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INHIBITION OF THE GASTRIC ($H^+ + K^+$)-ATPASE BY FENOCTIMINE

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Abstract—The effects of fenoctimine, an inhibitor of gastric acid secretion, on the microsomal ($H^+ + K^+$)-ATPase were studied. In the micromolar concentration range, fenoctimine inhibited hydrolysis of ATP and *p*-nitrophenyl phosphate by the ($H^+ + K^+$)-ATPase. Inhibition was reversible and noncompetitive with substrate. The apparent K_i was dependent on the concentration of membranes, being increased by added liposomes or high microsomal membrane concentrations. Over the concentration range that ($H^+ + K^+$)-ATPase was inhibited, fenoctimine increased the turbidity of microsomal suspensions. The effects of fenoctimine were not specific for the gastric ($H^+ + K^+$)-ATPase, since the hydrolytic activities of the ($Na^+ + K^+$)-ATPase and mitochondrial ATPase were also inhibited by the drug. These results suggest that inhibition of hydrolysis may not be the direct result of an interaction between the ($H^+ + K^+$)-ATPase and fenoctimine but the secondary effect of a fenoctimine-induced perturbation of the microsomal membrane.

Fenoctimine, 4-(diphenylmethyl)-1-[(octylimino)-methyl] piperidine, has been shown recently to be an inhibitor of gastric acid secretion in rats, dogs, guinea pigs, and man [1, 2]. Studies with isolated oxyntic cells have demonstrated that fenoctimine is not a histamine H_2 receptor antagonist and that it appears to act at a more distal step than cAMP, as it blocks the stimulation of aminopyrine uptake by dibutyrl-cAMP [3]. These results suggested that fenoctimine might act by directly inhibiting the H^+ pump in the apical membrane of the oxyntic cell. This pump has been isolated [4] and shown to be an ($H^+ + K^+$)-ATPase [5]. In the present work, we have examined the inhibition of hydrolysis of both ATP and *p*-nitrophenyl phosphate (pNPP) by fenoctimine. While we found that fenoctimine inhibited the hydrolysis of both ATP and pNPP, we obtained no evidence for a specific interaction between fenoctimine and the gastric ATPase. The data suggest that fenoctimine inhibits the ATPase indirectly by disrupting the membrane environment necessary for optimal ATPase activity.

METHODS

Vesicle preparation. Gastric microsomes were prepared from hog fundic mucosa as previously described [6]. Briefly, crude microsomes were harvested from mucosal scrapings as the membrane fraction that sedimented between 14,600 *g* for 15 min and 143,000 *g* for 45 min. These membranes were further purified by centrifugation for 4 hr at 131,000 *g* in a sucrose density gradient. The purified microsomes were collected from the interface between 20% and 27% sucrose and stored in 1-ml aliquots at −20° until use. Mitochondria were isolated from the gastric mucosa of rabbits as described previously [7]. ($Na^+ + K^+$)-ATPase from dog kidney was purchased from Sigma.

Assays. ATPase was measured from the liberation of inorganic phosphate according to the method of Sanui [8]. All points represent the mean of at least two separate determinations. The ($H^+ + K^+$)-ATPase assay medium contained 1 mM $MgSO_4$, 1 mM ATP, 10 mM Pipes buffer [piperazine-*N*,*N*^{1}-bis(2-ethanesulfonic acid)] at pH 6.8 in 1.0 ml at 37°. When present, KCl was 150 mM and valinomycin was 10 μM. Valinomycin and fenoctimine stock solutions were made up in ethanol and added so that the total ethanol concentration was less than 0.5%, which did not inhibit ATP hydrolysis. The ($Na^+ + K^+$)-ATPase assay medium contained 100 mM NaCl, 20 mM KCl, 1 mM $MgSO_4$, 1 mM ATP, and 10 mM Tris–HCl, pH 7.5, at 37°. The assay medium for the mitochondrial ATPase contained 1 mM $MgSO_4$, 1 mM ATP, and 10 mM Pipes, pH 6.8, at 37°, and 5 μg protein/ml.

The hydrolysis of *p*-nitrophenyl phosphate by the ($H^+ + K^+$)-ATPase was assayed by three methods. (A) Duplicate assays were performed in a total volume of 1.0 ml containing 5 mM $MgSO_4$, 5 mM KCl, 5 mM pNPP and 10 mM Tris–HCl at pH 7.5 and 37°. Reactions were quenched with 1.5 ml of 0.5 M NaOH and centrifuged, and the optical density was read at 410 nm. (B) Aliquots (1.0 ml) from 15-ml reaction mixtures were removed and quenched at various times. Quenched samples were assayed as described for method A. (C) pNPPase was also monitored continuously at 410 nm with a Varian 210 spectrophotometer. Reaction mixtures were identical to those used above except that the total volume was 3 ml. When present, fenoctimine was added as an ethanol solution. The ethanol concentration did not exceed 0.5%, which did not inhibit pNPP hydrolysis. The pNPPase activity of the ($Na^+ + K^+$)-ATPase was assayed by method A with an assay mixture that

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contained 10 mM KCl, 5 mM $MgSO_4$, 5 mM pNPP, and 10 mM Tris–HCl at pH 7.5 and 37°.

Turbidity was measured with a Varian 210 spectrophotometer. Fenoctimine at various concentrations was added to vesicles suspended in 3.0 ml of the pNPPase assay medium (without pNPP). The change in optical density was determined from the difference in O.D. before, and 1.0 min after, the addition of fenoctimine.

Protein was determined by the method of Lowry *et al.* [9].

RESULTS

As shown by the representative results in Fig. 1, fenoctimine was a potent inhibitor of the microsomal $(H^+ + K^+)$-ATPase from pig gastric mucosa. In conformance to earlier experiments [10], the gastric microsomal ATPase activity was Mg^{2+}-dependent and subject to stimulation by K^+; further stimulation was achieved with K^+ plus valinomycin. In three separate experiments, where microsomes were tested at 8.9 μg protein/ml, the respective ATPase activities were inhibited by fenoctimine with mean values ± S.D. for half-maximum inhibition occurring at 6.3 ± 0.8 μM (Mg^{2+}), 25 ± 2 μM (K^+), and 14 ± 1 μM (K^+/val). Likewise the K^+-pNPPase activity of the same enzyme was inhibited by fenoctimine as demonstrated in Fig. 2; the apparent half-maximum inhibition for four experiments was 6.0 ± 0.5 μM fenoctimine. Hill plots of the data, such as those represented in Figs. 1 and 2, have slopes of 2 or greater, suggesting that more than 1 equivalent of fenoctimine interacted with each ATPase. Inhibition studies at various substrate concentrations revealed that the inhibition by fenoctimine was noncompetitive with respect to both ATP and pNPP (data not shown).

The specificity of fenoctimine was examined by assaying its effects on other membrane ATPases. The results of Fig. 3 show that fenoctimine inhibited the ATPase and pNPPase activities of the $(Na^+ + K^+)$-ATPase. Under these conditions the apparent K_i values for fenoctimine inhibition of the $(Na^+ + K^+)$-ATPase were similar to the values obtained for the $(H^+ + K^+)$-ATPase. The Mg^{2+}-ATPase activity of a mitochondrial rich fraction isolated from rabbit gastric mucosa was also assayed. Fenoctimine inhibited the hydrolysis of ATP with an apparent K_i of 6 μM (data not shown).

During the course of these studies, we noted variation in the inhibition constant for fenoctimine as a function of the concentration of membranes in the assay. As shown in Table 1, increasing the concentration of vesicles resulted in an increase in the concentration of fenoctimine necessary for half-maximum inhibition of the $(H^+ + K^+)$-ATPase. Half-maximum inhibition occurred at 16, 25 and 35 μm fenoctimine with 3, 10 and 30 μg protein/ml, respectively. Table 1 also shows a similar finding obtained with added liposomes, where the addition of 20 μg/ml of asolectin liposomes to 9.0 μg/ml of microsomal vesicles increased the concentration of fenoctimine necessary for half-maximum inhibition from 25 to 80 μM.

In addition to its effects on ATPase activity, we noted that fenoctimine produced a visible increase in turbidity of vesicular suspensions. Figure 4 docu-

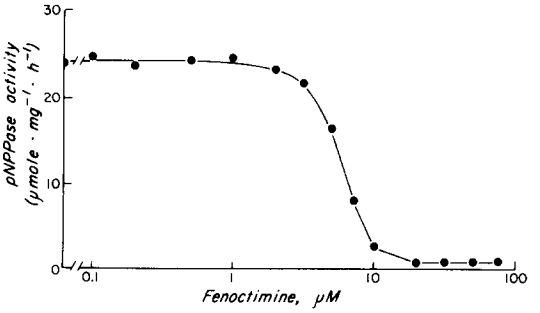

Fig. 2. Inhibition of pNPP hydrolysis by fenoctimine. Hydrolysis of 5 mM pNPP by gastric microsomes (8.9 μg protein/ml) at pH 7.5 was measured as described in Methods. Data are for a representative experiment of four.

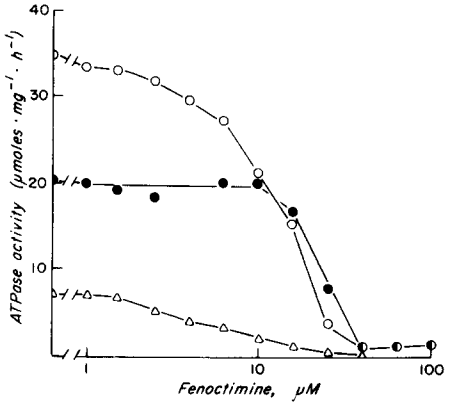

Fig. 1. Inhibition of ATP hydrolysis by fenoctimine. Hydrolysis of 1.0 mM ATP by gastric microsomes (8.9 μg protein/ml) at pH 6.8 was measured as described in Methods. Key: (△) Mg^{2+}-ATPase ([K^+] = 0); (●) K^+-ATPase ([K^+] = 150 mM); and (○) (K^+/val)-ATPase ([K^+] = 150 mM, [val] = 10 μM). Data are for a representative experiment of three.

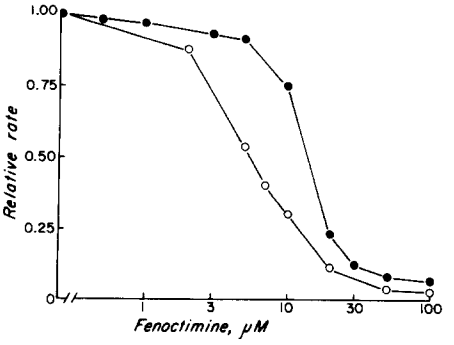

Fig. 3. Inhibition of $(Na^+ + K^+)$-ATPase-dependent hydrolysis of ATP (●) and pNPP (○) by fenoctimine. ATPase was assayed as described in Methods at 10.1 μg protein/ml; the uninhibited rate was 48 μmoles·(mg protein)$^{-1}$·hr^{-1}. pNPP was assayed as described in Methods at 9.3 μg protein/ml; the uninhibited rate was 28 μmoles·(mg protein)$^{-1}$·hr^{-1}.

Table 1. Effect of vesicle and liposome concentration on inhibition of K^+-ATPase by fenoctimine

Vesicles (μg protein/ml)	Liposomes (μg asolectin/ml)	K_i^{app} (μM)
3	—	16
10	—	25
30	—	35
9	—	25
9	20	80

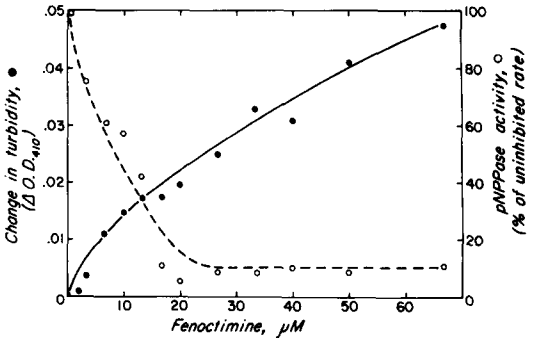

Fig. 4. Comparison of fenoctimine-induced change in turbidity (●) and inhibition of pNPP hydrolysis (○). Vesicles (14 μg protein/ml) were incubated in 3.0 ml of pNPPase assay medium, as described in Methods. Fenoctimine was added, and the change in O.D. at 410 nm was measured; then 5 mM pNPP was added, and the rate of hydrolysis was assayed. The uninhibited pNPPase rate was 23 μmoles·(mg protein)$^{-1}$·hr^{-1}.

ments this phenomenon as an increase in optical density of a suspension of gastric microsomes with increasing concentrations of fenoctimine. Also shown in the figure are the relative changes in pNPPase activity over the same range of fenoctimine concentrations. The turbidity, or light scattering, changes suggest fenoctimine-induced vesicle aggregation.

As an increase in turbidity would be consistent with irreversible denaturation of the gastric microsomes by fenoctimine, we tested for the reversibility of fenoctimine inhibition by measuring the recovery of activity upon dilution. These experiments were complicated by the fact that vesicle dilution decreased the apparent K_i for fenoctimine inhibition. Table 2 gives the results of a representative experiment for pNPP hydrolysis. First, control activities were measured at two vesicle concentrations, 17.5 and 1.75 μg/ml, and at three concentrations of fenoctimine, 0, 1.65 and 16.5 μM; specific activities for these conditions are given. Vesicles at 17.5 μg/ml and 16.5 μM fenoctimine were assayed and then reassayed after a 10-fold dilution into buffer so that the final fenoctimine concentration was 1.65 μM. For three separate experiments, the specific activity of the diluted vesicles was 12.0 ± 1.6 μmoles·mg^{-1}·hr^{-1}. This value was significantly larger than the specific activity prior to dilution (6.1 ± 0.6 μmoles·mg^{-1}·hr^{-1}), showing that inhibition by fenoctimine was reversed by dilution. The activity after dilution was significantly less than control activity under the same conditions, indicating that activity was not totally recovered by dilution. Control dilution experiments at constant fenoctimine concentrations gave activities that were not significantly different from the activities obtained by direct assay, showing that dilution *per se* did not affect the specific activity. These results and the results of similar studies with ATP hydrolysis (data not shown) establish that fenoctimine inhibition can be at least partially reversed by dilution.

DISCUSSION

This study shows that fenoctimine inhibited the $(H^+ + K^+)$-ATPase of gastric microsomes. Inhibition was noncompetitive with substrate. As both the mitochondrial ATPase and the $(Na^+ + K^+)$-ATPase were also inhibited, fenoctimine is not a specific inhibitor of the gastric $(H^+ + K^+)$-ATPase. Inhibition of the $(H^+ + K^+)$-ATPase was characterized by large Hill coefficients and a pronounced dependence of the apparent K_i on the concentration of membranes in the assay. Over the same concentration range that fenoctimine inhibited the $(H^+ + K^+)$-ATPase, the agent also promoted an increase in the turbidity of microsomal suspensions. The latter observation is characteristic of vesicle aggregation. These facts suggest that fenoctimine inhibited the $(H^+ + K^+)$-ATPase as a consequence of some alteration, or disruption, of the microsomal membrane. The disruptive effect of fenoctimine was not simply a deter-

Table 2. Fenoctimine inhibition of pNPP hydrolysis

		pNPPase Activity*	
	Fenoctimine (μM)	Membrane concn (17.5 μg/ml)	Membrane concn (1.75 μg/ml)
Control	0	19.0 ± 1.3	21.3 ± 1.5
experiment	1.65	15.6 ± 1.1	16.2 ± 0.7
	16.5	5.8 ± 0.7	2.8 ± 0.4
Dilution	16.5	6.1 ± 0.6	
experiment	1.65		12.0 ± 1.6†

* All values are given as specific activity in μmoles·mg^{-1}·hr^{-1}; values are mean ± S.D. ($N = 3$).

† Vesicles originally assayed at 17.5 μg/ml and 16.5 μM fenoctimine were diluted 10-fold into buffer without fenoctimine in order to achieve the stated conditions.

gent action to dissolve membranes. Fenoctimine worked at very low concentrations and, in fact, caused an increase in turbidity rather than a clarification, which is characteristic of detergents. Fenoctimine was also not an irreversible denaturant of gastric microsomes as dilution of inhibited microsomes resulted in at least a partial recovery of activity.

Finally, the results of this study do not indicate that the proposed mode by which fenoctimine inhibited the isolated gastric $(H^+ + K^+)$-ATPase necessarily reflects the mechanism by which fenoctimine inhibits gastric acid secretion *in vivo*. At the present time, the mode of action by which fenoctimine inhibits gastric acid secretion is unknown, and there is no evidence that fenoctimine directly inhibits the gastric proton pump *in vivo* or disrupts membranes within the gastric mucosa. Further studies are clearly necessary to determine if either of these effects of fenoctimine occurs *in vivo*. However, the overriding message of the present study is that fenoctimine is not a specific inhibitor of the $(H^+ + K^+)$-ATPase as it inhibits several membrane ATPases.

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