracentrifugation. The membranes were resuspended by glass/Teflon homogenization in 3 to 5 ml of 20 mM Tris-HCl, pH 8.0, without protease inhibitors and stored at −70°C.

PHM catalytic domain was solubilized from membrane-bound bifunctional PAM by limited tryptic digestion. Atrial membranes were brought to room temperature, adjusted to a final protein concentration of 7 to 10 mg/ml with 20 mM Tris-HCl, pH 8.0, and dispersed by glass/Teflon homogenization. All subsequent manipulations were performed at ambient temperature to prevent the formation of cryoprecipitate. Trypsin (Worthington Biochemical, Freehold, NJ) was added at a ratio of 1:150 (protein w/w), and proteolysis was carried out for 2 min. Digestion was terminated by the addition of protease inhibitors as above without EDTA. The digest was prepared for hydrophobic interaction chromatography (HIC) by adding an equal volume of 100 mM K_2HPO_4, pH 7.6, 2 M $(NH_4)_2SO_4$. Insoluble material was removed by ultracentrifugation at $300{,}000g_{av}$ for 15 min at 22°C. Protein concentrations were estimated by Lowry assay (Lowry et al., 1951), using BSA as the standard.

HIC. The supernatant from trypsinized atrial membranes was applied to an HR 10/10 phenyl Superose column (Pharmacia/LKB Biotech. Inc., Piscataway, NJ) equilibrated with 1 M $(NH_4)_2SO_4$, 50 mM K_2HPO_4, pH 7.6. Proteins were eluted at 1 ml/min with a discontinuous decreasing $(NH_4)_2SO_4$ gradient from 1 to 0 M. The initial stage of the gradient was from 1 M to 150 mM over 30 min, followed by isocratic holds at 150 mM and 50 mM for 5 min and 10 min, respectively, before a final step to 0 mM. Chromatography was performed at ambient temperature on a Pharmacia/LKB system equipped to measure absorbance at 280 nm and conductivity through the flow cell. Fractions (1 ml, in borosilicate glass tubes) containing maximal PHM activity by assay were pooled (average total volume, 8 ml) and desalted by gel filtration (Sephadex G-25 M PD-10 columns, Pharmacia Biotech AB, Uppsala, Sweden) into 20 mM Tris, pH 8.0.

Anion Exchange Chromatography. Desalted HIC-purified PHM was applied to an HR 5/5 Mono-Q (MQ) anion exchange column (Pharmacia/LKB) equilibrated with 20 mM Tris-HCl, pH 8.0. Following a 10-min isocratic hold for sample application, proteins were eluted at 1 ml/min with an increasing gradient of 0 to 200 mM NH_4Cl in 20 mM Tris-HCl, pH 8.0, over 30 min. Fractions (1 ml, collected in polypropylene tubes) determined to have maximal activity by assay were pooled (average total volume, 6 ml). Kinetic analyses were performed on aliquots of peak activity diluted 1:1 with 2×-concentrated assay diluent (described below) containing protease inhibitors without EDTA and stored at −70°C.

PHM Activity Assay and Kinetic Analysis. PHM activity was assayed as previously described (Perkins et al., 1990) in a total volume of 40 μl 150 mM 2-[*N*-morpholino]ethanesulfonic acid, pH 5.0, containing 0.5 μM $CuSO_4$, 1 mM ascorbic acid, 300 μg/ml catalase (Sigma Chemical Co.), 0.5 μM α-N-Ac-Tyr-Val-Gly and [^{125}I]iodo-α-N-Ac-Tyr-Val-Gly (30,000–60,000 cpm). Samples were diluted for assay in 10 mM Tris-HCl, pH 7.0, containing 0.2 mg/ml BSA and 1% Triton X-100 (Surfact-Amps X-100, Pierce Chemical Co, Rockford, IL). Kinetic analyses were performed over a range of 1 to 60 μM α-N-Ac-Tyr-Val-Gly using a minimum of five concentrations in duplicate. Enzyme dilution and/or incubation times were adjusted so that maximal conversion of substrate to product remained within the linear range of the assay (less than 20%). Kinetic data were analyzed using the EnzFitter program (Elsevier Biosoft, Cambridge, UK).

Reversed Phase HPLC. Chromatography was performed using a 4.6 × 250 mm Vydac C_4 column (The Nest Group Inc., Southboro, MA) on a Hewlett-Packard (Wilmington, DE) series 1100 HPLC system equipped with HP ChemStation software and a diode array detector. The system was operated at a flow rate of 1 ml/min, and a column temperature of 40°C. Initial conditions were 97.5% solvent A (0.1% trifluoroacetic acid and 2.5% acetonitrile in water) and 2.5% solvent B (0.08% trifluoroacetic acid in acetonitrile). Following sample loading, initial conditions were maintained for 5 min, after which a linear gradient to 42.5% solvent B was developed over the next 10 min. Isocratic conditions were maintained at 42.5% solvent B for 5 min followed by an increase to 92.5% solvent B over 2.5 min. Elution profiles were monitored at 280 and 214 nm, and peaks were collected manually.

Amino Acid Sequencing. PHM protein from anion exchange chromatography (1–2 μg) was concentrated to approximately 100 μl by lyophilization. The sample was brought to 4 M guanidine HCl containing 10 mM dithiothreitol and incubated for 3.5 h at 50°C. After cooling to room temperature, iodoacetamide (Sigma Chemical Co) was added to a final concentration of 4 mg/ml. The alkylation reaction was stopped after 20 min by snap freezing on dry ice. The reduced and alkylated protein was separated from reactants by HPLC, as described above. The collected fraction was concentrated by lyophilization and applied to a precycled glass filter coated with Polybrene matrix (Perkin-Elmer, Foster City, CA). NH_2-terminal amino acid sequencing was performed by automated Edman degradation on an Applied Biosytems (San Fransisco, CA) model 476A protein sequencer.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Silver Staining. Proteins were resolved in precast 12% polyacrylamide gels (Novex Experimental Technology, San Diego, CA) using the Tris-glycine SDS buffer system described by Laemmli (1970). Samples were concentrated by lyophilization and reconstituted in nonreducing loading buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% SDS, 20% glycerol, and 0.003% bromphenol blue). A 10-K molecular weight ladder (Gibco BRL, Gaithersburg, MD) was used to estimate M_r values. Silver staining was performed using the SilverXpress system (Novex Experimental Technology). Highly purified PHM catalytic domain was quantified from densitometric scans of silver-stained gels using a standard curve generated from known amounts of recombinant PHM (rat amino acids 42–356) run in adjacent lanes of the same gel.

Immunoblot Analysis and Anti-PHM-Specific Antibodies. For immunoblot analysis, samples were reduced by boiling for 5 min in loading buffer containing 2.5% β-mercaptoethanol, resolved by SDS-PAGE (described above) and transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA) as described previously (Towbin et al., 1979) using 10% methanol in the transfer buffer. Membranes were blocked with a solution of 50 mM Tris-HCl, pH 7.6, 0.8% NaCl, 0.1% Tween 20 (TBST) containing 1% (w/v) nonfat dried milk and 1% horse serum (blocking buffer). Blots were incubated for 2 to 18 h with anti-PHM antibodies diluted in blocking buffer and then extensively washed with TBST. Immunoreactive proteins were visualized with an enhanced chemiluminescent reagent system (ECL, Amersham Searle Corp, Arlington Heights, IL). Rabbit polyclonal antibodies were generated to synthetic PHM peptides [Ab100, rat (r)PAM-1(293–315); Ab246, rPAM-1(116–131)] or to purified recombinant PHM protein [Ab475 and Ab1761, rPHM(37–382)]. The specificity of Ab475 has been mapped to a single epitope located within rPHM(370–382) (Eipper et al., 1995). Ab1761 has not been characterized with respect to epitope specificity; however, it recognizes both PHM size isoforms with equal intensity. All antibodies were used at a dilution of 1:1000, except Ab1761 which was diluted 1:10,000.

Northern Blot Analysis. Northern blots were prepared from total RNA isolated from atrium pairs (RNAgents, Promega, Madison, WI). RNA was fractionated by denaturing formaldehyde agarose gel electrophoresis and transferred to nylon membrane as described previously (Sambrook et al., 1989). PAM mRNA was visualized radiographically with a random-labeled rat PAM-1 cDNA probe (base pairs 351–1681). Quantification of PAM mRNA was standardized by stripping the blots and reprobing with a cDNA probe derived from frog ribosomal RNA (28S). Autoradiographic signals were scanned, digitized, and analyzed statistically by Duncan's multiple-range comparison test after two-way ANOVA.

Results

Purification of PHM Catalytic Domain. Groups of rats were treated with either dexamethasone, disulfiram, or the two in combination as described in *Materials and Methods* (control animals received vehicle only). Purification of PHM catalytic domain from atria was performed identically and in parallel for the four groups. Soluble monofunctional PHM was generated from membrane-bound bifunctional PAM by limited proteolysis with trypsin. Figure 1 shows immunoblot analysis of isolated atrial membranes for each treatment group before trypsinization (lanes 1–4) and following digestion (lanes 5–8). Three major forms of PAM are evident in the pretryptic membrane fractions and represent PAM-1 (120 K), PAM-2 (105 K), and either PAM-3 or a processed form of PAM-1 or PAM-2 (100 K) (Maltese and Eipper, 1992). Limited tryptic digestion produced a major immunoreactive product of approximately 37 K indicated by the arrows in Fig. 1. Time-course and dose-response experiments were performed to determine optimal conditions for trypsinization (data not shown). No differences in the sensitivity of PAM to proteolytic digestion were observed among the treatment groups. Limited tryptic digestion consistently resulted in a 1.5- to 2-fold increase in PHM activity for each treatment group, suggesting that PHM is inhibited sterically either by the membrane environment or by its association with peptidyl-α-hydroxyglycine α-amidating lyase in full-length PAM.

The soluble PHM catalytic domain was fractionated by HIC. PHM was retained on the column and eluted as a single peak of activity between 150 to 50 mM $(NH_4)_2SO_4$ (Fig. 2). Immunoblot analysis demonstrated direct correlation between PHM activity and the presence of immunoreactive PHM protein (Fig. 2, inset). No differences in chromatographic behaviors were noted for PHM prepared from the four treatment groups. Peak PHM activity from HIC was pooled, desalted by gel filtration, and subjected to anion exchange chromatography. PHM activity eluted in a biphasic manner between 50 to 100 mM NH_4Cl (Fig. 3). PHM protein and activity from the four treatment groups behaved similarly on anion exchange chromatography. The activity profile correlated directly with the elution of two PHM isoforms (lower band 36.3 K; upper band 38 K) that were readily visualized by SDS-PAGE and silver staining (Fig. 3, inset). Thus, both isoforms of PHM are active, with the lower band constituting the major component of the first activity peak (fraction 19) and the upper band constituting the major component of the second activity peak (fractions 23 and 24). All treatment groups consistently demonstrated both isoforms, and no differences in the ratio of lower band to upper band were evident among the groups. More detailed characterization of the isoforms is presented below.

Figure 4 shows the progressive purification of PHM catalytic domain from atrial membranes to an essentially homogeneous preparation following anion exchange chromatography. Attempts to separate the isoforms by reversed phase

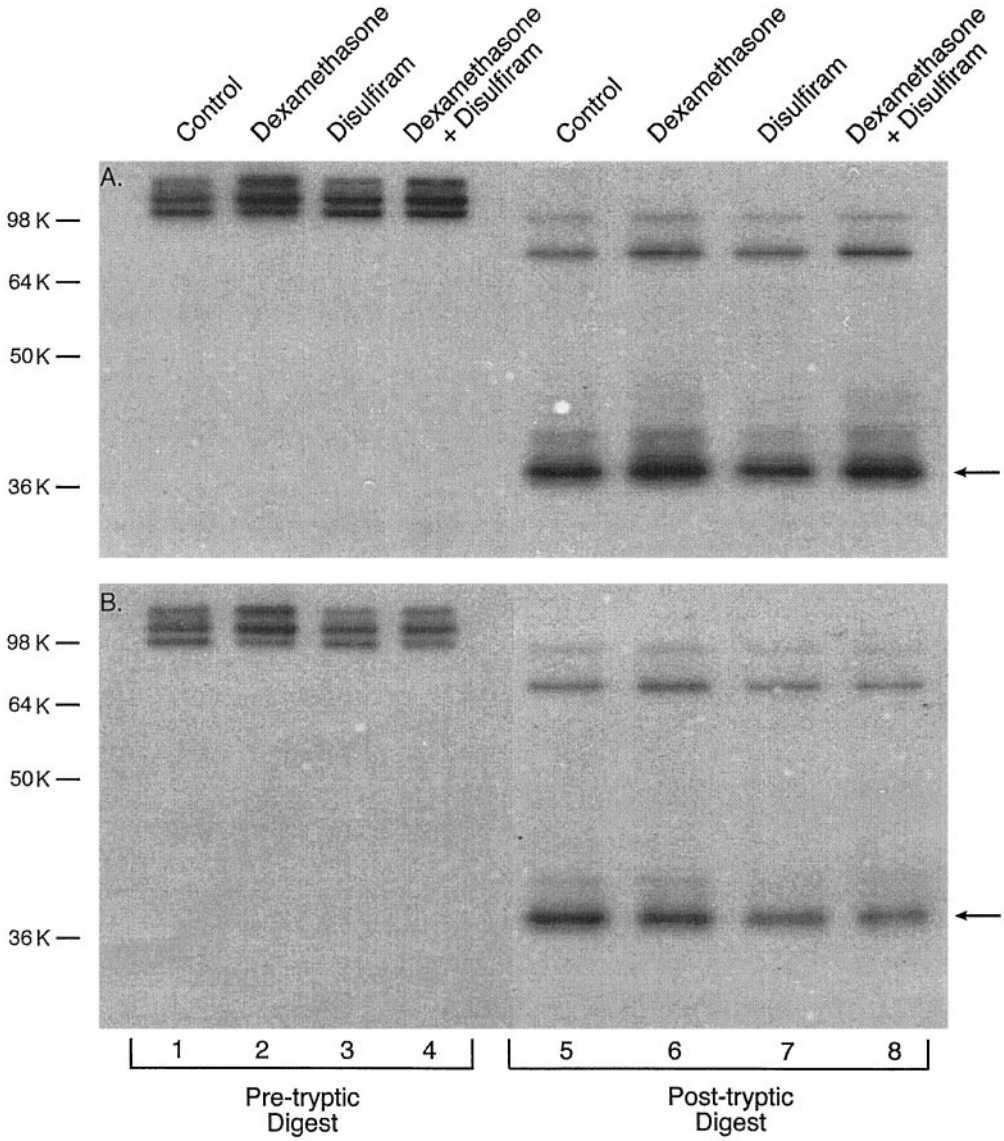

Fig. 1. Tryptic processing of membrane-bound PAM to soluble PHM. Atrial membranes were prepared from each treatment group and subjected to limited tryptic digestion as described in *Materials and Methods*. Samples for electrophoresis were taken before (lanes 1–4) and following (lanes 5–8) digestion with trypsin. Proteins were fractionated by SDS-PAGE, transferred to PVDF membrane, and probed with anti-PHM antibody Ab1761. A, samples were loaded at equivalent protein, 1.85 μg/lane; B, samples were loaded at equivalent activities, 172 pmol AcYV-amide formed/h for pre-tryptic samples and 334 pmol AcYV-amide formed/h for post-tryptic samples. Arrows indicate the location of soluble PHM catalytic domain.

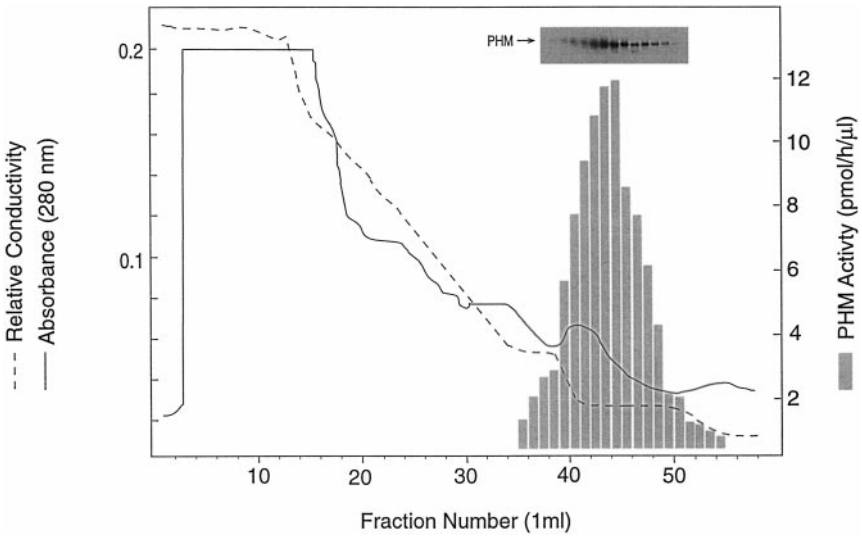

Fig. 2. Purification of PHM by hydrophobic interaction chromatography. PHM catalytic domain was prepared from atrial membranes by limited tryptic digestion as described in *Materials and Methods*. The elution gradient was initiated concurrently with the loading of the digest (8.2 ml), and fractions were collected at 1-min intervals. Inset, immunoblot analysis for fractions across the activity peak using anti-PHM antibody Ab1761. The profile shown is for PHM catalytic domain from control animals and is representative of all treatment groups.

HPLC using C_4 and C_{18} columns were unsuccessful; however, HPLC was an effective method for concentrating PHM protein.

A summary of parallel purifications of four treatment groups is presented in Table 1. On average, atrial PHM was purified 460-fold with 11% yield. Both dexamethasone and disulfiram treatments resulted in post-tryptic atrial membrane preparations with significantly higher specific activities compared to control. When administered individually, dexamethasone and disulfiram produced almost 2-fold increases in specific activity. When administered together, the combination resulted in a 3-fold increase, indicating an additive effect. Highly purified preparations (Table 1, MQ peak pool), however, showed increased specific activities only for PHM isolated from disulfiram-treated groups. These results indicate that dexamethasone and disulfiram increase PHM specific activity by different mechanisms (discussed further below).

Characterization of PHM Isoforms. NH_2-terminal amino acid sequencing was performed on HPLC-purified preparations that contained both isoforms. The analysis produced a single sequence: SFXNE(C)LGXIGP (where (X) represents an unidentified residue, and (C) indicates cysteine not identified because of alkylation). This sequence is that of rat PAM (Ser^{42}-Pro^{53}), and the data showed no evidence of a second amino terminus; therefore, both PHM isoforms have identical NH_2 termini, beginning at Ser^{42}. These termini were generated by tryptic cleavage at Lys^{41} during the limited proteolytic digestion used to solubilize PHM for purification.

The COOH termini of the isoforms were mapped by immunoblot analysis using differential antibody recognition (Fig. 5). The location of antibody epitopes within PHM catalytic domain is depicted above the blots. Antibodies Ab246 and Ab100 were generated to synthetic peptides corresponding to rPAM (116–131) and rPAM (293–315), respectively. Antibody Ab475 has been mapped to an epitope within rPAM (370–382) (Eipper et al., 1995). Antibodies Ab246 and Ab100 recognized both PHM isoforms, whereas Ab475 recognized only the larger isoform. These results demonstrate that the larger isoform contains an intact Ab475 epitope, whereas the smaller is truncated within or before this epitope. Because the COOH termini were also generated by tryptic digestion, cleavage sites can be deduced from the known sequence. The putative COOH terminus for the larger isoform is Lys^{383}. Cleavage at this site would preserve the Ab475 epitope and result in a polypeptide with a calculated M_r = 38,070, in good agreement with SDS-PAGE estimates. Cleavage at the next tryptic site (Lys^{401}) would result in a protein too large to be consistent with SDS-PAGE data. Similarly, the COOH-terminal residue for the smaller isoform is most likely Lys^{368}. This cleavage site eliminates the Ab475 epitope and generates a protein of appropriate size (calculated M_r = 36,340). Although cleavage at the next site toward the NH_2 terminus (Arg^{344}) would result in a protein considerably smaller than that observed on SDS-PAGE, alternative sites within the Ab475 epitope (Lys^{373} or Lys^{375}) cannot be definitively ruled out by this analysis. Nonetheless, assuming NH_2 termini at Lys^{383} (upper band) and Lys^{368} (lower band), the calculated difference between the two forms, 1730 Da, is in good agreement with the difference observed on SDS-PAGE. The 15 amino acid extension on the larger isoform contains three glutamate residues that likely account for the longer retention of this isoform on anion exchange chromatography (Fig. 3).

Induction of PAM Expression by Dexamethasone. Immunoblot analysis of atrial homogenates (Fig. 6) clearly shows that the two dexamethasone-treated groups (lanes 2 and 4) had significantly more immunoreactive PAM-1 (120 K) and PAM-2 (105 K) than either the control or disulfiram-treated groups (lanes 1 and 3, respectively). The identity of the band at 100 K has not been confirmed but could represent PAM-3 or a processed form of PAM-1 or PAM-2 (Maltese and Eipper, 1992). The apparent ratio of PAM-1 to PAM-2 expression did not change with treatment, indicating that the increased specific activity was not due to the up-regulation of a particular isoform.

Increased PAM expression by dexamethasone was also evident in isolated atrial membranes (Fig. 1A, lanes 2 and 4) and was reflected in higher amounts of 37 K PHM catalytic domain following tryptic digestion (Fig. 1A, lanes 6 and 8, arrow). In contrast, samples loaded at equivalent activities showed no difference between dexamethasone treatment and control (Fig. 1B, lanes 5 and 6, arrow) indicating that increased specific activity in post-tryptic digests (Table I) was due to the presence of additional PHM protein. The elevated

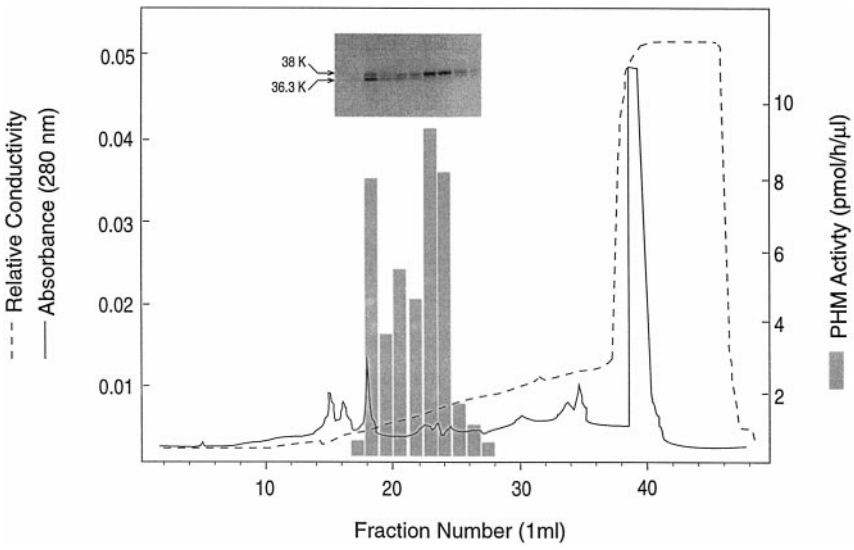

Fig. 3. Purification of PHM by anion exchange chromatography. Pooled peak activity from hydrophobic interaction chromatography (7.5 ml) was desalted by gel filtration and fractionated on a Mono-Q anion exchange column as described in *Materials and Methods*. Following loading of the sample, the gradient was initiated, and fractions were collected at 1- min intervals. Inset, silver-stained SDS-PAGE of fractions across the activity peak. The profile shown is for PHM catalytic domain from disulfiram-treated animals and is representative of all treatment groups.

protein expression associated with dexamethasone treatment remained evident through purification to homogeneity. Greater amounts of immunoreactive PHM were recovered following HIC for the groups administered dexamethasone (Fig. 7A, lanes 2 and 4), and the final yields of highly purified PHM catalytic domain were increased more than 2-fold (Table I, MQ peak pool). The dexamethasone-induced increase in PAM protein expression was accompanied by elevated levels of PAM mRNA (Fig. 8). Importantly, there was no induction of mRNA encoding PAM with disulfiram treatment, as reported previously (Mueller et al., 1993). These results indicate that increased gene transcription or enhanced mRNA stability is responsible, at least in part, for dexamethasone-induced elevation of PAM expression.

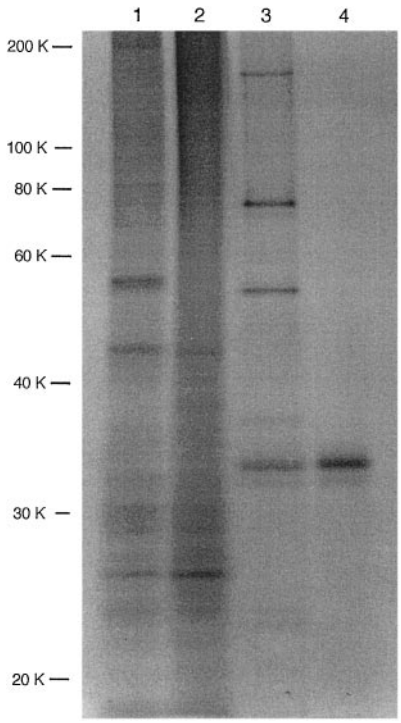

Fig. 4. Purification of PHM from atrial membranes. Proteins were fractionated by nonreducing SDS-PAGE (12% acrylamide gel) and visualized by silver staining. Lane 1, pretryptic atrial membranes (1.5 μg); lane 2, post-tryptic atrial membranes (1.5 μg); lane 3, HIC activity peak pool (0.46 μg); lane 4, anion exchange activity peak pool (0.09 μg). The samples shown are for a dexamethasone + disulfiram treatment group.

Increased PHM Specific Activity by Disulfiram. In contrast to dexamethasone treatment, the increased specific activity of PHM induced by disulfiram was not due to higher levels of protein expression. Post-tryptic digests of atrial membranes (Fig. 1A) demonstrated little quantitative difference in PHM protein between control and disulfiram treatments (lanes 5 and 7) or between dexamethasone and dexamethasone + disulfiram treatments (lanes 6 and 8). Yet, disulfiram treatment significantly increased PHM specific activity in both cases (Table 1). These data suggest that the intrinsic activity of the protein itself had changed. The increased specific activity induced by disulfiram treatment is illustrated by immunoblot analysis presented in Fig. 1B. When samples with equivalent amounts of activity were analyzed, significantly less immunoreactive PHM protein was evident for disulfiram-treated groups in both pre- (lanes 3 and 4) and post-tryptic digests (lanes 7 and 8); i.e., less disulfiram-activated PHM protein was required to attain a level of activity comparable with control or dexamethasone treatments. Disulfiram, therefore, acts by increasing the specific activity of PHM protein itself. This effect remained evident following HIC purification (Fig. 7B, lanes 3 and 4) and was retained through purification to homogeneity. Following anion exchange chromatography, specific activities for highly purified PHM catalytic domain from disulfiram-treated groups were, on average, increased 2-fold (Table 1, MQ peak pool). Optimal concentrations of cofactors (copper and ascorbate) required for PHM activity were not altered by either disulfiram or dexamethasone treatment (data not shown).

Kinetic analyses performed on these samples demonstrated that enzyme isolated from the disulfiram-treated groups had higher maximal velocities (V_{max}) compared with control or dexamethasone treatment groups (Table 2). K_M values for PHM were unaffected by either dexamethasone or disulfiram administration. Figure 9 presents immunoblot analysis of highly purified PHM normalized by maximal velocity (V_{max}) for each treatment group. The averaged relative signal intensities indicate that at least 50% more PHM protein was required from the control and dexamethasone groups to attain activity parity with PHM isolated from the

TABLE 1
Summary of PHM purifications

	Total activity	Total Protein[a]	Specific Activity	Fold Purification	Yield
	pmol/h	*μg*	*pmol/h/μg*		*%*
Control group					
Post-tryptic digest	168,000	22,950	7.4		100
HIC peak pool	26,300	114	231	31	16
MQ peak pool	14,300	3.7[b]	3865	522	9
Dexamethasone group					
Post-tryptic digest	354,900	26,550	13.4		100
HIC peak pool	87,200	136	641	48	25
MQ peak pool	38,500	9.4[b]	4096	306	11
Disulfiram group					
Post-tryptic digest	406,300	28,350	14.3		100
HIC peak pool	75,900	158	479	33	19
MQ peak pool	44,900	4.7[b]	9553	668	11
Dexamethasone + disulfiram group					
Post-tryptic digest	548,100	26,550	20.6		100
HIC peak pool	102,000	158	644	31	19
MQ peak pool	67,500	9.5[b]	7105	345	12

[a] Determined by Lowry protein assay, except as noted.
[b] Determined by densitometric scan of silver-stained SDS gel.

groups treated with disulfiram. Thus, the increased specific activity resulting from disulfiram treatment is directly attributable to an elevated V_{max} of the enzyme with no change in K_M. Because this effect was retained through limited proteolysis of PAM and multiple purification steps, it seems reasonable to conclude that disulfiram treatment induced either a covalent modification or a stable conformational change within the PHM catalytic domain.

Discussion

The bioactivation of many neural and endocrine peptides is dependent upon α-amidation. PHM can be the limiting step in the formation of α-amidated peptide messengers, and therefore constitutes an important site for biologic control. We have used in vivo administration of dexamethasone and disulfiram to investigate the regulation of PHM in rat atrium, the tissue in which PHM is most abundant. Although both treatments increase the activity of PHM, each does so by a distinctly different mechanism. Dexamethasone increases the level of PAM protein in atrium through a mechanism involving gene activation. Importantly, the kinetic properties of PHM purified from dexamethasone-treated animals do not differ from control enzyme. In contrast, disulfiram treatment elevates α-amidating activity by inducing an increase in the V_{max} of PHM without altering protein expression. This effect of disulfiram on PHM activity was previously documented in crude tissue extracts (Mueller et al., 1993). The novel data presented here show that the disulfiram-induced increase in PHM's V_{max} is retained through limited proteolysis and purification to homogeneity. Thus, it is reasonable to conclude that PHM undergoes covalent modification in response to disulfiram treatment. Alternatively, it is possible that a stable conformational change in the protein could account for its altered activity. Finally, a disulfiram-mediated increase in V_{max} is also evident when PAM expression is up-regulated by dexamethasone indicating that the two mechanisms can function independently, but may also work concurrently, to maintain levels of α-amidated peptides.

The effects of glucocorticoids on PAM expression are tissue specific, and for those tissues examined to date, only cardiac atrium demonstrates up-regulation both in vivo, as shown here, and in vitro (Thiele et al., 1989). In contrast, glucocorticoid treatment decreases PAM mRNA levels and activity in mouse AtT-20 corticotrope tumor cells (Thiele et al., 1989; Maltese et al., 1996), and decreases PAM secretion in cultured rat medullary thyroid carcinoma cells (Birnbaum et al., 1989). Additionally, Grino and coworkers (Grino et al., 1990) reported that adrenalectomy increases hypothalamic PAM mRNA levels in rats, an effect that was reversed by the

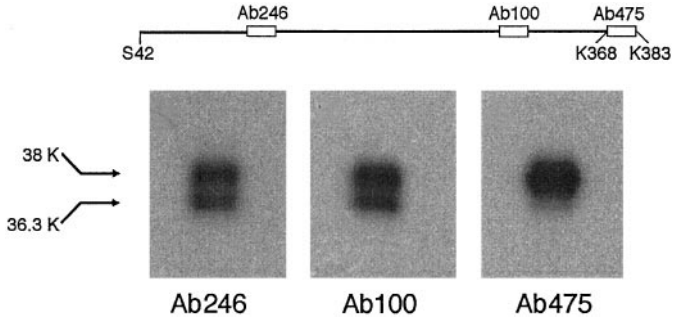

Fig. 5. Immunoblot analysis for PHM isoforms. Equal aliquots of an anion exchange chromatography fraction (dexamethasone treatment group) containing similar amounts of the 38 K and 36.3 K forms of PHM were run in parallel lanes on a 12% SDS polyacrylamide gel and transferred to PVDF membrane. Individual blots were probed with the indicated anti-PHM antibodies. The locations of antibody epitopes within PHM catalytic domain and the positions of key amino acids are shown schematically above the blots.

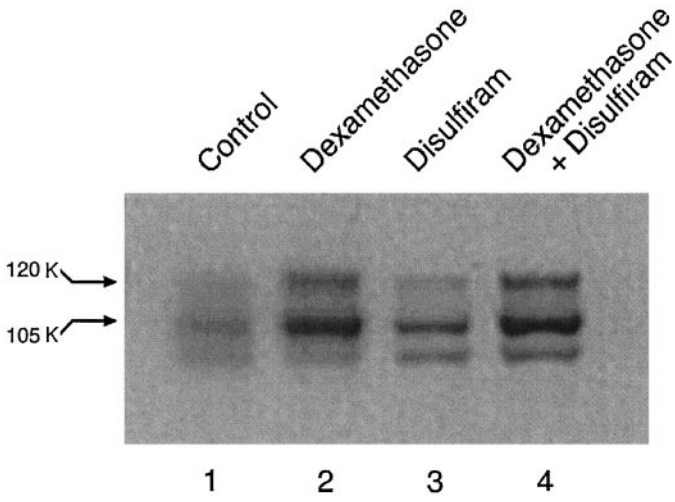

Fig. 6. PAM expression in atrial homogenates. Homogenates were prepared for each treatment group (n = 40) as described in *Materials and Methods*. Aliquots were taken for analysis just before the first ultracentrifugation step in the preparation of atrial membranes. Proteins were fractionated by SDS-PAGE, transferred to PVDF membrane and probed with anti-PHM antibody, Ab1761. Lanes were loaded at equal percentages (0.0025%) of total homogenate protein: lane 1, 3.3 μg; lane 2, 2.9 μg; lane 3, 3.0 μg; lane 4, 2.7 μg. Immunoreactive bands migrating at 120 K and 105 K represent PAM-1 and PAM-2, respectively.

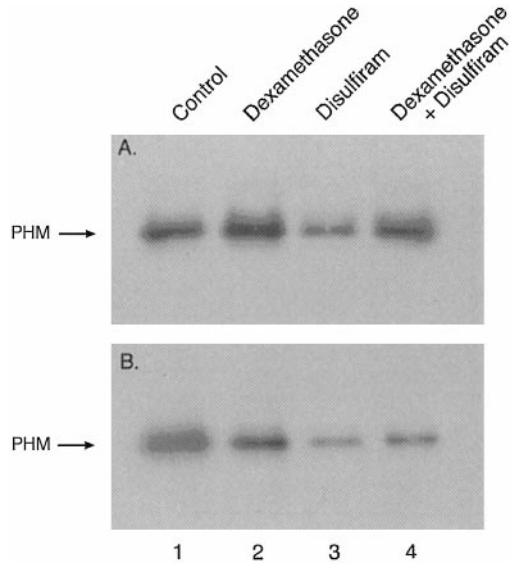

Fig. 7. Immunoblot analysis for PHM following hydrophobic interaction chromatography. For each treatment group, the peak activity fractions from hydrophobic interaction chromatography were pooled (8 ml) and assayed for PHM activity. Aliquots from each pool were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with anti-PHM antibody Ab1761. A, samples were loaded at equivalent volume, 1.0 μl per lane (protein: lane 1, 18 ng; lane 2, 22 ng; lane 3, 21 ng; lane 4, 23 ng); B, samples were loaded at equivalent activities, 105 pmols AcYV-amide formed/h per lane (protein: lane 1, 18 ng; lane 2, 13 ng; lane 3, 11 ng; lane 4, 11 ng).

administration of corticosterone. Thus, negative feedback by glucocorticoids on PAM expression in the paraventricular nucleus may serve to regulate the α-amidation of corticotropin-releasing hormone and arginine vasopressin, hypothalamic hormones that coordinate the activity of the hypothalamic-pituitary-adrenal axis (Stratakis and Chrousos, 1995; Webster et al., 1997).

Induction of PAM expression by dexamethasone could be mediated by glucocorticoid response element half-sites (Gronemeyer, 1992) located at nucleotides −2026 to −2021 and −2344 to −2339 in the promoter region of the PAM gene (Hand et al., 1996). Alternatively, glucocorticoids may function indirectly via transcription factors acting at other regulatory elements or possibly through enhanced mRNA stability. Supporting the notion of an indirect pathway are the findings that dexamethasone-induced changes in PAM expression require de novo protein synthesis and that the induction of PAM mRNA in cultured atrial myocytes requires more than 6 h of exposure to glucocorticoid (Thiele et al., 1989). Because the structural and functional analyses of the PAM promoter are incomplete, understanding the precise mechanism by which glucocorticoids activate PAM gene expression will require additional study.

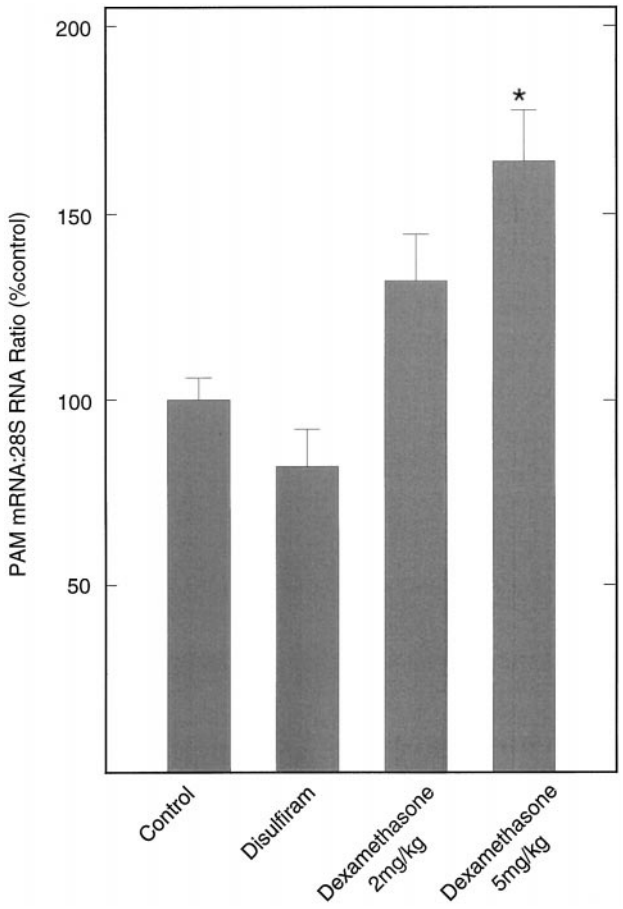

Fig. 8. Induction of atrial PAM mRNA by dexamethasone. Groups of rats (n = 8) were treated daily with either disulfiram (400 mg/kg; 7 days) or dexamethasone (5 days; doses indicated). Total atrial RNA was isolated from each group and subjected to northern blot analysis as described in *Materials and Methods.* Quantification of PAM mRNA was standardized to the amount of ribosomal RNA (28S) present and expressed as a percentage of control values. Error bars indicate the S.E.M. (*$P \leq .005$). The findings are representative of two separate experiments.

TABLE 2
Kinetic constants for purified PHM

Treatment Group	K_M[a]	V_{max}[a]	Turnover[b]
	μM	*pmol/ng/min*	*pmol/pmol/s*
Control	11.6 ± 0.8	6.6 ± 0.3	4.2
Dexamethasone	10.8 ± 0.7	7.4 ± 0.4	4.7
Disulfiram	12.0 ± 0.5	10.4 ± 0.4[c]	6.6[c]
Dexamethasone + disulfiram	11.3 ± 0.3	9.7 ± 0.4[c]	6.1[c]

[a] S.E.M. is given for average of four independent analyses.
[b] Quantification of PHM catalytic core is based on M_r = 38,000.
[c] Significantly different from control ($p \leq .01$).

Although the level of PAM expression is unaltered by disulfiram administration, the V_{max} of PHM is markedly increased in response to the treatment. This change appears to be mediated by a physiologic mechanism designed to upregulate α-amidation under conditions when tissue levels of α-amidated peptides become diminished (Mueller et al., 1993). Disulfiram treatment can lower tissue stores of α-amidated peptides to less than 5% of control values, presumably by chelating Cu^{2+}, an essential prosthetic group for PHM. Under these conditions, when peptidergic transmission is compromised, a compensatory physiologic mechanism apparently induces a biochemical modification that increases the catalytic efficiency of PHM. These changes occur in parallel over the effective dose range for in vivo disulfiram treatment (Mueller et al., 1993). Although levels of amidated peptides in vivo remain low, presumably due to limiting Cu^{2+}, PHM's enhanced V_{max} is evident experimentally when the enzyme is assayed under optimal concentrations of Cu^{2+} in vitro. The mechanism underlying the enhanced V_{max} is complex and cannot be demonstrated by direct application of disulfiram to either purified enzyme or cultured cells expressing PHM protein (our unpublished observations). It is likely, therefore, that the response to disulfiram is mediated by a multicellular sensor-effector feedback loop. This proposal for copper-based regulation of peptide α-amidation is supported by the observations that PAM-specific activity is increased in animals maintained on a low copper diet (Mains et al., 1985; our unpublished observations) and in human subjects with Menkes disease (Prohaska et al., 1997), an X-linked recessive disorder of copper transport.

The possibility that an active metabolite of disulfiram may ultimately mediate the increase in PHM's V_{max} remains open. In vivo, disulfiram undergoes rapid metabolism to produce *S*-methyl-*N*,*N*,-diethylthiolcarbamate sulfoxide (DETC-MeSO), a metabolite having increased activity in inhibiting aldehyde dehydrogenase (Hart and Faiman, 1992) and covalently modifying glutamate receptors by carbamoylation (Nagendra et al., 1997). Direct application of DETC-MeSO or two other metabolites of disulfiram, diethyldithiocarbamate-methyl ester and *S*-methyl *N*,*N*-diethylthiolcarbamate to PHM in vitro, however, does not alter the activity of the enzyme (our unpublished observations). Each metabolite was evaluated in concentrations ranging between 0.2 and 200 μM by either direct incorporation into the assay of PHM activity or preincubation with enzyme protein before assay for activity. The doses span the range recently reported for DETC-MeSO to inactivate glutamate receptors in vitro by carbamoylation (Nagendra et al., 1997). Because there is no direct interaction to explain the response of PHM to disulfiram treatment in vivo, it the appears most likely that a

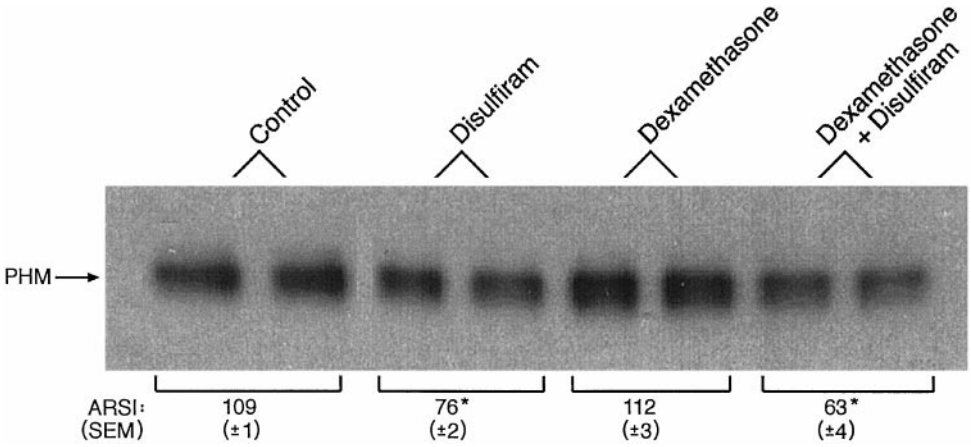

Fig. 9. Immunoblot analysis for PHM following anion exchange chromatography. High specific activity fractions from anion exchange chromatography were pooled for each treatment group. The maximum velocities for each pool were determined by kinetic analysis, and equivalent amounts of maximal activity (24 pmol/min) were loaded in duplicate. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with anti-PHM antibody Ab1761. The intensity of the immunoreactive PHM in each lane was measured by densitometry. The averaged relative signal intensity (ARSI ± S.E.M., arbitrary units) for four independent measurements is indicated below each pair of duplicates (*significantly different from control, $P < .005$).

multicellular mechanism mediates the increase in PHM's V_{max}.

The mechanism mediating the increased V_{max} of PHM in atrium appears to be generally applicable to the physiology of PHM in all tissues. The response of PHM in atrium to disulfiram treatment is representative of changes that occur in the V_{max} of PHM in the anterior and intermediate lobes of the pituitary (Mueller et al., 1993). The atrium produces adrenomedullin (Miller et al., 1996) and thyrotropin-releasing hormone (Shi et al., 1996), both of which require α-amidation for biologic activity. Nevertheless, the expression of PAM in heart seems to greatly exceed the amount necessary for amidating the comparatively small amount of peptide substrate present, suggesting that the primary function for PHM in the atrium may not be amidation. In this regard, detailed studies of PAM's subcellular localization and trafficking patterns reveal that it is distributed widely and readily moves from endoplasmic reticulum through the *trans*-Golgi network to the cell membrane and is recycled via the endosomic pathway (Oyarce and Eipper, 1995). Thus, atrial PAM might serve as an uptake, storage and/or intracellular transport protein for Cu^{2+} in the heart. On the other hand, it is possible that the high levels of PHM activity normally found in blood (Eipper et al., 1985; Kapuscinski et al., 1993) originate in the atrium. Accordingly, regulatory modifications occurring within the atrium may serve to control the actions of PHM at sites distant from the heart.

The findings presented here demonstrate the existence of a novel mechanism for sustaining levels of α-amidated peptides required for intercellular communication. This mechanism serves to coordinate the activity of PHM with the use of its products and constitutes an efficient means for maintaining homeostasis under conditions when the turnover of α-amidated peptides can vary greatly. This phenomenon in which the kinetic properties of PHM are regulated physiologically by covalent modification may be shared by other copper-dependent enzymes. There is evidence that dopamine β-monooxygenase exhibits a similar form of regulation when its products, norepinephrine and epinephrine, are depleted pharmacologically (Wong and Wang, 1994) or when Cu^{2+} is limiting (Kaler et al., 1993). A common mechanism for regulating these two enzymes would be consistent with the remarkable structural and functional similarities that exist between them (Southan and Kruse, 1989).

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