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Correlation between Acid Secretion and Proton Pump Activity during Inhibition by the Proton Pump Inhibitors Omeprazole and Pantoprazole

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ABSTRACT. Omeprazole and pantoprazole are known to be irreversible, SH-acting inhibitors of gastric H^+,K^+-adenosine triphosphatase (H^+,K^+-ATPase). Both drugs concentration-dependently and pH-dependently inhibited K^+-dependent *p*-nitrophenyl phosphatase (K^+-pNPPase) activity in purified rabbit gastric microsomes. The potency of omeprazole was about three times that of pantoprazole in the pH ranges tested. Both drugs also inhibited acid secretion, as determined by [^{14}C]aminopyrine accumulation in isolated rabbit gastric glands, with the potency ratio being about 5 (omeprazole over that of pantoprazole). Under conditions in which acid secretion was inhibited completely by the drugs, the total K^+-pNPPase activity in the digitonin-permeabilized glands was scarcely reduced, showing an apparent discrepancy between the acid secretion and the proton pump activity. The isolated glands were stimulated with secretagogues for 30 min in the presence of the inhibitors, homogenized, and then separated into fractions in which K^+-pNPPase activity was measured. Omeprazole exclusively inhibited the activity in the low-speed fraction, which was rich in the apical membranes, whereas pantoprazole did not inhibit activity in any fraction. When the time of treatment with the inhibitors was increased up to 5 hr, the inhibition of the total K^+-pNPPase activity in the glands reached a plateau at an inhibition rate lower than 50% within 2 hr. This suggested that no continuous recycling of the proton pump was occurring during stimulation. The inhibitory effect of both drugs on the permeabilized gland preparation was less potent than that on the purified enzyme, especially at the higher pH, and it appeared to be partially reversible. The extent of the reduction in potency was more prominent for pantoprazole. It is concluded that a lower amount of proton pump activity needs to be inhibited by pantoprazole than by omeprazole to achieve the same extent of acid secretion inhibition. This appears to be due to the nature of pantoprazole, i.e. the requirement of low pH for activation and the partial reversibility of the inhibition. BIOCHEM PHARMACOL **58**;8:1349–1359, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. omeprazole; pantoprazole; H^+, K^+-ATPase; gastric glands; acid secretion; rabbit

Activation of gastric acid secretion is achieved by two concomitant functional changes: (a) tubulovesicles that contain an inactive proton pump, H^+,K^+-ATPase†, fuse with the apical secretory membrane, and (b) the apical membrane acquires permeability to KCl [1–3]. Proton pump inhibitors, which block the final step of acid secretion, are quite useful as remedies for peptic ulcer (especially the H2 blocker-resistant type), since they effectively suppress acid secretion stimulated by any types of secretagogues [4]. Proton pump inhibitors have been classified into two types. One is reversible and the other is irreversible; the former type includes 2-methyl-8-(phenyl-methoxy)-imidazo-1,2-pyridine-3-acetonitrile (SCH28080) and 3-butyryl-8-methoxy-4-(2-tolylamino)quinoline (SK&F 96067), and the latter type consists of benzimidazole derivatives: omeprazole, lansoprazole, rabeprazole (E3810), pantoprazole, and others. The latter types share a common mechanism, i.e. the inactive form turns into a thiophilic cyclic sulfenamide (an active form) in the acidic space of the gastric lumen and forms covalent S—S bonds with SH groups at the luminal side of ATPase [4–6]. Therefore, it is suspected that this type of inhibitor suppresses the enzyme actually doing the pumping [7]. In addition, the recovery from complete suppression after the cessation of drug intake is quite slow, since it depends on *de novo* synthesis of the enzyme [8]. However, there are a few reports examining the precise relationship between inhibition of enzyme activity and acid secretion [9, 10], and there are discrepancies among them.

Of the irreversible proton pump inhibitors, pantoprazole has the property of not affecting P450, in contrast to omeprazole or lansoprazole [11, 12]. Pantoprazole is more

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† *Abbreviations*: H^+, K^+-ATPase, H^+, K^+-adenosine triphosphatase; K^+-pNPPase, K^+-dependent *p*-nitrophenyl phosphatase; IBMX, isobutylmethylxanthine; and PIPES, piperazine-1,4-bis(2-ethanesulfonic acid).

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stable in acidic conditions and requires a lower pH for activation than other benzimidazole derivatives [5, 6, 13, 14]. The methyl in the pyridine ring of omeprazole or lansoprazole is replaced by methoxy in pantoprazole, and this substitution increases its chemical stability under neutral to acidic conditions, resulting in an increased selectivity for the proton pump [15]. This is supported by the observation that the rate of inhibition of H^+,K^+-ATPase by pantoprazole is exceptionally slower in microsomes under acid-transporting conditions than the rate of other inhibitors: rabeprazole, omeprazole, and lansoprazole [6]. However, there have been a limited number of reports making a precise comparison between these two drugs in terms of enzyme inhibition *in vitro* and antisecretory activity *in vivo*, and it is still uncertain how these differences are related to the inhibition of acid secretion *in vivo*.

Pantoprazole is now on the market in Europe and is under clinical trial in Japan. Therefore, it is quite important to assess the possible differences in clinical effects of these drugs from differences in their pharmacodynamics. Although it is considered to be common to the basic mechanism of action of the irreversible type of proton pump inhibitors, in fact the precise position of SH in the site of action as well as the degree of reversibility is different among the derivatives [4–6, 16]. Moreover, although it generally has been accepted that no recovery of the inhibited proton pump occurs *in vivo*, possible recovery of this activity has been suggested in the case of E3810 [10, 17] and omeprazole [18]. In the present study, experiments were conducted to investigate the relationship between the inhibition of acid secretion and enzyme activity, using purified proton pump and isolated rabbit gastric glands.

MATERIALS AND METHODS

Materials

Pantoprazole, omeprazole, and SCH28080 were synthesized and provided by the Daiichi Pharmaceutical Co. Ltd. [^{14}C]Aminopyrine was purchased from New England Nuclear. Other chemicals of reagent grade were purchased from the Sigma Chemical Co. or Wako Pure Chemical. New Zealand White rabbits weighing about 2.5 kg were obtained from the Shiraishi Co.

Isolation of Rabbit Gastric Glands and Measurement of Their Functions

Isolated gastric glands were prepared from rabbits by a combination of high-pressure perfusion and collagenase digestion [19]. Acid secretion of the glands was monitored by the accumulation of a weak base, [^{14}C]aminopyrine [20]. Maximal stimulation by histamine (0.1 mM) + IBMX (50 μM) was performed for 30 min at 37°. In general, stimulation of acid secretion was expressed as the increment of the aminopyrine ratio above the resting value, and the effects of drugs on the agonists were expressed as percentages of the control values, i.e. treated with vehicle alone. Test drugs all were dissolved in dimethylsulfoxide such that the final concentration of the vehicle was less than 0.5%, v/v.

Fractionation of the homogenate of isolated rabbit glands was performed using a method reported previously [21]. In brief, resting (0.1 mM cimetidine for 30 min) or stimulated (histamine + IBMX for 30 min) glands in the absence or presence of proton pump inhibitors were homogenized. After removal of cell debris (40 *g*, for 5 min), the homogenate was fractionated sequentially into three pellets, P1 (4,000 *g*, for 10 min), P2 (14,500 *g*, for 10 min), and P3 (100,000 *g*, for 45 min), and the supernatant.

Permeabilized glands were prepared basically according to Hersey *et al.* [22]. Isolated glands were suspended in 250 mM sucrose, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid/NaOH (pH 7.4), protease inhibitor mixture (1.5 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 1.5 μM aprotinin), 20 μg/mL of digitonin, and incubated for 10 min, washed with the buffer without digitonin for 10 min, and suspended in the buffer.

Purification and Enzymatic Assay of H^+,K^+-ATPase

H^+,K^+-ATPase was purified from the microsomes of resting rabbit gastric mucosal homogenate by a sucrose density gradient as described [23]. In the present study, microsomes floating on the 21% sucrose layer were used exclusively. When the effects of pretreatment with ATP + valinomycin were assessed, "tight" vesicles, freshly purified and confirmed by acridine orange quenching, were used, whereas "leaky" vesicles, subjected to freezing–thawing cycles, were used for the usual assays. When the effect of glutathione was examined, the vesicles were lyophilized to make sure that glutathione was accessible to the luminal side of the enzyme. In general, K^+-pNPPase activity was measured in a total volume of 1 mL containing 7.5 mM Tris, pH 7.5, 2.5 mM $MgSO_4$, 0.1 mM ouabain, with or without 20 mM KCl, and 5 mM sodium *p*-nitrophenyl phosphate. When the pH of the medium was lowered, 7 mM PIPES/Tris (pH 6.0) or 7 mM Tris-acetate (pH 5.0) was employed instead of Tris–HCl, and protease inhibitor mixture was included. The net increase by KCl was considered to be due to K^+-pNPPase activity.

The ATPase assay was performed in a total volume of 1 mL containing 10 mM PIPES, pH 6.5, 1 mM $MgSO_4$, 0.1 mM ouabain, with or without 20 mM KCl, and 1 mM ATP. When a preparation other than microsomes was assayed, 5 μg/mL of oligomycin was added to exclude mitochondrial ATPase. Liberated phosphate was quantitated as described [24].

Acridine orange quenching was performed using purified enzyme or digitonin-permeabilized glands in a solution containing 125 mM KCl, 50 mM sucrose, 0.4 mM ATP, 0.4 mM $MgSO_4$, 25 μM EDTA, 10 mM phosphocreatine, 1.5 μM acridine orange, and 4 mM Tris–HCl, pH 6.8. When permeabilized glands were used, 5 μg/mL of oligomycin was included. Fluorescence changes (excitation: 493 nm, emission: 540 ± 6 nm band pass filter) were monitored

TABLE 1. IC_{50} Values and potency ratio of omeprazole to pantoprazole on K^+-pNPPase activity of purified gastric microsomes under various conditions

Treatment		IC_{50} (μM)		
Preincubation	Assay pH	Omeprazole	Pantoprazole	Potency ratio
pH 7.5	7.5	78.0	212	2.7
pH 6.0	6.0	5.8	14.0	2.4
pH 5.0	5.0	1.3	4.3	3.3
pH 5.0	7.5	1.4	9.2	6.6
ATP + valinomycin	7.5	1.4	33.0	24.0

Each IC_{50} value was calculated from three to four concentration-response curves performed in duplicate.

continuously with a spectrophotofluorometer (CAF-110; JASCO Corp.) at 37°.

Statistical Analysis

Parametric data were expressed as means ± SEM. The IC_{50} was calculated from the concentration–response curves with the aid of a computer program (Graphpad Inplot). Multiple comparisons were analyzed by ANOVA and Dunnett's *post hoc* test using a computer program (Super ANOVA, ABACUS Concepts). The level of significance was set uniformly at $P < 0.05$, and no further calculation of P values was performed.

RESULTS

Effects of Proton Pump Inhibitors on K^+-pNPPase of Purified Gastric Microsomes

The potency of the K^+-pNPPase inhibitors omeprazole and pantoprazole was measured at pH 7.5, 6.0, and 5.0 in purified gastric microsomes. The IC_{50} values were calculated from each curve and listed in Table 1. Figure 1 shows the plotting of IC_{50} values against assay pH. In accordance with previous reports [5, 13], both omeprazole and pantoprazole showed increases in their potency as the pH went down. At each pH tested, the potency ratio of omeprazole to pantoprazole was consistently about 3.

To confirm that the inhibition of the enzyme was irreversible even when the assay pH was increased, we measured K^+-pNPPase activity at pH 7.5 after the gastric vesicles were preincubated with the inhibitors at pH 5.0. As shown in Fig. 1 and Table 1, the IC_{50} values of omeprazole and pantoprazole were 1.4 and 9.2 μM, respectively. At pH 7.5, omeprazole showed values approximately equal to that at pH 5.0, whereas pantoprazole showed values about twice as large as that at pH 5.0. This indicates that the effect of omeprazole was completely irreversible, while that of pantoprazole was partially reversible in the purified enzyme preparation.

The above experiments were performed by changing the pH of the assay medium. This does not exactly mimic the actual mechanism of inhibition by the drugs, i.e. the luminal side of the proton pump is lowered exclusively, and the inhibitor accumulates in the acidic space. Lowering the pH outside the vesicle also reduces the enzyme activity, making the effects of the drugs difficult to assess. Thus, we lowered the pH of the inside of the vesicle by the addition of ATP and valinomycin, then the inhibitors were added, and finally the enzyme activity was measured at pH 7.5. As shown in Fig. 2, in the presence of a negative control (nigericin) that exchanges H^+ with K^+, ATP was hydrolyzed (no proton gradient was formed), and the potency of the inhibitors was weak. On the other hand, in the presence of valinomycin, where a pH gradient is expected to be formed, the potency of both inhibitors was increased. Omeprazole showed a marked decrease in IC_{50} compared with that at pH 7.5, whereas pantoprazole showed a relatively small decrease in IC_{50} (Table 1). This is consistent with the result of Besancon *et al.* [6] using H^+,K^+-ATPase activity as proton pump activity. This also suggests that the pH attained by ATP and valinomycin was not low enough to alter pantoprazole to its active form.

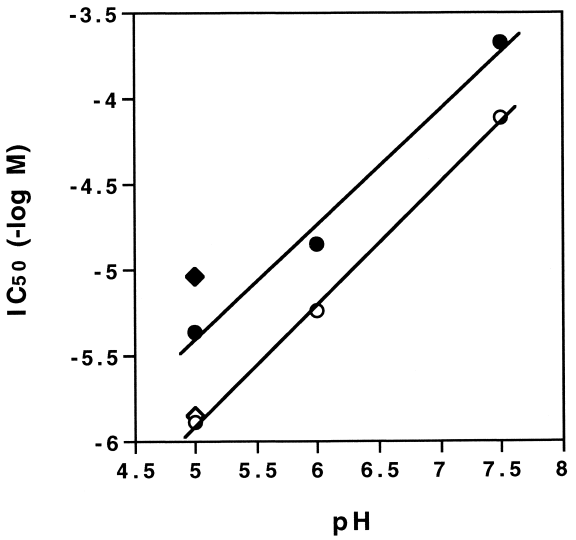

FIG. 1. pH-dependent changes in IC_{50} values of omeprazole (open symbols) and pantoprazole (closed symbols) on the K^+-pNPPase activity of purified H^+, K^+-ATPase. Purified gastric vesicles were preincubated with omeprazole or pantoprazole in the assay buffer at the respective pH at 37° for 30 min, and then the enzyme activity was assayed at the same pH (circles) or at pH 7.5 (diamonds). Note that the IC_{50} of pantoprazole, but not omeprazole, was increased by raising the assay pH from 5.0 to 7.5. Each IC_{50} value was calculated from three to four concentration–response curves performed in duplicate.

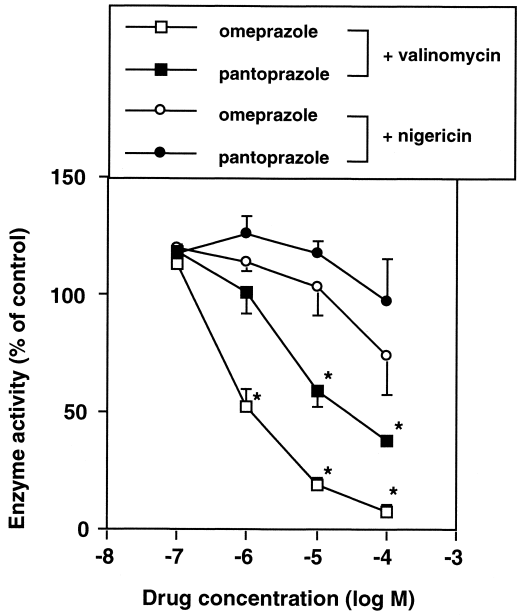

FIG. 2. Influence of luminal acidification on the inhibitory effects of omeprazole and pantoprazole on K^+-pNPPase. Purified gastric vesicles were preincubated in the presence of ATP + valinomycin (luminal side acidified) or ATP + nigericin (luminal side not acidified) at 37° for 10 min, and the vesicles were incubated further with omeprazole or pantoprazole for 30 min. After the removal of the agents by centrifugation, the enzyme activity was assayed at pH 7.5. Data are expressed as a percentage of the vehicle control value (44–65 μmol/hr/mg protein). Means ± SEM of three independent assays performed in duplicate are shown. Key: (*) significantly different from control at $P < 0.05$.

Inhibition of Acid Secretion and K^+-pNPPase in Isolated Glands

Figure 3A shows the effects of the inhibitors on aminopyrine accumulation (acid secretion) in isolated gastric glands maximally stimulated by histamine + IBMX. Omeprazole and pantoprazole both concentration-dependently inhibited aminopyrine accumulation in the glands. The IC_{50} value of each was 0.64 and 3.0 μM, respectively (Table 2).

TABLE 2. IC_{50} Values and potency ratio of omeprazole to pantoprazole on [^{14}C]aminopyrine accumulation, acridine orange quenching, or K^+-pNPPase activity of intact or permeabilized gastric gland preparation

Preparation	IC_{50} (μM)		Potency ratio
	Omeprazole	Pantoprazole	
Intact glands			
Aminopyrine accumulation	0.64	3.0	4.7
K^+-pNPPase (pH 7.5)*	220	-†	-‡
Permeabilized glands			
Acridine orange quenching	6.4	24	3.7
K^+-pNPPase			
pH 7.5	-†	-†	-‡
pH 5.0	106	368	3.5
pH 5.0 → 7.5§	450	-†	-‡

* Glands were treated with the inhibitors under stimulation and then permeabilized with digitonin to measure K^+-pNPPase activity at pH 7.5.

† The IC_{50} values could not be calculated because of poor inhibition.

‡ The potency ratio could not be estimated because of the lack of IC_{50} value.

Each IC_{50} value was calculated from three to four concentration-response curves performed in duplicate.

§ Glands were permeabilized with digitonin, treated with the inhibitors at pH 5.0, and then K^+-pNPPase activity was measured at pH 7.5.

It is a very interesting question how much of the proton pump in parietal cells was inhibited when acid secretion was suppressed. To answer the question, we permeabilized the glands with digitonin after the glands were treated with histamine + IBMX in the absence or presence of the inhibitors, and then the K^+-pNPPase activity was measured at pH 7.5. Surprisingly, 10 μM omeprazole and 100 μM pantoprazole, both of which were enough for complete inhibition of acid secretion (Fig. 3A), reduced the enzyme activity to only 25 and 21% of control (Fig. 3B), respec-

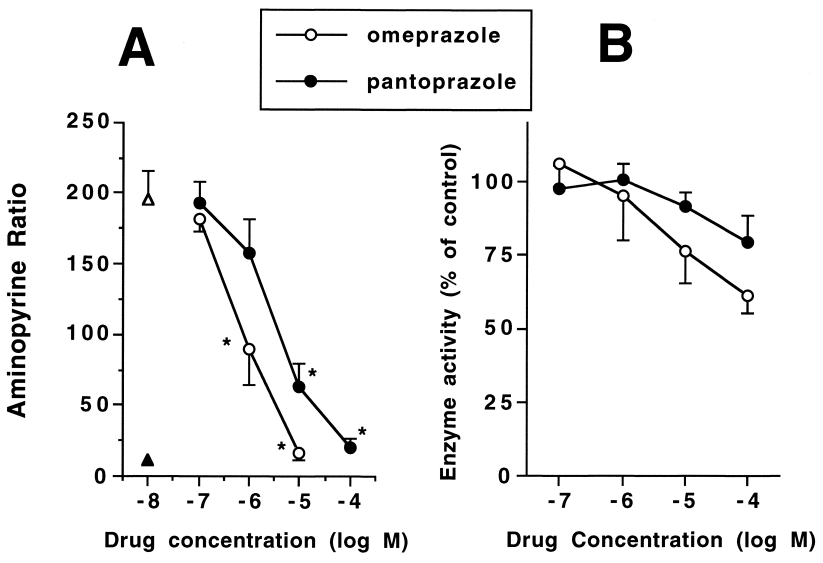

FIG. 3. Effects of omeprazole and pantoprazole on [^{14}C]aminopyrine accumulation and on K^+-pNPPase activity in isolated rabbit gastric glands stimulated by histamine and IBMX. (A) Isolated glands were stimulated by 0.1 mM histamine and 50 μM IBMX for 30 min at 37° in the presence of various concentrations of omeprazole (○) or pantoprazole (●). Aminopyrine ratios without secretagogues (▲) and with secretagogues without inhibitors (open triangle) also are shown. Absolute values of the aminopyrine ratio were calculated and are shown as means ± SEM of three independent assays performed in duplicate. Key: (*) significantly different from control at $P < 0.05$. (B) Isolated glands were treated as in (A) and permeabilized with digitonin, and K^+-pNPPase was measured at pH 7.5. Data are expressed as a percentage of the vehicle control value (5.6 to 9.5 μmol/hr/mL of suspended glands). Means ± SEM of three independent assays performed in duplicate are shown.

tively, and with very high IC_{50} values (Table 2). Although free inhibitors were washed out after digitonin treatment, the inhibition should persist until the assay point, since the inhibition is achieved by the formation of covalent bonds between the inhibitor and the enzyme. Here we found a discrepancy in that the K^+-pNPPase activity was almost unabated even when the acid secretion was suppressed completely.

Examination of the Cause of the Discrepancy between K^+-pNPPase Activity and Acid Secretion

There could be three possible causes of the above-mentioned discrepancy: (a) K^+-pNPPase activity cannot be measured in the permeabilized glands because of an unknown factor(s); (b) the proton pump can recover from inhibition by omeprazole or pantoprazole at the cellular level; or (c) a very small compartment of enzyme, participating in acid secretion, is inhibited preferentially by the drugs in the gastric gland.

At first, we examined the effects of the inhibitors on digitonin-permeabilized glands using the acridine orange quenching technique. Gastric glands, prestimulated and then permeabilized by digitonin, have an ability to transport H^+ in the presence of ATP, and this can be monitored by the quenching of acridine orange [22]. As shown in Fig. 4, the quenching by stimulated glands persisted more than 10 min. Although the recording was noisy due to light scattering by the glands as large particles, the net proton gradient could be estimated at the end of the experiment by the addition of nigericin, a cation-exchanging ionophore. Omeprazole, added when the quenching reached its maximum, inhibited the pump activity and the fluorescence recovered due to proton leakage. The IC_{50} values of omeprazole and pantoprazole from the inhibition curves were calculated to be 6.4 and 23.7 μM, respectively, showing 1/10 of the potency obtained in intact glands (Table 2). From these results, it was confirmed that proton pump inhibitors, which were added to the digitonin-permeabilized glands, were also effective on the functional proton pump on the apical membrane. The reduction of their effect might reflect the lower capacity of the permeabilized glands to produce acid compared with intact glands.

In the next experiment, the K^+-pNPPase assay was performed using digitonin-permeabilized glands. Employing the routine K^+-pNPPase assay conditions, omeprazole or pantoprazole was added to the glands, and a K^+-pNPPase assay was performed at pH 7.5 but no inhibition was observed, suggesting a large reduction in the potency of the inhibitors in the glands. Next, permeabilized glands were preincubated with the inhibitors at pH 5.0, and K^+-pNPPase activity was assayed at that pH. The IC_{50} values of omeprazole and pantoprazole were 106 and 368 μM, respectively (Table 2), the potency being less than 1/100 of that in the purified enzyme. It had been observed that when the purified enzyme was preincubated with the inhibitors at

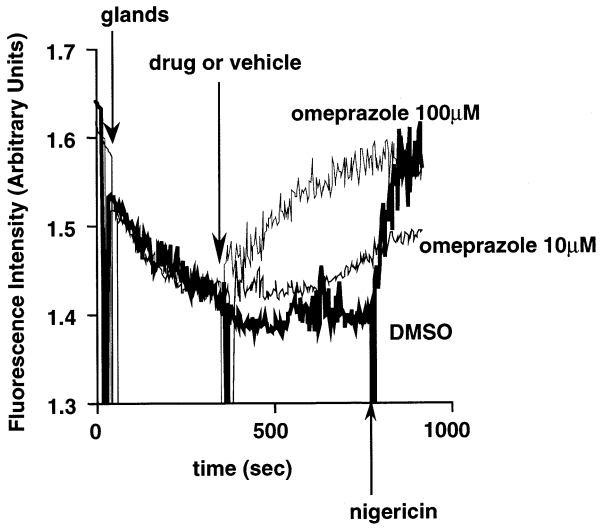

FIG. 4. Inhibitory effect of omeprazole on acridine orange quenching by isolated gastric glands. Isolated rabbit gastric glands were stimulated by 0.1 mM histamine and 50 μM IBMX for 30 min at 37° and permeabilized with digitonin. A suspension of glands (about 6 mg protein) was added to the buffer containing KCl, ATP, phosphocreatine, and acridine orange at the first arrow and observed during valinomycin-independent acridine orange quenching. In the control test (the lower thick line), dimethylsulfoxide was added at the second arrow to confirm the maintenance of the pH gradient formed, and nigericin was added to estimate the net gradient formed at the third arrow. When omeprazole was added at the second arrow (thin lines), the quenching was recovered gradually. Typical traces were performed for three independent experiments, with identical results.

pH 5.0 and the assay pH was increased to 7.5, no or small change in inhibition was found compared with the case in which assay pH was kept at 5.0 (Table 1). In contrast, when the permeabilized glands were treated with the drug at pH 5.0 and K^+-pNPPase activity was measured after the pH was adjusted to 7.5, the inhibitory activity was reduced markedly (Fig. 5, Table 2). The reduction in the potency was more prominent for pantoprazole compared with omeprazole. It was obvious from these results that the effect of the proton pump inhibitors was reduced and partially reversible in the isolated gastric gland preparation.

To determine if there were any problems in the assay system using digitonin-permeabilized glands, we employed another type of inhibitor, SCH28080. As shown in Fig. 6, the IC_{50} value of SCH28080 on the K^+-pNPPase activity in the permeabilized glands was not larger, but significantly smaller than that obtained in the purified enzyme (1.9 ± 0.2 vs 5.2 ± 0.6 μM; $N = 4$; $P < 0.05$). Therefore, it is concluded that the assay of K^+-pNPPase activity in the permeabilized glands represented the total proton pump activity in the glands, and that the discrepancy in the inhibitory effect between permeabilized glands and purified enzyme was specific for the SH-acting type of inhibitor.

It would be reasonable to suppose that the attenuation in the potency of the inhibitors in the glands is due to an

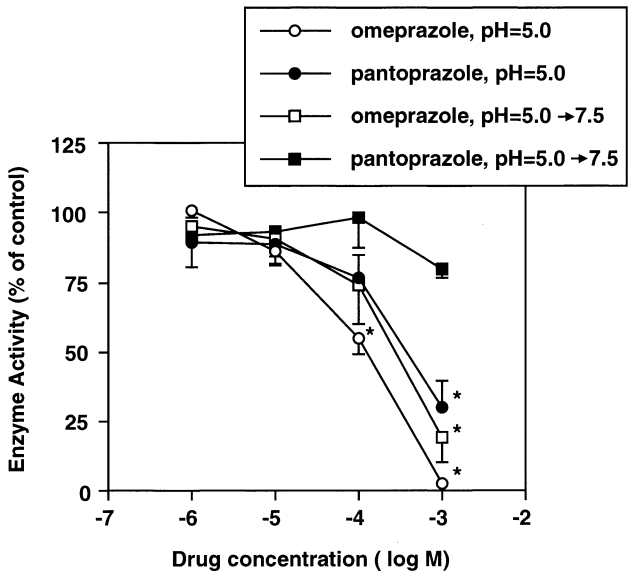

FIG. 5. Effects of omeprazole and pantoprazole on K^+-pNPPase activity in isolated, permeabilized gastric glands. Isolated glands were permeabilized with digitonin and incubated with the inhibitors in buffer at pH 5.0 for 30 min at 37°. The K^+-pNPPase activity in the glands then was assayed at pH 5.0 or 7.5. Data are expressed as a percentage of the vehicle control value (1.0 to 2.4 μmol/hr/mL of glands at pH 5.0 and 5.5 to 6.7 μmol/hr/mL of glands at pH 7.5). Means ± SEM of three independent assays performed in duplicate are shown. Key: (*) significantly different from control at $P < 0.05$.

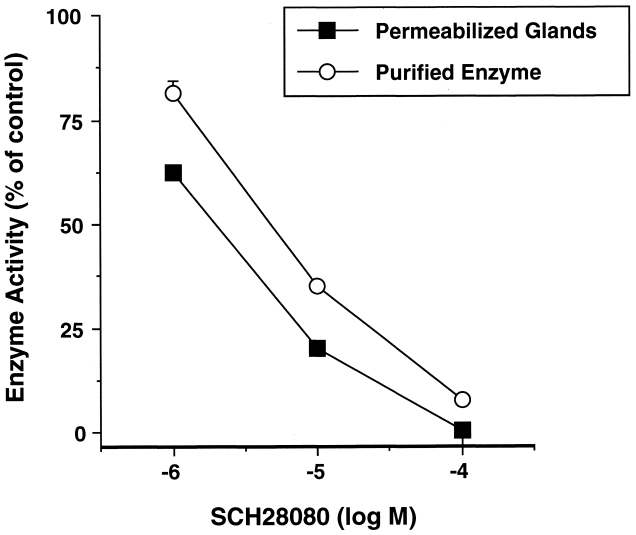

FIG. 6. Inhibitory effects of SCH28080 on K^+-pNPPase activity in isolated, permeabilized gastric glands and in purified microsomes. K^+-pNPPase activity in the presence of various concentrations of SCH28080 is expressed as a percentage of the vehicle control value (glands: 5.0 to 7.8 μmol/hr/mL of glands, microsomes: 23 to 36 μmol/hr/mg protein). Means ± SEM of four independent assays performed in duplicate are shown. The IC_{50} values of SCH28080 for the permeabilized glands and for the purified enzyme are 1.9 ± 0.2 and 5.2 ± 0.6 μM, respectively, and these are significantly different at $P < 0.05$.

endogenous SH-compound such as glutathione. We measured K^+-pNPPase activity of purified enzyme at pH 5.0 in the presence of various concentrations of glutathione and found that the concentration of glutathione needed to reduce the activity of omeprazole to that in the glands was 200 μM. This means that 200 μM glutathione would need to exist around the enzyme inside the parietal cell if the reduction of the potency of the inhibitor were to be attributed exclusively to endogenous glutathione.

We then treated the enzyme with the inhibitors at pH 5.0 and neutralized to pH 7.5 in the presence of this concentration of glutathione, but no recovery was observed. We supposed that the glutathione could not penetrate into the luminal side of the vesicle, the location of the reaction sites. Therefore, we used lyophilized microsomes, which are permeable to glutathione. As shown in Fig. 7, the protective effect of glutathione appeared to be augmented in the lyophilized preparation, showing that the inhibitory effects of pantoprazole and omeprazole were lost at 0.1 mM in the presence of 200 μM glutathione at pH 5.0, but both inhibitors completely inhibited the enzyme activity at 1 mM. After the enzyme was incubated with the inhibitors in the presence of glutathione at pH 5.0, the pH was raised to 7.5 and incubated further for 15 min. It was found that inhibition by pantoprazole, but not omeprazole, was partially reversed by raising the pH in the presence of glutathione (Fig. 7).

Examination of the Compartment of the Enzyme Participating in Acid Secretion

As another possibility for the discrepancy, we hypothesized that a specific compartment of enzyme, participating in acid secretion, was inhibited preferentially by the drugs in the

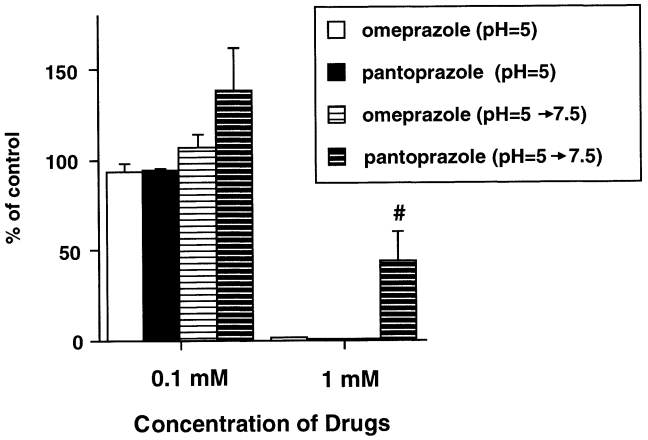

FIG. 7. Recovery of K^+-pNPPase activity by neutralizing assay pH in the presence of glutathione. Purified, lyophilized gastric vesicles were preincubated with omeprazole or pantoprazole in buffer at pH 5.0 at 37° for 30 min. Then the pH was adjusted to 7.5, and 200 μM glutathione was added before the enzyme assay was performed at pH 7.5. Data are expressed as a percentage of the vehicle control (32–44 μmol/hr/mg protein). Means ± SEM of three independent assays performed in duplicate are shown. Key: (#) significantly different from 1 mM omeprazole at $P < 0.05$.

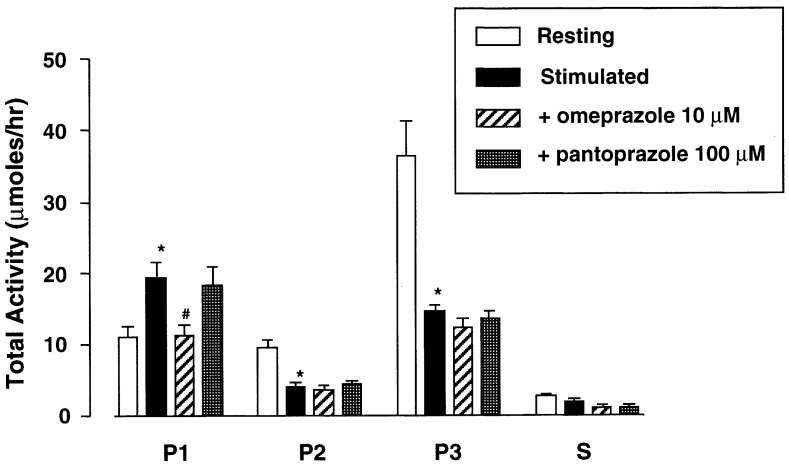

FIG. 8. K^+-pNPPase activity in the various fractions of gastric glands stimulated in the presence of omeprazole or pantoprazole. Isolated glands were stimulated by 0.1 mM histamine and 50 μM IBMX for 30 min at 37° in the presence or absence of 10 μM omeprazole or 100 μM pantoprazole, homogenized, and fractionated into P1 (pellet of 4,000 g, for 10 min), P2 (pellet of 14,500 g, for 10 min), P3 (pellet of 100,000 g, for 45 min), and S (supernatant of the last centrifugation). K^+-pNPPase activity was assayed for each fraction, and the total activity (specific activity times total protein amount) is shown. As the distribution of the protein was constant among the treatment groups, the pattern was almost the same as that of the specific activity. Means ± SEM of three independent assays performed in duplicate are shown. Key: (*) significantly different from resting control value at $P < 0.05$; and (#) significantly different from stimulated control value at $P < 0.05$.

gastric gland. Isolated glands were stimulated with histamine + IBMX in the absence or presence of 10 μM omeprazole or 100 μM pantoprazole, both of which are enough to abolish acid secretion, and the glands were homogenized, fractionated, and assayed for K^+-pNPPase activity in each fraction.

Results are shown in Fig. 8. As reported previously [21], the enzyme activity in the microsomal fraction was reduced markedly, while that in the low-speed pellet correspondingly increased. This has been interpreted by supposing that there is a translocation of proton pump from the tubulovesicles to the apical membrane associated with the stimulation [2, 3]. Administration of omeprazole reduced the enzyme activity only in the low-speed pellet, a reduction down to the resting level.

We have already reported that omeprazole never affects the redistribution of the proton pump from the tubulovesicles to the apical membrane [25]. Therefore, it was interpreted that omeprazole exclusively inhibited the active, working enzyme that was recruited from the tubulovesicular compartment to the apical membrane compartment in the gastric cell. However, in the case of pantoprazole, no significant reduction of enzyme activity was observed in any fraction, including the low-speed pellet. When the enzyme activity of the fractions was added and then divided by the sum of the protein amount in all of the fractions, the specific activity of the total homogenate was obtained. The values (μmol/mg protein/hr) were 5.79 ± 1.2 for stimulated control, 4.05 ± 0.6 for omeprazole-treated, and 5.10 ± 1.1 for pantoprazole-treated glands (mean ± SEM, N = 3), namely, the inhibition rate was only 30 and 12% by omeprazole and pantoprazole, respectively. This showed very little reduction in the activity by the inhibitor, and is consistent with the results obtained by the assay using digitonin-permeabilized glands (Fig. 3B).

If the membrane recycling continues during the stimulation and thus the proton pump molecules in the apical membrane keep being replaced by those in the tubulovesicles, the total enzyme activity in the glands would be abolished by prolonged treatment with the proton pump inhibitors. We treated the isolated glands with histamine + IBMX in the absence or presence of 10 μM omeprazole or 100 μM pantoprazole (both of which are enough to abolish acid secretion for as long as 5 hr), then permeabilized the glands with digitonin and assayed the K^+-pNPPase activity. As shown in Fig. 9, the inhibitory rate of K^+-pNPPase activity by 10 μM omeprazole or 100 μM pantoprazole reached a plateau within 2 hr of treatment, about 45 and 35% of control, respectively, and no increase was observed thereafter. At each point, except at 30 min of treatment,

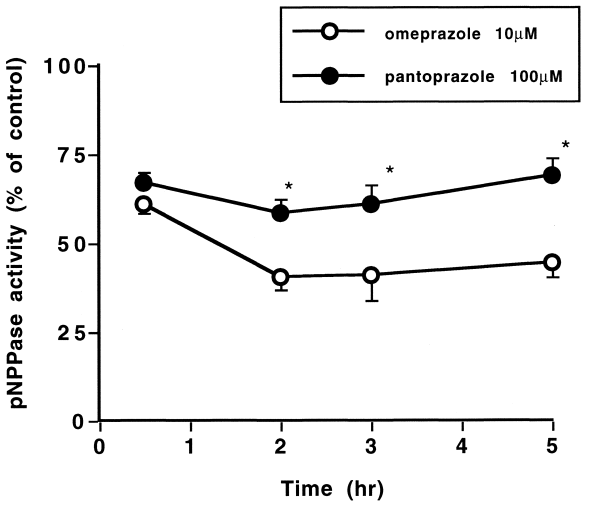

FIG. 9. Time course of inhibition by omeprazole or pantoprazole on the K^+-pNPPase activity in isolated permeabilized gastric glands. Isolated glands were stimulated by 0.1 mM histamine and 50 μM IBMX for the indicated time at 37° in the presence of 10 μM omeprazole (open circles) or 100 μM pantoprazole (closed circles). The glands then were permeabilized with digitonin, and K^+-pNPPase was measured at pH 7.5. Data are expressed as a percentage of the vehicle control (4.4 to 6.8 μmol/hr/mL of glands). Means ± SEM of 3–4 independent assays performed in duplicate are shown. Key: (*) significantly different from omeprazole at the indicated time ($P < 0.05$).

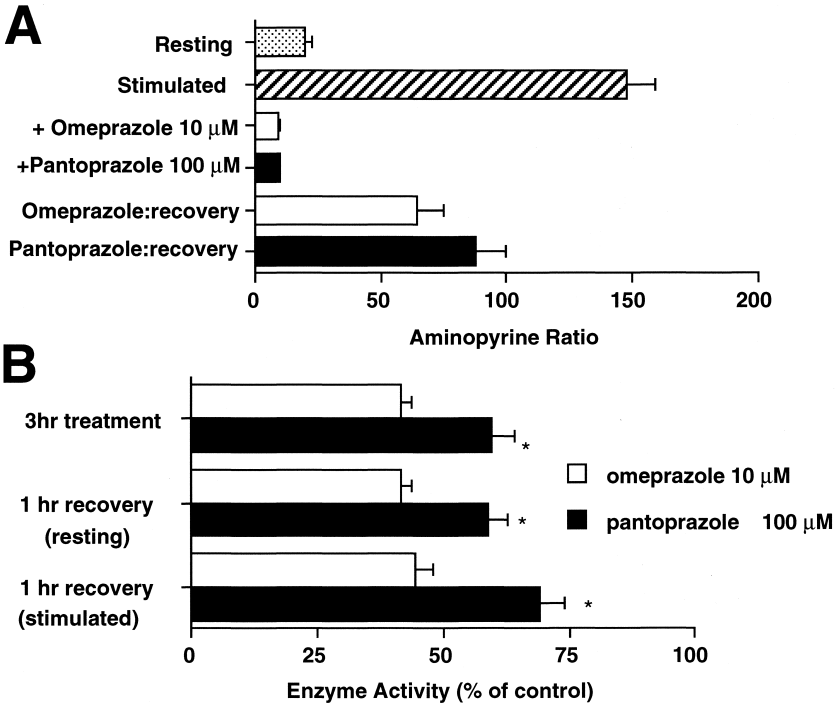

FIG. 10. Recovery of [^{14}C]aminopyrine accumulation and the K^+-pNPPase activity in isolated rabbit gastric glands treated with omeprazole or pantoprazole. (A) Isolated glands were stimulated by 0.1 mM histamine and 50 μM IBMX for 2 hr at 37° in the presence or absence of 10 μM omeprazole or 100 μM pantoprazole, washed three times, and incubated with [^{14}C]aminopyrine for 30 min at 37° with or without secretagogues. Stimulated glands without inhibitor returned to the resting state by this procedure (Resting). Stimulated glands without inhibitor retained an ability to be restimulated by histamine + IBMX (Stimulated). When the glands treated with secretagogues + omeprazole or pantoprazole were incubated with [^{14}C]aminopyrine without washing, a lower aminopyrine ratio than resting was recorded. When the glands treated with secretagogues + omeprazole or pantoprazole were washed and restimulated, significantly higher ratios were observed (Recovery). Absolute values of the aminopyrine ratio are shown as means ± SEM of three independent assays performed in duplicate. (B) Isolated glands were treated with 0.1 mM histamine + 50 μM IBMX for 2 hr at 37° in the presence or absence of 10 μM omeprazole or 100 μM pantoprazole, washed three times, further incubated with or without secretagogues, and then permeabilized with digitonin to measure K^+-pNPPase activity at pH 7.5. Data are expressed as a percentage of the control value of the glands treated without inhibitor (3.1 to 4.5 μmol/hr/mL of glands). Means ± SEM of three independent assays performed in duplicate are shown. Key: (*) significantly different from the omeprazole-treated group at $P < 0.05$.

the inhibitory rate of pantoprazole was significantly lower than that of omeprazole.

To test the possibility that the ability to recycle was injured during the long stimulation, we conducted the experiments shown in Fig. 10. Isolated glands were stimulated with histamine + IBMX for 2 hr, washed, and incubated further for 30 min with or without histamine + IBMX in the presence of [^{14}C]aminopyrine. The glands without secretagogues showed an aminopyrine ratio typical of resting glands, whereas those with secretagogues showed a large value typical of stimulated glands. These results indicate that the wash-out of the secretagogues and the re-stimulation of the glands both were working. When the glands were treated for 2 hr with histamine + IBMX in addition to 10 μM omeprazole or 100 μM pantoprazole and were incubated further with [^{14}C]aminopyrine for 30 min without washing, the aminopyrine ratio was lower than the resting control, confirming that these inhibitors were enough to abolish acid secretion throughout the experiment. When the glands were incubated for 2 hr with histamine + IBMX + 10 μM omeprazole or 100 μM pantoprazole, washed, and re-stimulated with histamine + IBMX without inhibitors for 30 min, there was an obvious increase in the aminopyrine ratio, which showed that the glands treated with omeprazole or pantoprazole still had an ability to secrete acid (Fig. 10A). Next, the glands were incubated for 2 hr with histamine + IBMX in the presence of 10 μM omeprazole or 100 μM pantoprazole, washed, incubated further for 30 min with or without histamine + IBMX, and the K^+-pNPPase was assayed after permeabilization by digitonin. As shown in Fig. 10B, no recovery in the K^+-pNPPase was observed either with or without secretagogues. Therefore, it was concluded that the recovery of the acid secretory response seen in Fig. 10A was not due to the recovery of enzyme activity by washing, but to the recruitment of the proton pump from the compartment where the enzyme was not inhibited irreversibly.

DISCUSSION

It has been established that when the proton pump inhibitors belonging to the class of benzimidazole derivatives are activated in the acid environment, they chemically modify the SH residues of H^+,K^+-ATPase and subsequently inhibit its enzyme activity irreversibly. Scott *et al.* [7] reported that omeprazole specifically bound to H^+,K^+-ATPase on the intracellular canaliculi where acid secretion was occurring. This means that only a small compartment of the proton pump was inhibited when the acid secretion was suppressed. It would be expected that the enzyme on the

tubulovesicles would have been turned into an inhibited form after the irreversibly modified enzymes were retrieved from the apical membrane, and subsequently the whole enzyme activity would attain perfect inhibition gradually if the recycling persisted in the presence of the inhibitor. However, few experiments have been available to figure out the relationship between the total activity of the proton pump and the acid secretory capacity in the same preparation. In most cases [8, 9, 17], enzyme activity was measured in a small part of the total mucosal activity, mainly in the microsomes, which consist of tubulovesicles. In the present study, we showed for the first time the relationship between acid secretory capacity and total proton pump activity in the gastric glands.

When the proton pump activity in the gastric mucosal fractions is measured, especially in the fractions other than microsomes, it is critical to consider possible contamination by other ATP-hydrolyzing enzymes. As for the Na^+,K^+-ATPase, it is eliminated easily by the addition of ouabain in the assay medium. However, mitochondrial ATPase cannot be ignored, especially in the assay of the low-speed pellet containing apical membranes as well as mitochondria, since mitochondrial ATPase appears to be activated by K^+ [21]. Considering the observation that oligomycin-resistant activity was lower than 10% of total K^+-ATPase activity in the low-speed pellet [21, 23], it is practically impossible to quantify the proton pump activity by K^+-activated hydrolysis of ATP in the fractions other than microsomes. K^+-pNPPase activity has been considered to be part of the reaction of Na^+,K^+-ATPase or H^+,K^+-ATPase. In fact, it was reported that inhibition of H^+,K^+-ATPase by omeprazole occurs along with inhibition of K^+-pNPPase activity by this drug [26]. The main advantage to measuring K^+-pNPPase is that no inhibitors other than ouabain are necessary for estimating proton pump activity in the impure fraction, since mitochondrial ATPase lacks phosphatase activity [21, 22]. Therefore, we measured K^+-pNPPase activity routinely to assess the proton pump activity in the present study. Some of the present results were evaluated by measuring ATPase activity in the presence of oligomycin, and this confirmed that the changes in K^+-pNPPase were parallel with those in K^+-ATPase (data not shown). The IC_{50} values of the inhibitors available in a previous report [13] were close to those obtained in the present study.

The most interesting finding in the present study was the discrepancy between acid secretion and enzyme activity, i.e. little inhibition of the proton pump activity was observed when the acid secretion was suppressed completely by 10 μM omeprazole or by 100 μM pantoprazole. The interpretation could be that only a small proportion of the proton pump participates in the actual secretion. This is consistent with the results of immunoelectron microscopy by Scott *et al.* [7], which indicate that omeprazole specifically inhibits enzyme actively working on the apical membrane and that recruitment from the apical membrane to the tubulovesicles is barely occurring on this time scale.

Even so, there remains a riddle. If these concentrations of inhibitors, 10 μM omeprazole or 100 μM pantoprazole, are present around tubulovesicles, the total activity should be almost abolished, judging from the inhibition curve of purified enzyme at pH 5.0, since the pH inside the tubulovesicles must be lower than 5.0 even in the resting state. Actually, the inhibition curves for these inhibitors on the enzyme activity in the permeabilized glands were shifted to the right compared with that for the purified enzyme. Therefore, it is suggested that the enzyme is protected from the inhibitor by an endogenous unknown factor(s). As an endogenous substance, glutathione is one of the most probable candidates. It has been reported that the inhibitory effect of an irreversible type of these drugs, E3810, appears to be reversible at the cellular level because of the action of glutathione, which is secreted by parietal cells into the lumen [17]. In the present study, the reversal of the enzyme was observed for pantoprazole but not for omeprazole by 200 μM glutathione (its physiological range of concentration). We conclude that the reduction in the potency of omeprazole and pantoprazole, as well as the reversibility of the inhibition of the drugs in the cell level, could be attributed to endogenous glutathione, at least in part.

In an early report [9], it was observed in the rat that the inhibition by omeprazole of acid secretion stimulated by carbachol plus pentagastrin for 3 hr completely correlates with both H^+,K^+-ATPase activity and the formation of phosphorylated intermediate. However, the enzyme activity was measured only in the light microsomal fraction. This small part of the fraction mainly consists of tubulovesicles and practically no apical membranes containing actually pumping enzymes [1, 8, 23]. Thus, it is difficult to believe these results unless the inhibited enzymes on the apical membrane were retrieved into the tubulovesicles by membrane recycling during the stimulation of acid secretion. However, based on the work by Im *et al.* [8], it appears that activation of membrane recycling is not needed to cause inhibition of the enzymes in the microsomal fraction, since they observed 80% inhibition of the activity in the microsomes 2 hr after the administration of omeprazole to unstimulated rats.

On the other hand, Tomiyama *et al.* [10] showed that lansoprazole completely abolishes the basal acid secretion in rats within 2 hr after dosing, whereas the enzyme activity in the microsomes persists unchanged for at least 4 hr, and over 80% inhibition finally is observed 10 hr after dosing. They also observed that the short-acting type of E3810 abolishes acid secretion within 1 hr of dosing, and the activity in the microsomes reaches zero within 4 hr. According to their explanation, the difference in the time course of inhibition of acid secretion and enzyme activity is due to the membrane recycling between tubulovesicles and apical membranes, and the rate of recycling is affected by proton pump inhibitors. They postulated that the small difference in the structure of the drugs produces changes in

the structure of the α-subunit of the H^+,K^+-ATPase–drug complex, and subsequently affects the rate of endocytosis of the pump. However, according to the study of Gedda *et al.* [18], the rate of membrane recycling is never changed by treatment with proton pump inhibitors, and this supports our present results.

When acid secretion was abolished in the isolated glands, enzyme inhibition by omeprazole was limited in the apical membrane fraction and was not observed at all in the microsomal fraction within 30 min of treatment. This is again consistent with the work by Scott *et al.* [7] using an immunoelectron microscopic technique, and suggests that omeprazole specifically inhibits H^+,K^+-ATPase in its function of secreting protons on the apical membrane. Also, little recruitment of the pump from the apical membrane to the tubulovesicles occurs within this time course. In contrast, no inhibition by pantoprazole was observed in any fraction. This means that the compartment of the proton pump participating in the acid secretion is a quite small part of the apical membrane compartment and/or some part of the enzyme is recovered from the inhibited state because of reversibility of the inhibition by pantoprazole.

It was totally unexpected that the inhibitory rate of omeprazole or pantoprazole on the total K^+-pNPPase activity in the gastric glands never increased even though the incubation time was prolonged from 2 to 5 hr. This indicates that membrane recycling does not persist during stimulation of acid secretion. Also, the proton pump is inserted into the apical membrane transiently, and no retrieval occurs until the stimulation terminates. This point is an interesting and important issue in parietal cell physiology. Further work will be necessary from another approach.

It is indicated from the present study that pantoprazole inhibits acid secretion without apparent reduction in the total proton pump activity, possibly due to its requirement of low pH for activation as well as its reversibility of inhibition at the cellular level. It appears that a lower amount of proton pump activity needs to be inhibited by pantoprazole than by omeprazole to achieve the same extent of acid secretion inhibition. Thus, it could be stated from this point of view that pantoprazole is a more selective inhibitor of acid secretion. The difference between omeprazole and pantoprazole observed in the present study might manifest itself in the clinical field as the potential for the recovery of acid secretion, or in the side-effects due to the nonspecific inhibition of SH-enzymes other than the proton pump. To elucidate how this selectivity emerges in the clinical field, more work is clearly necessary, especially in clinical pharmacology.

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