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ACTIONS OF ANTISECRETORY AGENTS ON PROTON TRANSPORT IN HOG GASTRIC MICROSOMES

HIDEAKI NAGAYA, HIROSHI SATOH and YOSHITAKA MAKI
Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan

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Abstract—The properties of K^+-stimulated ATP hydrolysis (K^+-ATPase) and vesicular accumulation of H^+ (H^+ accumulation) in hog gastric microsomes were investigated. The microsomes consisted of smooth surfaced vesicular particles, 70–300 nm in diameter. Both the activities of ATPase and the vesicular accumulation of H^+ were stimulated by K^+ in the presence of Mg^{2+}, and enhanced by the K^+-ionophore, valinomycin. However, there were differences in regulation of K^+-ATPase and H^+ accumulation by K^+ ions, i.e. K^+ at concentrations higher than 10 mM decreased K^+-ATPase activity but further enhanced H^+ transport. This observation suggests that the two reactions are partly independent. The H^+ accumulation was inhibited by omeprazole, fenoctimine, spermine, and NaSCN, but not by cimetidine, prostaglandin E_2, and atropine. The inhibitory effect of omeprazole on H^+ accumulation paralleled the inhibition of K^+-ATPase, while fenoctimine, spermine, and NaSCN suppressed H^+ accumulation, without inhibiting K^+-ATPase, under appropriate concentrations. In addition, the spontaneous diffusion of H^+ across the microsomal membrane was markedly enhanced by fenoctimine, but not by the other agents used. These results indicate that (1) omeprazole inhibits H^+ accumulation by inhibiting K^+-ATPase, (2) fenoctimine suppresses H^+ accumulation mainly by increasing the loss of accumulated H^+ from the microsomal vesicles, (3) spermine and NaSCN reduce H^+ accumulation by inhibiting the transport of H^+ into microsomal vesicles.

The gastric proton pump has been isolated and proved to be an ($H^+ + K^+$)-ATPase [1–4]. Purified microsomes, derived primarily from the apical and tubulovesicular membranes of parietal cells, are capable of accumulating H^+ within the vesicle interior in exchange for K^+ at the expense of ATP under appropriate conditions; the process is mediated by gastric ($H^+ + K^+$)-ATPase.

It was reported that H^+ transport can be inhibited by some species of ions and polyamines, without affecting the rate of K^+-stimulated ATP hydrolysis [5–7]. In the present work, we also found that K^+ at high concentrations enhanced H^+ transport, independently of K^+-ATPase. In an attempt to characterize the H^+ transport mediated by ($H^+ + K^+$)-ATPase, effects of various antisecretory agents on K^+-ATPase, vesicular H^+ accumulation, and passive H^+ diffusion were studied.

MATERIALS AND METHODS

1. *Isolation of gastric microsomes.* Isolated hog stomachs were rinsed with tap water. The fundus was placed in 3 M NaCl to remove the superficial cells, cell debris and mucus. The oxyntic cell-enriched mucosa was scraped off and the scrapings were homogenized in medium consisting of 0.25 M sucrose, 1 mM EDTA and 5 mM Tris–HCl (pH 6.8), using Polytron® (Kinematica). The homogenate was differentially centrifuged to harvest the microsomal fraction. The crude microsomes suspended in 0.25 M sucrose were layered over 7.5% Ficoll (w/w) in 0.25 M sucrose and centrifuged at 100,000 *g* for 3 hr. Microsomal bands appearing at the interface of the 0.25 M sucrose and 7.5% Ficoll were collected, diluted with 0.25 M sucrose, and centrifuged at 100,000 *g* for 1 hr. The pellet was suspended in 0.25 M sucrose with a protein concentration of 0.5 mg/ml and stored at −20° until use. Protein was determined by the method of Lowry *et al.* [8] using bovine serum albumin as a standard.

2. *Electron microscopy.* Partially purified microsomes collected from the interface of the 0.25 M sucrose and 7.5% Ficoll were diluted and spun down at 100,000 *g* for 1 hr. The pellet was fixed for 1 hr with 3% glutaraldehyde buffered with 50 mM cocadylate (pH 7.4). After the glutaraldehyde had been removed by washing with 50 mM Tris–HCl (pH 7.4), the sample was fixed for 1 hr with 1% OsO_4 buffered with 50 mM cocadylate (pH 7.4) at 4°. The pellet was then washed in buffer, dehydrated in increasing concentrations of ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and observed under a TEM 300 electron microscope.

3. *Assay of* K^+*-ATPase.* The assay medium contained in a total volume of 1 ml, 70 mM Tris–HCl (pH 6.8), 5 mM $MgCl_2$, 20 μg/ml membrane protein, and various concentrations of test compounds, in the presence and absence of 10 mM KCl and 10 μM valinomycin. The assay was carried out at 37°. After a preincubation period of 1 hr, the reaction was initiated by adding Tris–ATP (final concentration of 2 mM) and the preparation was incubated for 20 min. The reaction was halted by adding 1 ml of 12% (w/v) trichloroacetic acid. Inorganic phosphate produced by ATP hydrolysis was determined according to Fiske and Subbarow [9]. The compounds to be tested were dissolved in methanol or distilled water, and 10 μl added to the reaction mixture.

4. *Vesicular accumulation of* H^+. The vesicular accumulation of H^+ was measured at room tem-

perature according to Lee and Forte [10]. The method makes use of change in fluorescence intensity (quenching) of acridine orange; the quenching is proportional to the amount of dye taken up by the microsomes. The amount of dye taken up is a sensitive measure of intravesicular H^+ concentration. Acridine orange fluorescence was monitored at 493 → 530 nm (excitation → emission) in an RF-502 spectrofluorometer (Shimadzu). The reaction mixture contained 70 mM Tris–HCl (pH 6.8), 5 mM $MgCl_2$, 150 mM KCl, 10 μM valinomycin, 10 μM acridine orange, 20–50 μg/ml membrane protein, and various concentrations of test compounds. After a preincubation of 1 hr, the reaction was initiated by adding Tris–ATP (final concentration of 2 mM).

5. *Passive diffusion of* H^+. The effects of test compounds on passive diffusion of H^+ were assessed according to Nandi and Ray [6]. Gastric microsomes (20–50 μg) were incubated in 1 ml of medium containing 150 mM KCl, 10 μM acridine orange, 10 mM succinate, and 5.6 mM Tris (pH 4.0) at 25°. After a preincubation period of 1 hr, the pH of the incubation medium was increased from 4.0 to 8.0 by adding 25 mM Tris base. Changes in fluorescent intensity of acridine orange in the medium were observed. Assuming that the vesicular interior equilibrated to about pH 4 in the low pH succinate–Tris medium, the addition of Tris base altering the external medium to pH 8 would thus impose a H^+ gradient of 4 units across the vesicles. When the Tris base is added, acridine orange abruptly accumulates, according to the pH gradient. Thereafter, as H^+ ions passively diffuse from the vesicles, dye is released into the external medium. Test compounds were added to the reaction medium in 10 μl at 3 min after the Tris base had been added.

6. *Drugs*. The following drugs were used: Tris–ATP and valinomycin (Sigma), acridine orange, atropine sulfate and NaSCN (Wako), spermine (Aldrich), nigericin (Calbiochem-Behringer), prostaglandin E_2 (PGE_2, Fuji), cimetidine (extracted from Tagamet® SK&F). Omeprazole and fenoctimine were synthesized in the Chemistry Laboratories of Takeda Chemical Industries. All other reagents used were the best grade available and were used without further purification.

RESULTS

1. *Electron microscopic study*

An electron micrograph of hog gastric microsomal fraction showed that the fraction consisted of vesicular particles of smooth-surfaced membrane free from ribosomes, 70–300 nm in diameter (Fig. 1). Although small vesicles contained electron dense materials, most of the large vesicles had no obvious contents. Almost all of the vesicles were tightly sealed.

2. *Effects of* Mg^{2+} *and* K^+ *on* $(H^+ + K^+)$*-ATPase*

Effect of Mg^{2+}. The effect of Mg^{2+} on K^+-ATPase and H^+ accumulation was investigated in the presence of valinomycin (Fig. 2). Both K^+-ATPase activity and H^+ accumulation were increased by Mg^{2+}, in a concentration-dependent manner. Each reaction reached a maximal level at 3 or 1 mM of Mg^{2+}, respectively. The increase of H^+ accumulation appeared to parallel that of K^+-ATPase activity.

Effect of K^+. The effect of K^+ on K^+-ATPase and H^+ accumulation is shown in Fig. 3. The activity of K^+-ATPase increased with the concentration of K^+ and reached a maximum at 10 mM. The activity was

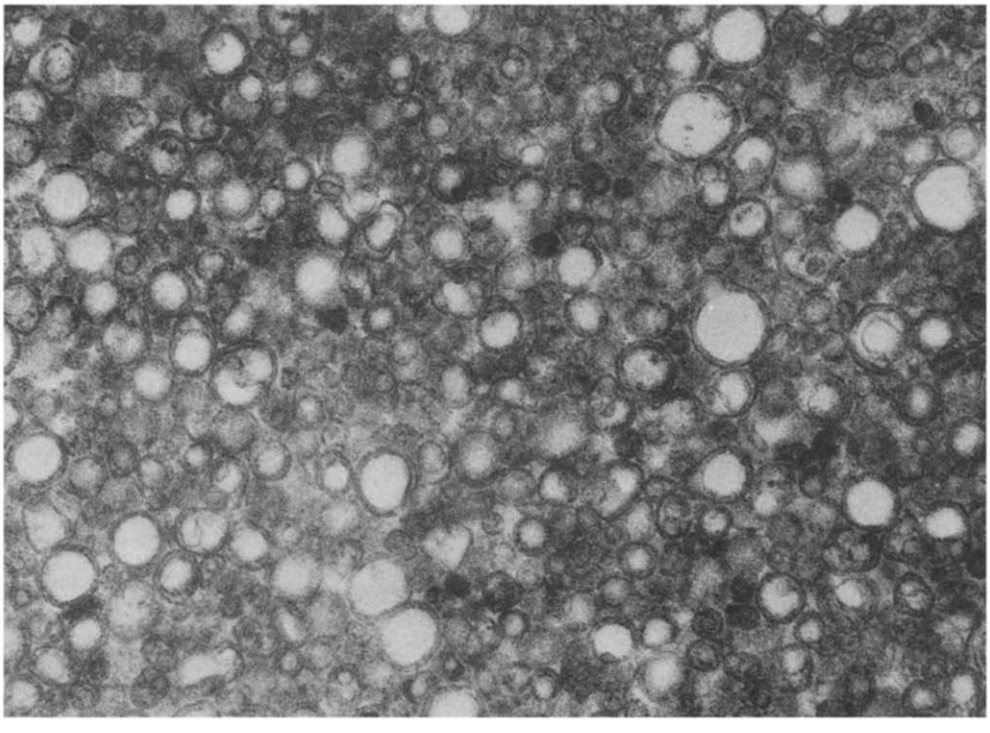

Fig. 1. Electron micrograph of hog gastric microsomal fraction (×41,700).

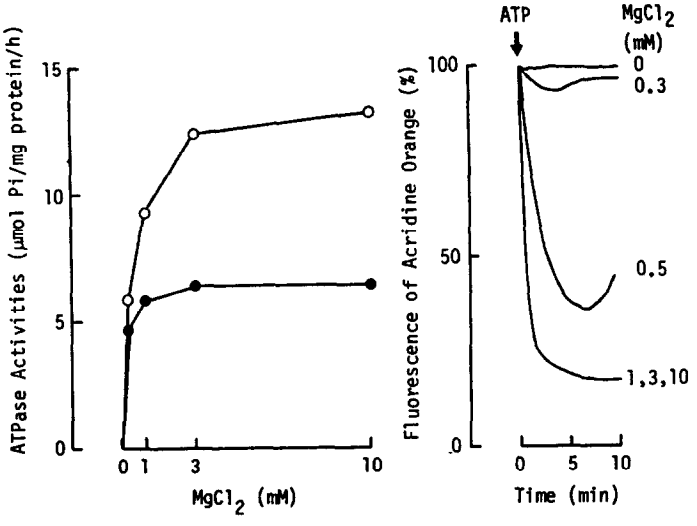

Fig. 2. Effect of Mg^{2+} on K^+-ATPase activity and vesicular accumulation of H^+ in hog gastric microsomes. The assays were carried out at 25°. Left: K^+-ATPase activity with (○) or without (●) 10 μM valinomycin was measured in the presence of 10 mM KCl. Right: The accumulation of H^+ in microsomal vesicles was assessed by quenching of acridine orange fluorescence. The assay was carried out in the presence of 100 mM KCl and 10 μM valinomycin.

markedly enhanced by valinomycin and a maximal value was also obtained at 10 mM. At concentrations over 10 mM, the activity of K^+-ATPase decreased (Fig. 3, left). In the presence of valinomycin, vesicular accumulation of H^+ was stimulated by K^+, in a concentration-dependent manner (Fig. 3, right). In contrast to K^+-ATPase, H^+ accumulation increased with increasing concentrations of K^+, up to 300 mM. The K^+-dependency of H^+ transport was also confirmed in the experiment in which K^+ was replaced by choline with the constant concentration of Cl^-.

Effects of K^+*-ionophores.* The effects of K^+-ionophores on $(H^+ + K^+)$-ATPase are shown in Fig. 4. Valinomycin (1–10 μM) increased the activity of K^+-ATPase in a concentration-dependent manner, but did not affect that of Mg^{2+}-ATPase. The activity of K^+-ATPase reached a maximum at 3 μM of valinomycin; it was 2.5 times higher than that observed

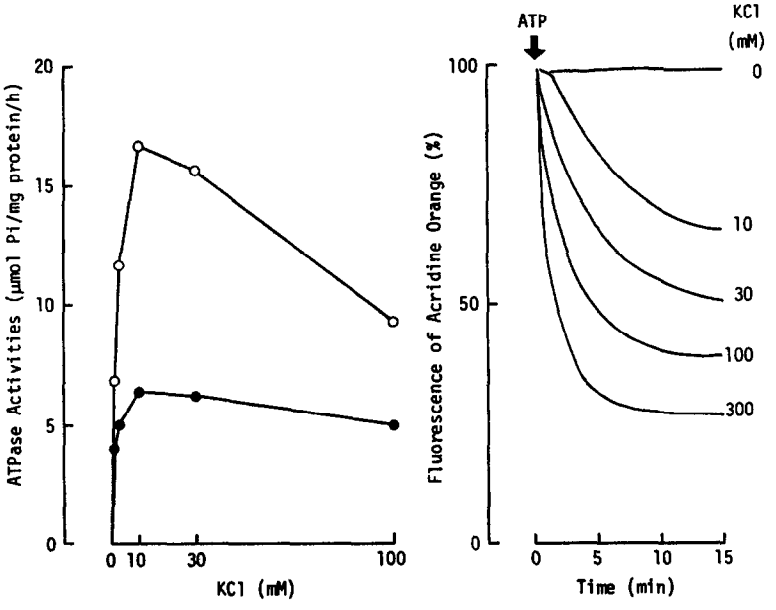

Fig. 3. Effect of K^+ on K^+-ATPase activity and vesicular accumulation of H^+ in hog gastric microsomes. The assays were carried out at 25°. Left: K^+-ATPase activity with (○) or without (●) 10 μM valinomycin was measured in the presence of 5 mM $MgCl_2$. Right: The accumulation of H^+ in microsomal vesicles was assessed by quenching of acridine orange fluorescence. The assay was carried out in the presence of 5 mM $MgCl_2$ and 10 μM valinomycin.

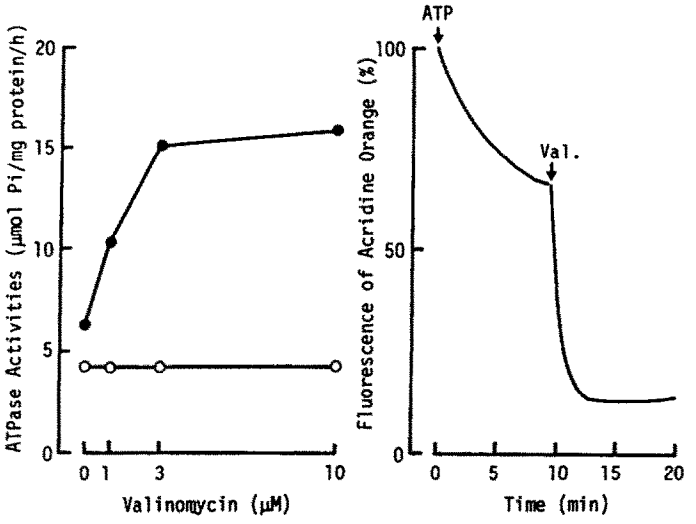

Fig. 4. Effect of valinomycin on Mg^{2+}-ATPase and K^+-ATPase activities and vesicular accumulation of H^+ in hog gastric micsosomes. The assays were carried out at 25°. Left: Each curve represents the activity of Mg^{2+}-ATPase (○) and K^+-ATPase (●). Right: The accumulation of H^+ in microsomal vesicles was assessed by quenching of acridine orange fluorescence. ATP (2 mM) and valinomycin (Val., 10 μM) were added, as indicated by arrows, to the reaction mixture containing 70 mM Tris–HCl buffer (pH 6.8), 5 mM $MgCl_2$, 150 mM KCl, 10 μM acridine orange and 20 μg/ml gastric microsomal vesicles, in 1 ml.

with KCl alone (Fig. 4, left). When ATP was added to the reaction mixture in the absence of valinomycin, the acridine orange fluorescence slowly decreased, by 30%. The addition of valinomycin to 10% of the initial level (Fig. 4, right). These fluorescence and the fluorescent intensity decreased to 10% of the initial level (Fig. 3, right). These results suggest that both K^+-ATPase and H^+ accumulation are stimulated by K^+ ions within the vesicles.

3. *Effects of antisecretory agents on* ($H^+ + K^+$)-*ATPase*

Effects of antisecretory agents on K^+-*ATPase.* The activity of K^+-ATPase was determined in the presence of valinomycin. Omeprazole (1–100 μM), fenoctimine (1–100 μM), and spermine (0.1–1 mM) inhibited the activity, in a concentration-dependent manner (Figs. 5–7). The IC_{50} values were 6.8, 6.2, and 610 μM, respectively. NaSCN (1–10 mM)

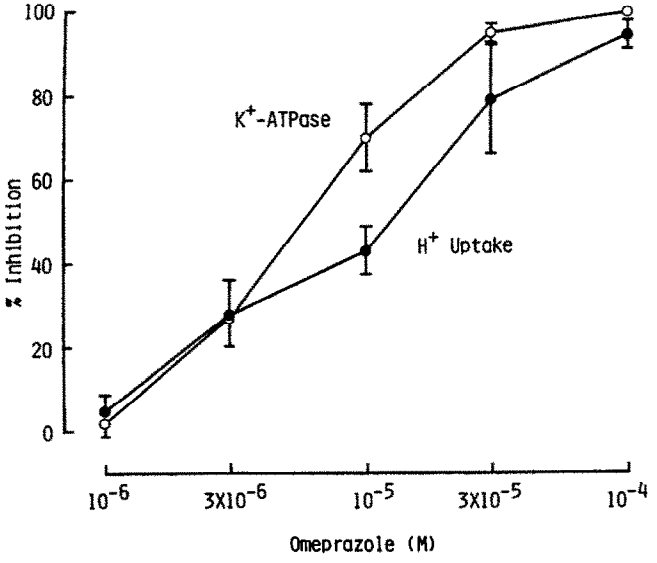

Fig. 5. Effect of omeprazole on K^+-stimulated ATP hydrolysis (K^+-ATPase) and vesicular accumulation of H^+ (H^+ uptake) in hog gastric microsomes. Results are expressed as % inhibition of control. Each point represents a mean value and standard error of three or four experiments.

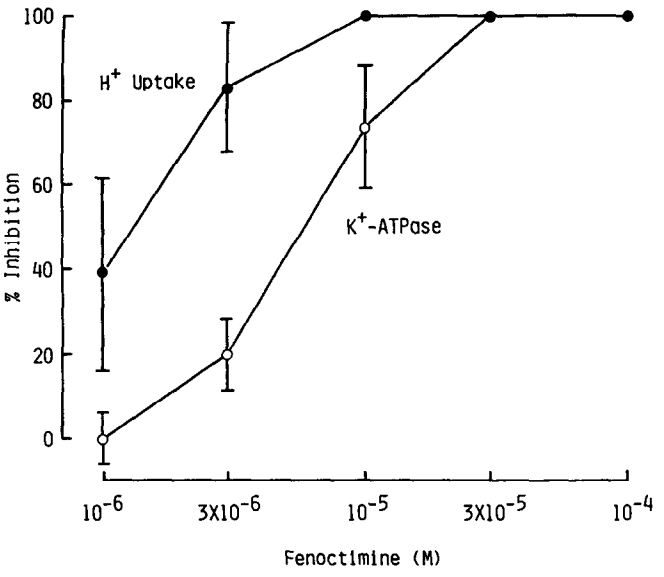

Fig. 6. Effect of fenoctimine on K^+-stimulated ATP hydrolysis (K^+-ATPase) and vesicular accumulation of H^+ (H^+ uptake) in hog gastric microsomes. Results are expressed as % inhibition of control. Each point represents a mean value and standard error of three or four experiments.

slightly depressed the activity (Fig. 8). Cimetidine (1–100 μM), PGE_2 (1–100 μM) and atropine (1–100 μM) did not inhibit the activity.

Effects of antisecretory agents on H^+ *accumulation.* The degree of vesicular accumulation of H^+ was assessed by the maximal value of quenching obtained after ATP had been administered. Omeprazole (1–100 μM), fenoctimine (1–100 μM), spermine (0.1–1 mM), and NaSCN (1–10 mM) inhibited the accumulation of H^+, in a concentration-dependent manner, with IC_{50} values of 15.5 μM, 1.5 μM, 160 μM, and 2 mM, respectively (Figs. 5–8). On the other hand, cimetidine (100 μM), PGE_2 (100 μM), and atropine (100 μM) did not affect H^+ accumulation.

Effects of antisecretory agents on passive diffusion of H^+. The effect of fenoctimine (1–100 μM) on passive H^+ diffusion from the gastric microsomal vesicles is shown in Fig. 9. The rate of passive diffusion of H^+ was markedly increased by fenoctimine, in a concentration-dependent manner. Fenoctimine even at a high concentration of 100 μM did not increase the fluorescent intensity of acridine orange in the absence of the microsomes. On the other hand, omeprazole (100 μM), spermine (1 mM), NaSCN (10 mM), cimetidine (100 μM), PGE_2

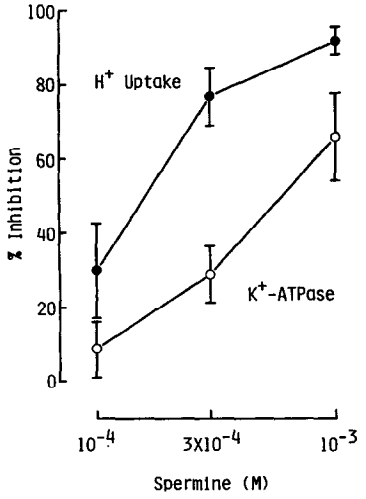

Fig. 7. Effects of spermine on K^+-stimulated ATP hydrolysis (K^+-ATPase) and vesicular accumulation of H^+ (H^+ uptake) in hog gastric microsomes. Results are expressed as % inhibition of control. Each point represents a mean value and standard error of three or four experiments.

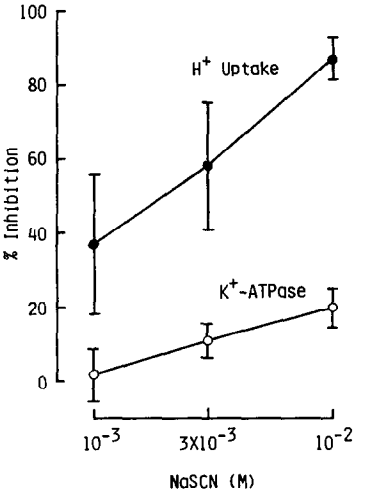

Fig. 8. Effect of NaSCN on K^+-stimulated ATP hydrolysis (K^+-ATPase) and vesicular accumulation of H^+ (H^+ uptake) in hog gastric microsomes. Results are expressed as % inhibition of control. Each point represents a mean value and standard error of four experiments.

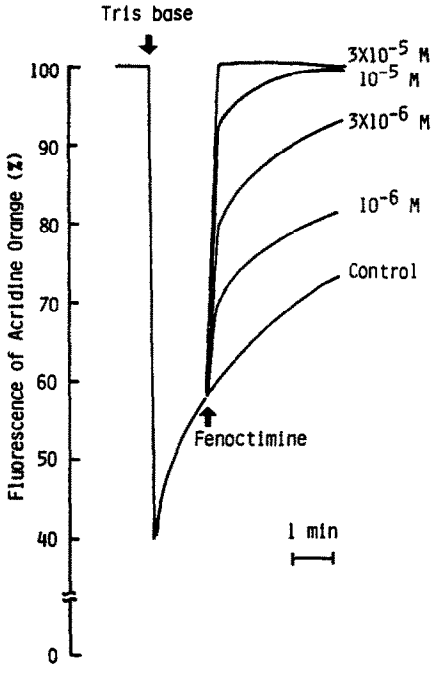

Fig. 9. Effect of fenoctimine on the spontaneous diffusion of H^+ from the gastric microsomal vesicles. Gastric microsomes were incubated in 1 ml of medium containing 150 mM KCl, 10 μM acridine orange, 10 mM succinate and 5.6 mM Tris at pH 4.0 for 1 hr at 25°. An artificial pH gradient was formed by adding 25 mM Tris base to change the external pH from 4 to 8. The addition of fenoctimine at various concentrations is shown by the arrow.

(100 μM), and atropine (100 μM) did not alter the rate of spontaneous diffusion of H^+.

DISCUSSION

The hog gastric microsomes used for the experiments were prepared according to the procedure of Wallmark *et al.* [11] with some modifications. Electron micrography revealed that the microsomes were tightly sealed with smooth surfaced membranes, as reported by Saccomani *et al.* [13]. The microsomes were able to hydrolyze ATP and thereby to accumulate H^+. Both ATP hydrolysis and H^+ accumulation were stimulated by K^+ ions and further enhanced by the K^+-ionophore, valinomycin. These results indicate that the microsomes possessed the proton pump mediated by K^+-ATPase. However, there are differences in the regulation of K^+-ATPase and H^+ accumulation by K^+ ions. In the presence of valinomycin, the H^+ transport was markedly increased by increasing the external concentration of K^+, up to 300 mM, while K^+-ATPase was decreased by K^+ ions, at concentrations exceeding 10 mM. Stewart *et al.* [13] and Ljungstrom *et al.* [14] reported that alkali metal ions suppressed K^+-ATPase and suggested that K^+ ions have dual effects on $(H^+ + K^+)$-ATPase, i.e. stimulation from the luminal side and inhibition from the cytosolic side. Nandi and Ray [6] reported that there were high and low affinity K^+ sites in $(H^+ + K^+)$-ATPase, and these were related to stimulation of K^+-ATPase and of H^+ accumulation, respectively. Our results also indicate that there is a K^+ site related to the inhibition of K^+-ATPase. However, we observed no inhibitory effect of K^+ ions on transport of H^+. In addition, the affinity for K^+ between K^+-ATPase and H^+ accumulation differed. Therefore, it is suggested that H^+ transport is partly independent of K^+-ATPase.

In the present work, we attempted to pharmacologically characterize H^+ transport mediated by $(H^+ + K^+)$-ATPase. For this purpose, the effects of K^+-ATPase and H^+ transport of seven antisecretory agents were studied from the point of concentration–inhibition relations. The effects on H^+ transport were assessed by both vesicular accumulation of H^+ and passive diffusion of H^+.

Omeprazole, a substituted benzimidazole, is a potent antisecretory agent that inhibits $(H^+ + K^+)$-ATPase [15, 16]. In our study, omeprazole suppressed both K^+-ATPase and H^+ accumulation, over the same concentration range. Omeprazole did not affect the rate of passive diffusion of H^+. These results support the findings by Wallmark *et al.* [15] that omeprazole may suppress H^+ transport by inhibiting K^+-ATPase.

Fenoctimine is reported to have an antisecretory effect in humans and in various laboratory animals [17–19]. The site of action was suggested to be beyond c-AMP in the sequence of acid secretion in rat parietal cells [17]. In the present study, fenoctimine inhibited H^+ accumulation, in a concentration-dependent manner, though it suppressed K^+-ATPase at higher concentrations. In addition, fenoctimine markedly increased the rate of passive diffusion of H^+, at lower concentrations. These results indicate that fenoctimine can suppress H^+ accumulation mainly by increasing the loss of accumulated H^+ from the microsomal vesicles. Recently, Reenstra *et al.* [20] showed that fenoctimine indirectly inhibits K^+-ATPase in gastric microsomes by inducing perturbation of the microsomal membrane. It is likely that fenoctimine acts on membranes to increase H^+ permeability without affecting K^+-ATPase, at lower concentrations; this action might be involved in the antisecretory effect.

At lower concentrations, spermine suppressed vesicular accumulation of H^+, without affecting K^+-ATPase. Unlike fenoctimine, spermine did not enhance the rate of spontaneous diffusion of H^+ from the vesicles. These results indicate that spermine can interfere with the transport of H^+ without inhibiting K^+-ATPase, thereby supporting the postulation of Ray *et al.* [5] that spermine exhibits an antisecretory effect only from the secretory side of the gastric mucosa, as an uncoupler of $(H^+ + K^+)$-ATPase. In our work, as spermine also inhibited K^+-ATPase at higher concentrations, the possibility cannot be excluded that the antisecretory effect of spermine involves the inhibition of K^+-ATPase.

NaSCN inhibited vesicular accumulation of H^+ but did not affect the activity of K^+-ATPase. In addition, NaSCN did not affect the rate of spontaneous diffusion of H^+. These results are consistent with those of Nandi and Ray [6], and suggest that NaSCN may suppress vesicular accumulation of H^+ by acting on $(H^+ + K^+)$-ATPase as an uncoupler.

Reenstra and Forte [21] reported that NaSCN suppressed the accumulation of H^+ by increasing the permeability to H^+. Further studies are needed to explain this discrepancy.

On the other hand, cimetidine, PGE_2 and atropine did not affect K^+-ATPase or H^+ transport in the gastric microsomes, a finding which supports the data of Fellenius *et al.* [22] and Im and Blakeman [23].

From the results described above, the effects of antisecretory agents on $(H^+ + K^+)$-ATPase can be classified as the inhibition of H^+ transport via inhibition of K^+-ATPase (omeprazole), the selective inhibition of H^+ transport to cause uncoupling of $(H^+ + K^+)$-ATPase (spermine, NaSCN), and the suppression of vesicular accumulation of H^+ by increasing the loss of accumulated H^+ (fenoctimine).

Recently Cl^- conductance has been thought to be one of the important factors to control $(H^+ + K^+)$-ATPase [24]. In this study we did not examine the effects of agents on Cl^- conductance, therefore the possibility that the agents affected the H^+ transport by influencing Cl^- conductance could not be excluded. This is the subject for further study.

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