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Peptide α-Amidation and Peptidylglycine α-Hydroxylating Monooxygenase: Control by Disulfiram

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SUMMARY

The final two steps in the biosynthesis of α-amidated bioactive peptides are catalyzed by peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL; EC 4.3.2.5). These enzymes are derived from the bifunctional precursor protein, peptidylglycine α-amidating monooxygenase. Because PHM is rate-limiting in peptide amidation and is copper-dependent, we examined the consequences of *in vivo* treatments with the copper-chelating drug disulfiram (Antabuse) on levels of α-amidated peptides and expression of PHM and PAL. Decreases in two amidated peptides (α-melanotropin and cholecystokinin) after disulfiram treatment were extremely pronounced outside the blood-brain barrier, with moderate decreases in the central nervous system. Unexpectedly, when assayed under optimal conditions *in vitro*, PHM activity was increased by disulfiram treatment, whereas PAL activity was unaltered. The increase in PHM activity in pituitary and atrium occurred within a few hours after the start of disulfiram treatment and was sustained up to 2 weeks after the cessation of treatment, whereas levels of α-amidated peptides remained low. Northern and Western blot analyses demonstrated that disulfiram had no influence on levels of peptidylglycine α-amidating monooxygenase mRNA or protein. Thus, inhibition of α-amidation by disulfiram *in vivo* occurs despite an increased V_{max} of PHM assayed *in vitro*. The increase in PHM activity may result from induction of a physiologic mechanism that normally regulates this rate-limiting enzyme.

α-Amidation is a common posttranslational modification of peptides utilized in intercellular communication. More than half of the known neuroendocrine peptides are α-amidated and in nearly all cases, this structural feature is essential for receptor recognition and signal transduction. α-Amidation is catalyzed by PAM, a bifunctional enzyme localized within secretory granules (1–3). In its largest form (110-kDa), PAM consists of a signal peptide followed by intragranular, transmembrane, and cytoplasmic domains (4). The two catalytic elements of the protein are both contained within the intragranular segment (Fig. 1). These sequentially catalyze the two-step formation of α-amidated peptides from their inactive glycine-extended precursors; PHM (EC 1.14.17.3) catalyzes the formation of a peptidyl-α-hydroxyglycine intermediate that is immediately converted into α-amidated product and glyoxylate by PAL (EC 4.3.2.5). In this sequence, PHM is rate limiting and requires ascorbate, molecular oxygen, and copper for activity. α-Amidation is a terminal modification in peptide biosynthesis and can itself be rate limiting in the overall production of α-amidated peptides (1, 5). This feature, demonstrated experimentally by limiting copper or ascorbate (6–12), highlights a regulatory role for PHM in determining the availability of bioactive peptide products.

In mammals, PAM is encoded by a complex single copy gene which gives rise to at least seven enzyme forms via alternative mRNA splicing (13, 14). This structural diversity is increased further by endoproteolytic processing at pairs of basic amino acids. Together, alternative splicing and proteolytic cleavage determine whether PAM proteins will be integral membrane proteins or soluble proteins and whether the two catalytic domains will be separated from each other. Increasing evidence indicates that PAM gene expression and mRNA and protein processing are selectively controlled during development and in different tissues (1, 14). Although the biologic importance of these phenomena is yet to be fully appreciated, their complexities suggest that the number of mechanisms for regulating peptide α-amidation could be high. At present, it is clear that separation from the transmembrane domain enables soluble PHM and PAL to undergo secretion upon exocytosis (1, 15,

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ABBREVIATIONS: PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; AL, anterior lobes; NIL, neurointermediate pituitary lobe; α-MSH, α-melanocyte-stimulating hormone; β-END, β-endorphin; CCK, cholecystokinin; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazineethanesulfonic acid; CNS, central nervous system.

16). Indeed, α-amidating activity is high in blood and measurable in cerebral spinal fluid (see ref. 1). Differential processing is an important determinant for intracellular routing (4, 16) and may also alter the kinetic parameters (1, 17) for the different molecular forms of PAM.

On the basis of structural homology it has been proposed that PHM and dopamine β-monooxygenase share a conserved catalytic domain that has evolved from a common precursor (13, 18). Functional similarities between the two enzymes support this conclusion. Both proteins are type II, copper, ascorbate, and molecular oxygen-dependent monooxygenases involved in the synthesis of intercellular messengers in secretory granules (19). It is likely that known consensus metal binding sites conserved between the two proteins (Fig. 1) convey copper dependence to both enzymes (18, 20).

The present investigation sought to take advantage of the requirement of PHM for copper as an approach to study its regulation *in vivo*. Disulfiram (tetraethylthiuram, Antabuse), a drug used clinically as an alcohol deterrent, chelates copper and inhibits α-amidation (6–10), as well as dopamine β-monooxygenase (21–23). Little is known of how this drug-imposed inhibition may influence the expression or activities of PHM or PAL over time. If α-amidation is subject to physiologic control, then compensatory changes in the expression or activity of PAM would be expected to occur in response to treatments that alter the biosynthesis or actions of its α-amidated products. In this report we describe the dose and time course effects of disulfiram on peptide α-amidation and on the activity and expression of PAM in pituitary, atrium, and brain. The findings indicate that the activity of PHM is regulated physiologically through direct modification of the enzyme.

Methods

Animals, Treatments, and Sample Collection

Mature male rats (Charles River) were housed at 22° under a 12-hr daily light cycle and received food and water *ad libitum*. Disulfiram (Sigma Chemical Company) was suspended in 0.9% saline containing 0.05% Tween 80 and was administered subcutaneously. After sacrifice (decapitation), atrium, AL, and NIL, hypothalamus, and cerebral cortex were quickly dissected and either processed immediately or snap frozen on dry ice and stored at −70° until assayed.

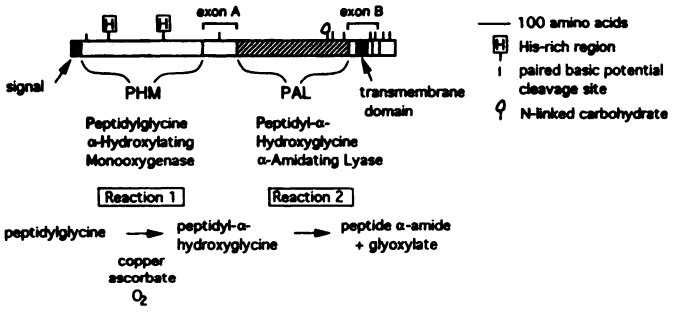


Fig. 1. Structural and functional organization of the rat PAM precursor protein. An NH_2-terminal signal peptide is followed by PHM; the monooxygenase domain contains two histidine clusters believed to mediate its copper dependence. A noncatalytic domain, *exon A*, separates PHM from PAL, which contains an *N*-gycosylation site. PAL is followed by a hydrophobic transmembrane domain and a hydrophilic C-terminal domain. Cleavage at some of the eight pairs of basic amino acids proceeds to varying degrees in different tissues. Predominant forms of tissue PAM include PAM-1 (contains exons A and B), PAM-2 (lacks exon A, contains B), and PAM-3 (lacks both). The reactions catalyzed by PHM and PAL are shown.

Radioimmunoassays

Immunoreactive α-MSH, CCK, and β-END were measured by radioimmunoassays. α-MSH and β-END were measured in acetic acid extracts (2N) made from tissues directly or in extraction mixtures initially prepared for enzyme analyses. CCK was measured in tissue extracts prepared with 90% methanol.

The α-MSH antiserum used (H-50) has an absolute requirement for C-terminal α-amidation (less than 1% cross-reaction with α-MSH-free acid, or peptides unrelated to α-MSH), whereas desacetyl-α-MSH and *N*,*O*-diacetyl-α-MSH are detected on an equimolar basis. The antiserum is sensitive to less than 5 fmol of synthetic α-MSH at a final dilution of 1:60,000 (24).

A new radioimmunoassay for CCK was developed using a rabbit antiserum (P45) raised against synthetic CCK-8 [CCK(26–33)]. The antiserum has an absolute requirement for C-terminal α-amidation and recognizes the N-terminal of CCK-8 without preference for sulfation state. CCK-8-free acid, and sulfated and desulfated CCK(26–29) are not detected (less than 1% cross-reaction), whereas CCK-8, desulfated CCK-8, and CCK(1–33) are detected on an equimolar basis. N-terminal shortening of CCK-8 by one [CCK(27–33)] or two amino acids [CCK(28–33)] reduces cross-reactivity to less than 8% and 1%, respectively. Human gastrin-1, an α-amidated 17 amino acid peptide that has the same 5 C-terminal residues as CCK-8, cross-reacts less than 8%. Using nonsulfated ^{125}I-CCK-8 (chloramine-T) and a final antibody dilution of 1:40,000, the assay detects less than 10 fmol of CCK-8. Gel filtration, ion exchange, and reverse phase high pressure liquid chromatography analyses of brain, pituitary, and intestine extracts demonstrated that the CCK and α-MSH assays do not cross-react with C-terminally extended precursors of the α-amidated peptides.

The β-END antiserum (C-55) recognizes α-*N*-acetylated and desacetyl forms of β-END(1–31), β-END(1–27), β-END(1–26), and β-lipotropin equally but exhibits less than 1% cross-reactivity with β-END(1–16), β-END(1–17), or peptides unrelated to β-END. At a final antiserum dilution of 1:50,000, the detection limit of this assay is less than 3 fmol (25).

Enzyme Assays

Sample preparation. Tissues were homogenized in 0.05 M MES (pH 6.0), 0.01 M mannitol containing protease inhibitors [leupeptin (2.0 μg/ml), benzamidine (16 μg/ml), lima bean trypsin inhibitor (10 μg/ml), and phenylmethylsulfonyl fluoride (15 μg/ml)]. The homogenates were then processed through three freeze-thaw cycles, followed by centrifugation at 400 × *g* for 10 min at 4°. The resulting supernatants were separated into soluble and crude particulate (membrane-bound) fractions by ultracentrifugation (435,000 × *g* for 15 min at 4°); pellets (membrane-bound fractions) were resuspended in 0.02 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0), 0.01 M mannitol, protease inhibitors, and Triton X-100 (1%). In cases where total tissue PHM or PAL activity were measured, the initial extraction buffer contained 1% Triton X-100.

PHM activity. PHM activity was measured under optimal conditions according to minor modifications of an established procedure (26). *The assay measures PHM activity in a manner that is independent of the activity of PAL.* A thorough assessment of copper optima was conducted for each sample to avoid any potential interference from disulfiram present in the tissue samples. Briefly, all samples (0.5 or 1 μg of protein) were assayed with 0.5 μM α-*N*-Ac-Tyr-Val-Gly and ^{125}I-α-*N*-Ac-Tyr-Val-Gly in 0.15 M NaMES (pH 5.0) containing 1 μM $CuSO_4$, 0.05 M ascorbate, and 100 μg/ml catalase in 40 μl. In some experiments, duplicate analyses of PHM activity were performed using α-*N*-Ac-Tyr-Phe-Gly as PHM substrate. Peptides terminating in -Phe-Gly exhibit greater reactivity with PHM compared to those ending in -Val-Gly (27), thus permitting activity analyses under conditions of different substrate affinity.

PAL activity. PAL activity in soluble and membrane-bound fractions (0.1–0.3 μg of protein) was measured using ^{125}I-labeled and 0.5 μM unlabeled α-*N*-acetyl-Tyr-Val-α-hydroxyglycine in 150 mM NaMES, 0.05% Triton X-100 (pH 5.0) as described (3).

Kinetic Analysis of PHM

PHM activity was assayed with either ^{125}I-α-*N*-Ac-Tyr-Val-Gly or ^{125}I-α-*N*-Ac-Tyr-Phe-Gly (20,000–30,000 cpm) and 0.1–100 μM unlabeled peptidylglycine. Eadie-Hofstee plots were constructed with 95% confidence intervals using the Hewlett Packard Statit Analysis System. Analysis of covariance revealed that all treatment conditions exhibited homogeneity in regression coefficients; differences in *x* and *y* intercepts were considered significant if 95% confidence intervals for the regression lines did overlap.

Northern blot analysis

Total RNA was prepared from tissues pooled from two (atrium) to six animals (AL, NIL, cortex) using the acid guanidinium isothiocyanate-phenol-chloroform procedure (28) (RNAgents, Promega, Madison, WI). Briefly, RNAs (5 or 10 μg) were fractionated by electrophoresis under denaturing conditions, transferred to nylon filters, baked, prehybridized, hybridized, and washed as described for SDS-PIPES buffer (29). The rat PAM-1 cDNA probe used (base pairs 351–1681, *Pst*I/*Bam*HI) was prepared by random prime biosynthesis with [α-^{32}P] dCTP (Amersham Corp., Arlington Heights, IL). Recovery of RNA was standardized by ethidium bromide staining before transfer, and by stripping the blots and reprobing with a cDNA probe derived from frog ribosomal RNA (15).

Western Blot Analysis

Western blot analysis of total cellular PHM protein was carried out as described (30). Samples consisting of tissues from two (atrium) to six (NIL and AL) animals each were solubilized in PHM extraction buffer containing 1.0% Triton X-100. Aliquots containing equal amounts of protein and known amounts of PHM activity were heat denatured in 2% SDS and 5% 2-mercaptoethanol and fractionated on SDS-polyacrylamide gels (10% acrylamide; 0.27% *N*,*N*′-methylenebisacrylamide). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and visualized with rabbit polyclonal antibodies directed against PHM or PAL, and enhanced chemiluminescence reagent (ECL, Amersham). Antibody 475 was generated to rat PAM-1 (37–382), the PHM domain, and antibody 471 was generated to rat PAM-1 (463–864), the PAL domain (4). Together, the antibodies detect all splice and endoproteolytic variants of PAM including soluble and membrane-bound bifunctional PAM, membrane-bound monofunctional PAL, and soluble monofunctional PHM and PAL.

Protein Assay

Data are standardized to protein content as determined by the BCA assay (Pierce Chemical Co., Rockford, IL).

Statistical Analysis

Statistical differences between treatment group means were determined using Duncan's new multiple-range comparison test after two-way analysis of variance (ANOVA) ($p < 0.05$).

Results

Effects on α-amidated peptides. Disulfiram treatment decreased concentrations of α-amidated peptides in the cerebral cortex, NIL, and duodenum in a time- and dose-related fashion (Fig. 2). Significant reductions were observed after 3 days treatment but not by 4 hr after a single administration. Greatest reductions were seen for concentrations of immunoreactive α-MSH in the NIL where levels were decreased to less than 5% of control values after 8 days treatment with 400 mg/kg disulfiram. In contrast, concentrations of immunoreactive β-END in the NIL were *essentially unaffected* by disulfiram treatment. Because α-MSH and β-END are derived from the same precursor (proopiomelanocortin) and co-stored in the same secretory granules of the NIL, this finding indicates that the effects of disulfiram in the NIL are selective to α-amidation and do not interfere with other steps *common to the biosynthesis of these two* proopiomelanocortin products. Levels of α-amidated CCK in the duodenum and cerebral cortex were decreased to approximately 10% and 50% of control levels, respectively. Presumably, this difference reflects ability of the blood-brain barrier to afford some protection to the CNS from the actions of disulfiram. Kinetics of the disappearance of α-MSH in the NIL during the high dose regimen are shown in Fig. 3; the log/linear rate of decrease revealed a $t_{1/2}$ of 2.4 days.

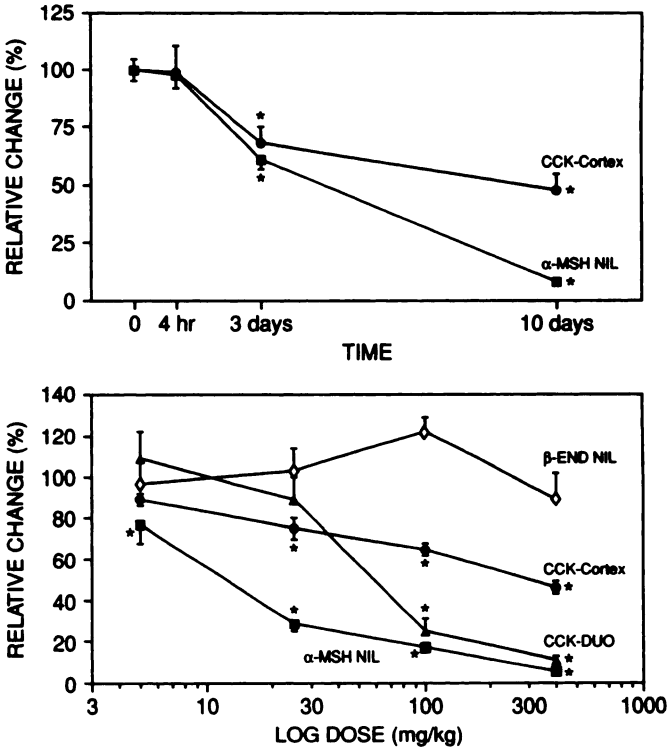


Fig. 2. Time course and dose response effects of disulfiram on tissue concentrations of immunoreactive β-END and α-amidated α-MSH and CCK. *Top panel*, Groups of rats (n = 8–9) were treated with a single dose or daily subcutaneous injections of disulfiram (400 mg/kg) for 3 or 10 days. Control animals received a single injection of vehicle; all samples were collected 4 hr after the last injection. Data are expressed as change relative to control values (100%). Values (mean ± standard error) in control animals were: NIL α-MSH = 6.1 ± 0.3 pmol/μg protein; Cortex CCK = 210 ± 10 pmol/g wet weight; $^*p \leq 0.05$ versus control. *Bottom panel*, Groups of rats (n = 6) were treated for 8 days with either vehicle or the indicated doses of disulfiram; samples were collected 24 hr after the last injection. Data are expressed as change relative to control values (100%). Values (mean ± standard error) in control animals were: NIL α-MSH = 7.8 ± 0.5 pmol/μg protein; NIL β-END = 17 ± 2 pmol/μg protein; Cortex CCK = 180 ± 10 pmol/g wet weight; Duodenum CCK = 22 ± 2 pmol/g wet weight; $^*p \leq 0.05$ versus control values.

The effects of disulfiram on tissue concentrations of α-amidated peptides persist for more than 2 weeks after the cessation of treatment. Fig. 4 shows the results of two separate experiments in which animals were treated daily for 7 days with either 100 mg/kg or 400 mg/kg disulfiram. Samples were then collected 1 to 14 days after the last injection. It should be

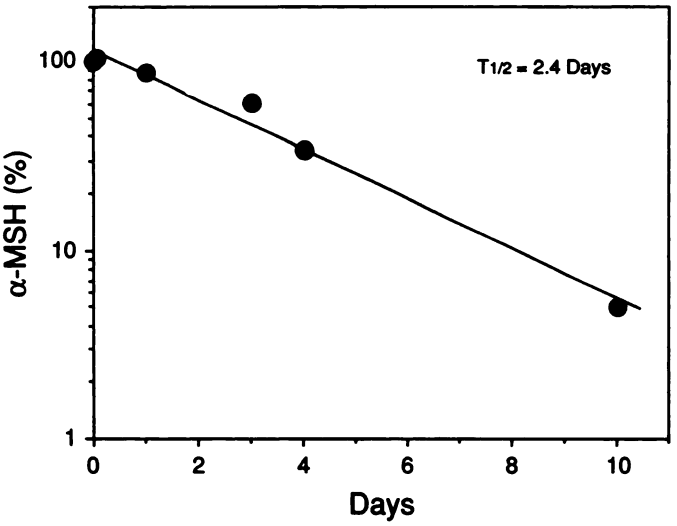


Fig. 3. Kinetics of disappearance of α-MSH from the NIL. Data plotted are derived from the same experiments depicted in Figs. 4 and 5 for animals treated with 400 mg/kg disulfiram for 90 min up to 10 days. *Line* is a least squares best fit line. See legends to Figs. 4 and 5 for additional details.

noted that the high dose treatment regimen produces long term toxicity resulting in progressive weight loss beginning at about 10 days, lower extremity neuropathy, and death by 3 weeks. Animals receiving the low dose regimen showed no ill effects of the disulfiram treatment (normal in body weight gain and general appearance).

Again, changes in tissue concentrations of α-amidated peptides were greatest for α-MSH in the NIL where levels were decreased to 20% and 4% of control values by the low and high doses of disulfiram, respectively. Following the low dose regimen, levels of α-MSH in the NIL were partially restored, yet remained significantly below control values by 7 and 14 days after the cessation of treatment (29% and 67%). Levels of α-MSH in the NIL of rats treated with the high dose were only 15% of control values at 10 days after the last injection. *Concentrations of β-END in the NIL were unaltered by disulfiram treatment except for a significant 25% reduction observed at 3 days after the cessation of the high dose.* Although the effects of disulfiram on decreasing levels of CCK in the cerebral cortex were similar for both dose regimens, no recovery of peptide levels was observed in rats receiving the high dose.

Effects on PAM activity. Based on the decline in tissue levels of α-amidated peptides, it was anticipated that reduced PHM activity would be measured *in vitro* when tissues from disulfiram-treated animals were examined. To address this question, extracts prepared from tissues of control and treated rats were assayed for PHM under optimized conditions. Fig. 5 shows the short term effects of daily treatment with disulfiram on soluble PHM activity in extracts of AL, NIL, atrium, and hypothalamus. Unexpectedly, disulfiram treatment consistently increased the activity of PHM in the AL, NIL, and atrium. This response was significant by 90 min in the NIL and atrium. The activity of soluble PHM in the hypothalamus was not appreciably altered during the 4-day treatment period; a similar observation was made for the cerebral cortex (not shown). These findings were consistent across all experiments for both soluble and membrane-bound PHM activity. In the same animals, changes in PHM activity induced by disulfiram preceded

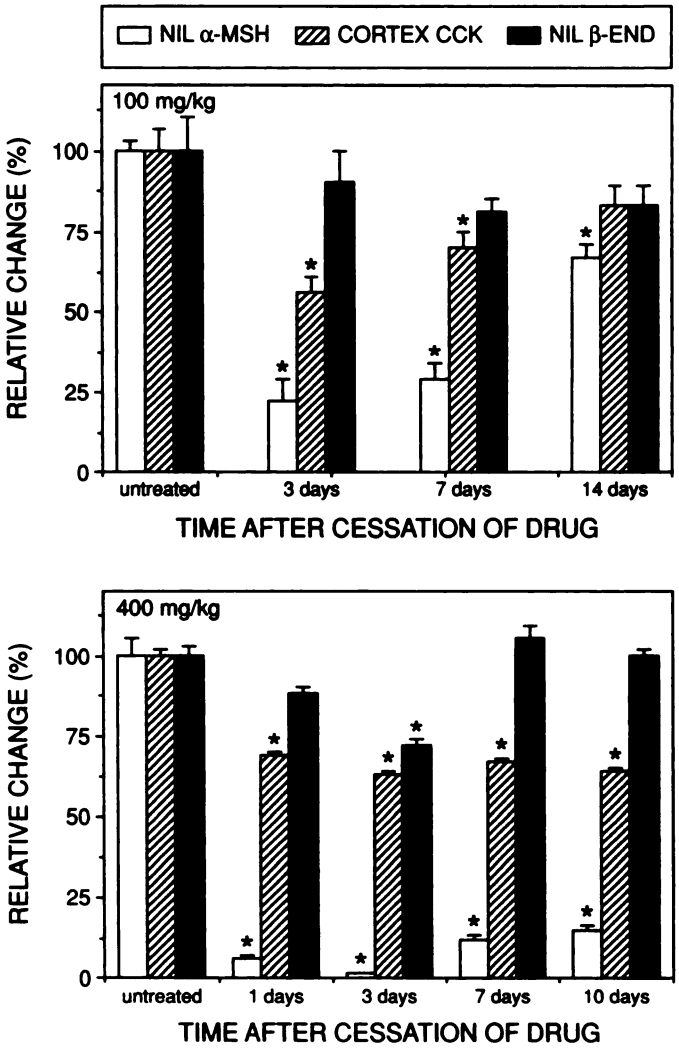
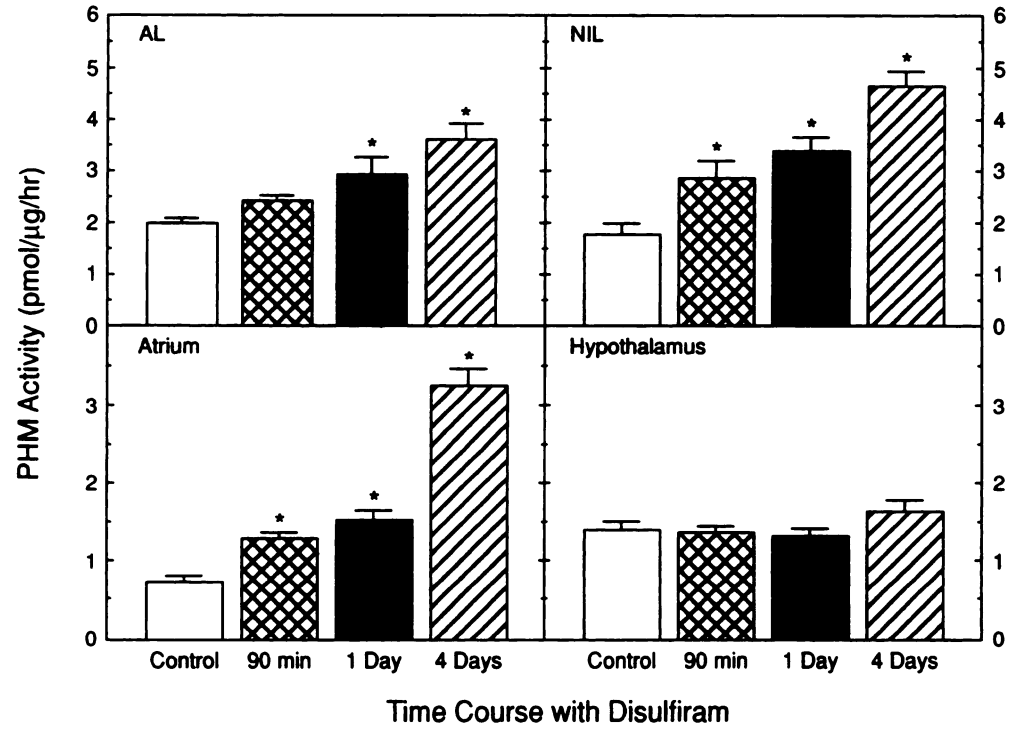


Fig. 4. Recovery from disulfiram treatment. Groups of rats (7–10) were treated daily for 7 days with either 100 mg/kg disulfiram (*upper panel*), 400 mg/kg disulfiram (*lower panel*), or vehicle. Samples were collected after the cessation of treatment at the days indicated. Tissue concentrations β-END and α-amidated α-MSH in the NIL and CCK in cerebral cortex are expressed as change relative to untreated animals (100%). In the low dose study, peptide contents in the NIL are based on immunoassay of soluble fractions of extracts prepared for PHM assay; total cellular protein was not measured. The effects of disulfiram on the content versus concentration of peptides are equivalent. Values (mean ± standard error) for control animals in the low dose study were: NIL α-MSH = 1.0 ± 0.1 nmol/lobe; Cortex CCK = 330 ± 20 pmol/g wet weight; NIL β-END = 2.0 ± 0.2 nmol/lobe. Values (mean ± standard error) for control animals in the high dose study were: NIL α-MSH = 4.0 ± 0.2 pmol/μg protein; Cortex CCK = 210 ± 10 pmol/g wet weight; NIL β-END = 9.0 ± 0.3 pmol/μg protein; $^*p \leq 0.05$ versus control values.

declines in tissue concentrations of α-amidated peptides by more than 24 hr; levels of α-MSH in the NIL were not appreciably altered by 1 day but were decreased to 34% of control values by 4 days disulfiram treatment (see Fig. 6). An analysis of the copper requirements for the PHM activity of each sample revealed the optimal added copper concentration for each tissue sample was the same (0.5–1 μM) at all time points (Fig. 5). As such, the possibility that disulfiram alters the copper requirement of PHM or that disulfiram present in tissue extracts interferes with the PHM assay were ruled out. The paradox of decreased α-amidation *in vivo* with increased PHM activity *in*



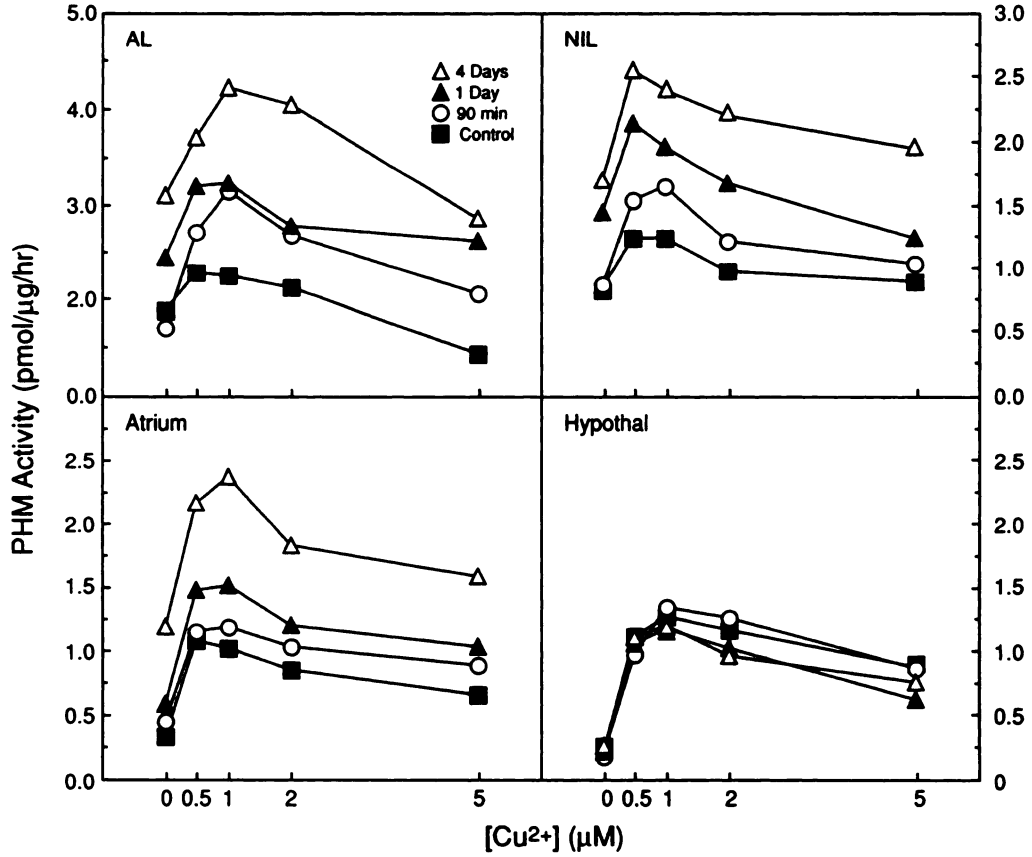


Fig. 5. *Top panel*, Short term effects of disulfiram on soluble PHM activity in extracts of pituitary AL and NIL, atrium, and hypothalamus. Groups of eight rats each were treated with vehicle or disulfiram (400 mg/kg, subcutaneously) for 90 min, 24 hr, or daily for 4 days. In all cases the last injection was administered 90 min before sample collection. The soluble fraction of each extract was assayed for PHM activity. Data are presented as group means ± standard errors. $^*p \leq 0.05$ versus control values. *Bottom panel*, Pooled samples were assayed with copper concentrations ranging from 0 to 5 µM copper.

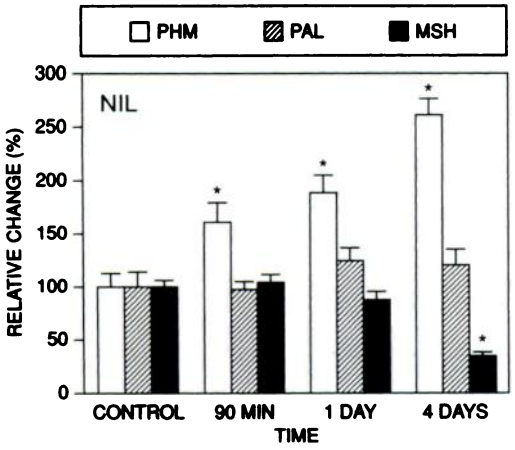


Fig. 6. Comparison of the effects of disulfiram on soluble PHM and PAL activities and concentrations of α-MSH in the NIL. Data are derived from the samples described in Fig. 5. Values (mean ± standard error) for untreated animals were: PHM = 1.8 pmol/μg/hr; PAL = 7.8 pmol/μg/hr; NIL α-MSH = 1.5 ± 0.1 nmol/lobe; *$p \leq 0.05$ versus control values.

vitro may be explained by a treatment-induced change in PHM that can only be realized under optimal copper conditions *in vitro*.

Because PAM is a bifunctional enzyme and only PHM exhibits a requirement for copper, the effects of disulfiram treatment on PHM activity were compared with the effects of disulfiram on PAL activity. As shown in Fig. 6, PAL activity was unaffected by the drug; increases in the activity of PHM (measured *in vitro*) and declines in NIL α-MSH content occurred in the expected time-related fashion. The fact that disulfiram treatment affects PHM activity without affecting PAL activity suggests that the affects of disulfiram are targeted to PHM via its copper prosthetic group.

Similar to the prolonged effects of disulfiram on tissue content of α-amidated peptides (Fig. 3), disulfiram-induced *in vitro* PHM activity persists following cessation of treatment (Fig. 7). Samples were collected 1 or 7 days after a 1-week treatment with 400 mg/kg disulfiram. The activity of PHM in the NIL, AL, and atrium was highest at 7 days posttreatment in both soluble and membrane-bound fractions. As shown here, and as seen in all other experiments involving different doses and treatment times, the action of disulfiram to increase PHM activity was reflected equally in both soluble and membrane-bound fractions, indicating that the different molecular forms of PHM are similarly affected by the drug. Some variations in the magnitude of response were observed for a given tissue among experiments (e.g., Figs. 5 and 7, atrium); however, qualitative changes were consistent across the entire study.

Under our standard assay conditions (low peptidylglycine substrate concentration), a change in the K_m or V_{max} of PHM could affect the activity measured. To determine which parameters are affected by disulfiram treatment, kinetic analyses were performed. The increase in PHM activity caused by disulfiram treatment was due to an increase in V_{max} with no change occurring in the K_m for its peptidylglycine substrate. A representative Eadie-Hofstee plot depicting this response is shown for soluble PHM from the NIL in Fig. 8. The same pattern of response was observed for all tissues affected by disulfiram (Table 1).

Effect on PAM expression. To examine the possibility that disulfiram treatment induced expression of the PAM gene,

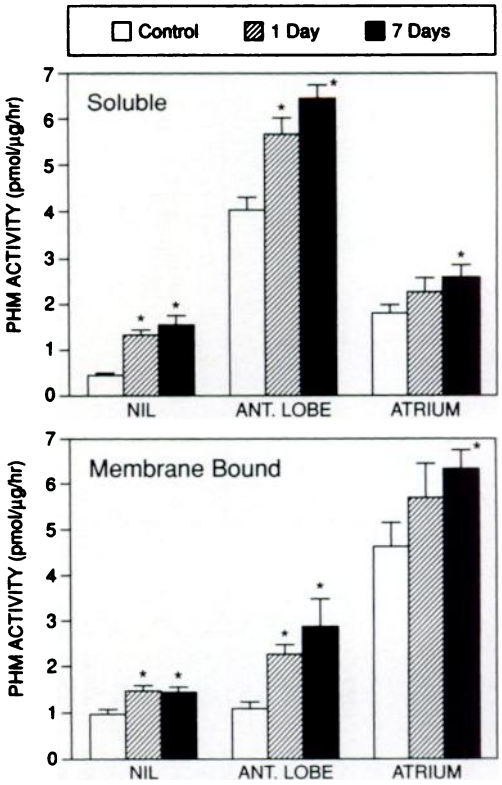


Fig. 7. Sustained effect of disulfiram on PHM activity in extracts of NIL, AL, and atrium. Groups of eight rats each were treated daily for 1 week with disulfiram (400 mg/kg, subcutaneously) or vehicle (untreated). Samples were collected either 1 or 7 days after the last injection of disulfiram. Soluble and membrane-bound PHM activity was measured *in vitro* under optimal conditions. Data are presented as group means ± standard error. *$p \leq 0.05$ versus control values.

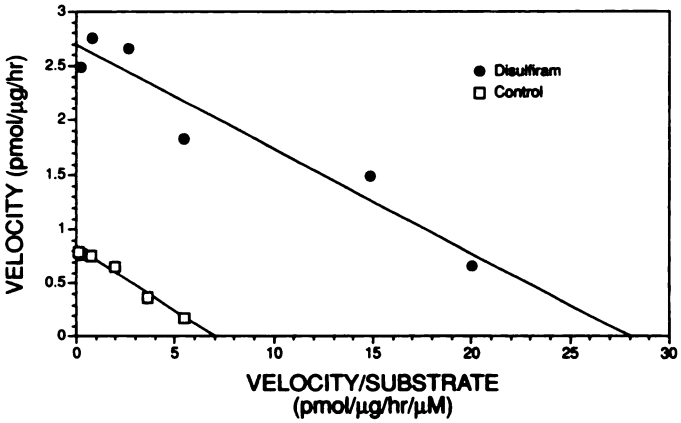


Fig. 8. Effects of disulfiram treatment on the kinetic properties of NIL soluble PHM activity. The Eadie-Hofstee plot shows results of analyses performed on group pools prepared from the individual untreated control and seven day disulfiram samples represented in the data of Fig. 6. An increase in V_{max} (*y* intercept) with no change in K_m (− slope) characterizes the effects of disulfiram on PHM activity in the NIL and all other tissues studied (see Table 1).

a series of Northern and Western blot analyses were performed to assess the effects of disulfiram on tissue levels of PAM mRNA and protein. Consistent with the failure of disulfiram to affect levels of PAL activity, these determinations revealed that tissue levels of PAM mRNA (Fig. 9) and protein (Fig. 10) were unchanged from control values by disulfiram, despite the increased levels of PHM activity. Further, disulfiram treatment

TABLE 1

Comparison of the effects of disulfiram treatment on the kinetic characteristics of soluble PHM from different tissues

The animals received either vehicle (untreated) or disulfiram (400 mg/kg) for 7 days; samples were collected 7 days after the last injection. Kinetic analyses were performed on soluble PHM pooled from eight animals.

Tissue	K_m	V_{max}
	μM	pmol/μg/hr
Neurointermediate pituitary		
Untreated	5.3	6.4
Disulfiram	7.7	21[a]
Anterior pituitary		
Untreated	7.7	39
Disulfiram	9.5	129[a]
Atrium		
Untreated	11	24
Disulfiram	13	58[a]
Hypothalamus		
Untreated	8.8	19
Disulfiram	8.1	20

[a] Significantly different from corresponding controls.

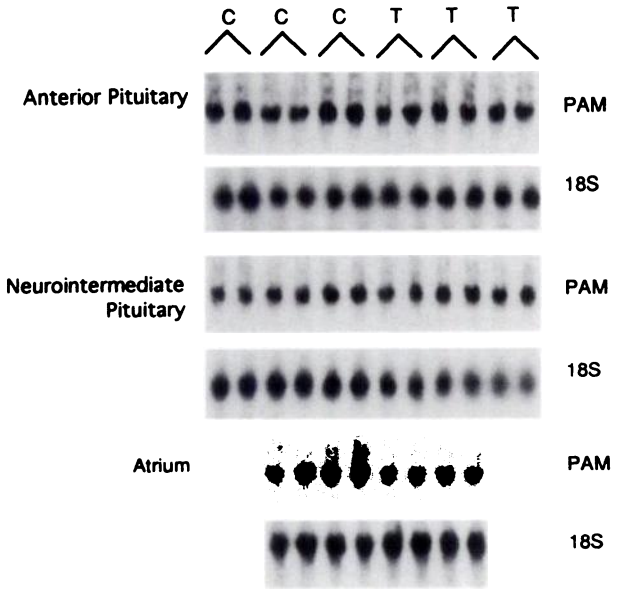


Fig. 9. Northern blot analysis of PAM mRNA in the AL, NIL, and atrium of untreated control (*C*) and disulfiram (400 mg/kg/day for 7 days)-treated (*T*) rats. Total RNA was prepared from pools of tissue from six (AL and NIL) or two (atrium) animals and fractionated (5 μg/lane) under denaturing conditions. Blots were probed with a cDNA to PHM and then stripped and reprobed with a cDNA to 18 S ribosomal RNA. Exposure times were adjusted for each blot to give bands of comparable intensity. Blots were densitized and analyzed statistically; no significant differences were detected. The findings are representative of two to three separate experiments depending on the tissue.

had no discernible effect on the distinctive electrophoretic patterns of PHM protein that characterize the alternative splicing and endoproteolytic processing of PAM precursors in AL, NIL, and atrium (14, 30). AL extracts contained intact PAM-1 (120 kDa), PAM-2 (105 kDa), and PAM-3 (95 kDa) and soluble PAM (75 kDa) and PHM (44–46 kDa) proteins derived from PAM-2 or PAM-3 and PAM-1, respectively. NIL extracts contained little intact PAM-1 and PAM-2 and were enriched in the soluble 75 kDa PAM and 44–46 kDa PHM proteins. Atrial extracts contained primarily intact PAM-1 and PAM-2. Analyses of equivalent Western blots analyzed with an anti-PAL antibody similarly revealed that disulfiram had

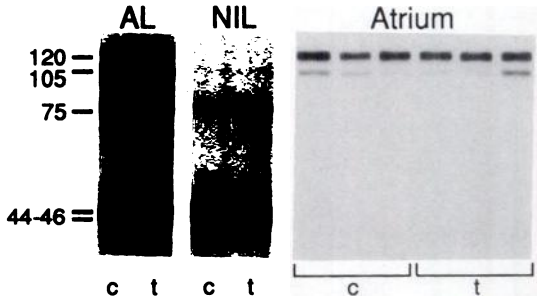


Fig. 10. Western blot analysis of PHM proteins in the NIL, AL, and atrium of untreated control and disulfiram-treated rats. Extracts were prepared from pools of tissue from animals treated daily with either vehicle (*c*) or disulfiram (400 mg/kg/day for 7 days) (*t*). Samples containing 50 μg (AL and NIL) or 20 μg of protein (atrium) and the following PHM activities were run in each lane: PHM activity for control versus disulfiram-treated samples: AL = 4.2 vs 8.7 pmol/μg/hr; NIL = 2.2 vs 5.3 pmol/μg/hr; atrium = 1.8 vs 3.7 pmol/μg/hr. PAM proteins were visualized with an antiserum to PHM (Methods). Exposure times were adjusted for each tissue to give bands of comparable intensity between tissues. The findings are representative of two to three separate experiments depending on the tissue.

no appreciable effect on the amount or molecular forms of PAL protein present in NIL, AL, and atrium (not shown).

Discussion

α-Amidation is an essential posttranslational modification in the bioactivation of many of the peptides utilized in intercellular communication. The process of α-amidation can be rate limiting in peptide production (1, 31) and as such, it is likely that the enzyme involved, PHM, is itself subject to important regulatory controls. To investigate this question we examined the short and long term consequences of *in vivo* inhibition of peptide α-amidation on activity and expression PHM. Disulfiram treatment decreases the synthesis and release of α-amidated peptides by inhibiting the activity of PHM (6–10). It was unknown, however, what cellular responses might be evoked to compensate for the drug-induced reduction in α-amidation. The present findings demonstrate that although peptide α-amidation is inhibited by disulfiram treatment *in vivo*, as indicated by pronounced declines in tissue concentrations of α-amidated peptides, PHM is altered in such a way that its activity is actually increased when assayed under optimal conditions *in vitro*. This increase in enzyme activity is sustained over time and is not due to an increase in levels of PAM mRNA or protein or alternative proteolytic processing of the PAM precursor. Rather, it appears that disulfiram treatment increases PHM activity through a modification of preexisting PHM protein resulting in an increase in V_{max}. This increase in V_{max} observed *in vitro* may reflect a physiologic mechanism for regulating α-amidation, one that occurs as a compensatory response to the sustained inhibition of α-amidation imposed by disulfiram *in vivo*.

The increased *in vitro* PHM activity caused by disulfiram treatment is rapid in onset. The progressive decline in tissue concentrations of α-amidated peptides occurs on a slightly slower time scale. In extracts of NIL and atrium, an increase in *in vitro* PHM activity was observed within 90 min after the administration of disulfiram. This time course is consistent with the rapid distribution of disulfiram following injection or oral administration (32–38). The ability of subcutaneously administered disulfiram to alter PHM activity so quickly indicates

that the acute effect of the drug is mediated through a modification of preexisting PHM protein. This conclusion is further supported by the observation that resting levels of PHM mRNA and protein are unaltered by disulfiram treatment in the same tissues where *in vitro* enzyme activity was markedly increased.

Measurable decreases in tissue concentrations of α-amidated peptides followed changes in *in vitro* PHM activity by more than 24 hr, with maximal reductions occurring after approximately 1 week of disulfiram treatment. The rate at which tissue stores of α-amidated peptides are depleted during disulfiram treatment may reflect their utilization and provide a measure of peptide turnover. Such an estimate would probably be most accurate for α-MSH in the NIL, where inhibition of PHM appears virtually complete. The blood brain barrier affords protection to the CNS from the actions of disulfiram; only modest alterations in amidated peptide content were noted in the CNS and PHM activity in brain was not significantly affected. Studies on the distribution of disulfiram and its metabolites in rodents have consistently shown that uptake into brain is the lowest of any organ, whether disulfiram is given acutely or chronically (32, 33, 35, 36, 38).

The effects of disulfiram treatment on α-amidation are remarkably long lasting. The increase in *in vitro* PHM activity and reductions in tissue stores of α-amidated peptides persisted for more than 7 days following a 7-day treatment with the low dose of disulfiram. It is evident from the established pharmacokinetics of disulfiram that this sustained effect on α-amidation is not due to the continued presence of disulfiram in tissues. Although disulfiram is highly lipophilic and would be expected to accumulate in body lipid compartments, this pattern of distribution is not observed due, in large part, to the very rapid metabolism of disulfiram *in vivo* (32–38). Essentially all of an administered dose of disulfiram is accounted for by metabolism and excretion within 24 hr after administration. As such, the overall tissue burden of disulfiram does not increase dramatically with chronic treatment (36–38). However, the possibility that preferential uptake of small amounts of disulfiram may occur into secretory granules cannot be discounted.

Although disulfiram has been used therapeutically in the treatment of alcoholism for more than 40 years, the mechanisms through which it inhibits metal-dependent enzymes remain to be determined (see ref. 36). Several mechanisms have been proposed and more than one may be involved in the *in vivo* inhibition of PHM by disulfiram. *In vivo*, disulfiram is rapidly reduced to diethyldithiocarbamate (32, 35), which is also an effective chelator of divalent metal ions, especially copper and zinc (32, 39; see ref. 36). Thus, chelation of an essential metal cofactor by either disulfiram or diethyldithiocarbamate could underlie the disulfiram-induced inhibition of copper-dependent PHM (1, 6) and dopamine β-monooxygenase (21–23). A mechanism based on chelation is consistent with the apparent specificity of disulfiram for the monooxygenase steps in the pathways leading to the formation of α-amidated peptides and norepinephrine. Disulfiram treatment rapidly increases tissue concentrations of inactive, glycine-extended peptide precursors (6–10), and dopamine (21–23), indicating that biosynthetic steps preceding those catalyzed by PHM and dopamine β-monooxygenase are functional during disulfiram treatment.

Direct, covalent modification of essential sulfhydryl groups, through the formation of mixed disulfide adducts (41–43) or internal disulfide bonds (40), has been demonstrated to occur in the inhibition of aldehyde dehydrogenase by disulfiram. The possibility that PHM is irreversibly altered in such a fashion would seem unlikely though, because enzyme activity was found to be inhibited *in vivo* (6) yet markedly increased when assayed under optimal copper concentrations *in vitro*. Further, exposure of purified bifunctional PAM to disulfiram *in vitro* (0.1–100 μM) was found to have no effect on PHM activity[1] indicating that direct modification of PHM by disulfiram is unlikely under the present experimental conditions. Recently, it was reported that disulfiram undergoes bioactivation to produce *S*-methyl-*N*,*N*-diethylthiolcarbamate sulfoxide, the most potent disulfiram-related inhibitor of aldehyde dehydrogenase (44). The possibility that this, or some other metabolite mediates the effects of disulfiram on PHM remains to be determined.

It is necessary to postulate at least two effects of disulfiram. First, disulfiram may inhibit PHM activity *in vivo* through copper chelation. Second, disulfiram treatment may induce an endogenous mechanism that normally regulates the activity of PHM. In this case enzyme activation would only be evident when PHM activity was assayed under optimal conditions *in vitro*. There is evidence that PHM is regulated physiologically, and in a manner consistent with the present interpretation. Dietary copper deficiency in rats (45) and pharmacologic inhibition of α-amidation in cell culture (46) both produce responses indicative of *in vivo* induction of PHM activity; copper deficiency was found to increase *in vitro* activity of PHM in extracts of anterior pituitary and submaxillary gland, and treatment with the PHM inhibitor, 4-phenyl-3-butenoic acid, was associated with a recovery of cellular PHM activity despite the continued presence of inhibitor. Phosphorylation is the most common mechanism for regulating enzyme activity and may mediate the response of PHM to disulfiram seen here. The possibilities that sulfation, acylation, and/or very limited proteolysis may be involved remain open. *Alternatively, disulfiram treatment could interfere with a normal process for PHM inactivation; we consider this possibility to be less likely because enzyme activity was linear in time and protein concentration.* By whatever mechanism(s), the long lasting effects of disulfiram treatment on PHM and on tissue concentrations of α-amidated peptides suggest that PHM is irreversibly modified in response to disulfiram treatment. The duration of the response following discontinuation of disulfiram treatment indicates that PHM itself turns over at a very slow rate, or that newly synthesized PHM is affected by prior disulfiram treatment. The present findings indicate that PHM is a target for drug action and that agents like disulfiram can be used to study the physiologic mechanisms that normally regulate the α-amidation of neural and endocrine peptides.

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[1] Unpublished data.

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