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SPECIFIC PROTON PUMP INHIBITORS E3810 AND LANSOPRAZOLE AFFECT THE RECOVERY PROCESS OF GASTRIC SECRETION IN RATS DIFFERENTLY

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Abstract—After a single subcutaneous administration (30 mg/kg) of proton pump inhibitor 2-[{4-(3-methoxypropoxy)-3-methylpyridin-2-yl}-methylsulfinyl]-1*H*-benzimidazole sodium salt (E3810), or lansoprazole in rats, time courses of inhibitory and recovery processes of acid secretion *in vivo* and pump enzyme activity in isolated microsomes were measured. The acid secretion rate which reflects H^+,K^+-ATPase activity in the secretory canalicular (apical) membrane was compared with that in the microsomal fraction which consists mostly of resting, intracellularly-pooled tubulovesicles. We found that the canalicular pump was first inhibited, followed by slow inhibition of the microsomal pump enzyme activity, with the rate of the latter process depending on the inhibitors. It took 2.5 hr for the half-maximal inhibition of the microsomal pump in E3810-treated rats, and 6 hr in lansoprazole-treated rats. The acid secretion and the microsomal enzyme activity completely recovered within 48 hr after the administration of E3810, but recovered by only 20% even 96 hr after the administration of lansoprazole. Incubation with dithiothreitol of isolated microsomes obtained from E3810-treated rats reactivated the enzyme activity, but not from rats treated with lansoprazole. These results suggest that dissociation of inhibitor from the pump and/or intracellular transport of the pump is affected differently by these inhibitors. Furthermore, it is possible that the half life of the proton pump protein is much longer (greater than 96 hr) than the previously proposed value of 30–48 hr.

Key words: E3810; lansoprazole; omeprazole; proton pump; H^+,K^+-ATPase; gastric acid secretion

Gastric acid secretion is inhibited specifically by omeprazole [1], E3810† [2] or lansoprazole [3]. These compounds are transformed into their active compounds in acidic environment such as the acidic intracellular lumen [4–7] and bind to SH-group(s) of gastric proton pump (H^+,K^+-ATPase) [8–10]. After inhibition by omeprazole, *de novo* synthesis of the proton pump was thought to restart acid secretion [11], its full recovery taking 3 days in humans and dogs [1, 2]. When omeprazole and E3810 were given as a single intraduodenal administration to dogs, the antisecretory activity of E3810 was slightly greater than that of omeprazole, and the duration of the activity was significantly shorter in the E3810-treated dogs than in the omeprazole-treated dogs when compared at the same doses (2 mg/kg and 4 mg/kg) [2]. It was suggested that this was due to dissociation of E3810 from the pump by endogenous glutathione in addition to *de novo* synthesis of the pump [2]. Accordingly, the conformational state of the omeprazole-bound pump differs greatly from that of the E3810-bound pump [10]. In the present study, E3810 and lansoprazole were given singly and subcutaneously to rats at a dose of 30 mg/kg, and the recovery process of acid secretion *in vivo* was compared with that of the pump enzyme activity in an isolated microsomal fraction of the gastric mucosa. We found that E3810 and lansoprazole affected these recovery processes differently, as they depend on dissociation of the inhibitor from the pump, *de novo* synthesis and intracellular transport of the gastric pump.

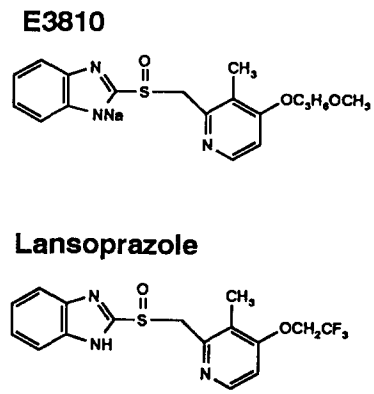

Fig. 1. Structures of E3810 and lansoprazole.

MATERIALS AND METHODS

Chemicals. E3810 (Fig. 1) was obtained from the

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† Abbreviations: E3810, 2-[{4-(3-methoxypropoxy)-3-methylpyridin-2-yl}-methylsulfinyl]-1*H*-benzimidazole sodium salt; FITC, fluorescein 5′-isothiocyanate; P_i, inorganic phosphate.

Eisai Co. (Tokyo, Japan), and lansoprazole (Fig. 1) from the Takeda Pharmaceutical Industrial Co. (Osaka, Japan). Male Wistar rats with body weight of approx. 300 g were used. FITC was obtained from Wako Pure Chemicals (Osaka, Japan). Lactate dehydrogenase (550 IU/mg at 25° in 50% glycerol) and pyruvate kinase (200 IU/mg at 25° in 50% glycerol) were obtained from Boehringer Mannheim Yamanouchi (Tokyo).

Gastric secretion in rats with chronic fistulas. Rats were provided with stainless steel cannulas, chronically implanted into the rumen of the stomach [12, 13]. Before each secretory test, the animals were fasted for 24 hr. The stomach was rinsed with saline (37°), and the animal was placed in a Bollman cage. One hour later, the inhibitor (30 mg/kg) or vehicle (saline) was administered by single subcutaneous injection (0.5 mL/300 g). E3810 was solubilized in saline, and lansoprazole was suspended in saline supplemented with 0.1% methyl cellulose (the pH of the suspension was adjusted to 8.0 by the addition of $NaHCO_3$. Gastric juice was collected by free flow from the cannula in 1 hr samples up to 8 hr. The sample volumes were measured and their acid content was determined by titration to pH 7.0 with 0.1 N NaOH.

Gastric secretion in pylorus ligated rats. The vehicle or inhibitor (30 mg/kg) was injected subcutaneously to rats which were not fasted. To obtain the acid secretory rate 24, 48, or 96 hr after the injection, rats were fasted for 24 hr before each acid test. The pylorus was then ligated under light ether anesthesia and the abdomen closed. Three hours later, an overdose of ether was given, and the stomach was removed. The gastric contents were centrifuged, and the acidity in the supernatant was determined.

Preparation of gastric microsomal fraction containing H^+,K^+*-ATPase.* Food and inhibitor were given to rats following schedules as described in Results. The microsomal fraction was prepared from each rat as described elsewhere [14]. In brief, rats were killed by an overdose of pentobarbital, and the stomach was removed. Scrapings of the corpus mucosa were homogenized in a solution containing 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EGTA and 2 mM HEPES/Tris (pH 7.4) in a glass–Teflon homogenizer. The crude homogenate was passed through a cotton gauze and centrifuged at 1600 *g* at 4° for 2 min. The supernatant was saved. The sediment was again homogenized and centrifuged. Both supernatants were collected together and centrifuged at 20,000 *g* at 4° for 11 min. The supernatant was centrifuged at 137,500 *g* for 40 min, and the microsomal fraction was obtained as the sediment [14]. For experiments involving the enzyme assay, the microsomal pellet fraction was suspended in a solution containing 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EGTA and 2 mM HEPES–Tris (pH 7.4), stored at −84° and used for analysis of the ATPase activities. For experiments investigating FITC fluorescence, the pellet was suspended in a solution containing 250 mM sucrose and 2 mM HEPES–Tris (pH 7.4).

Since rat gastric mucosa contains high levels of phospholipases, the microsomal vesicle membrane was reported to be leaky when prepared in the absence of EGTA and tight when prepared in its presence [14]. From our separate experiments of light scattering on membrane permeability as described elsewhere [15], we found that, although prepared in the presence of EGTA, the membrane of the present preparations was leaky to K^+,H^+ and Cl^- possibly because of insufficient inhibition of phospholipases.

Assays of the acid pump enzyme activity. Unless otherwise described, the $(Mg^{2+} + K^+)$-ATPase activity was assayed by the pyruvate kinase-lactate dehydrogenase-linked system in which hydrolysis of ATP is coupled with the oxidation of NADH [16, 17]. The final concentrations of reaction mixture were: 10 μg/mL of the microsomal fraction, 15 mM KCl, 2 mM $MgCl_2$, 0.8 mM phosphoenolpyruvate, 4 IU/mL of pyruvate kinase, 10 IU/mL of lactate dehydrogenase, 0.2 mM NADH, 0.2 mM MgATP and 40 mM Tris–HCl (pH 7.4). Enzyme activity was expressed as μmol P_i/mg of protein/hr. E3810 and lansoprazole did not interfere with the coupled-enzyme method, as found previously with E3810 and omeprazole [16]. The decrease in the amount of NADH was measured from the absorbance change at 340 nm (reference 500 nm) in an Aminco DW-2C UV-VIS spectrophotometer at 23°.

In the coupled-enzyme method, at least 0.5 mM K^+ is necessary for the activation of pyruvate kinase; therefore, this method could not be used to measure Mg^{2+}-ATPase activity. We measured Mg^{2+}-ATPase activity by the method of Yoda and Hokin [18] in a solution containing 10 μg/mL of the microsomal fraction, 0.2 mM MgATP, 2 mM $MgCl_2$ and 40 mM Tris–HCl (pH 7.4) at 23° for 5 min. When indicated, ATPase activities were measured in the presence and absence of 15 mM KCl, and K^+-ATPase activity (the acid pump enzyme activity) was calculated as the difference between $(Mg^{2+} + K^+)$-ATPase and Mg^{2+}-ATPase activities.

Labeling of H^+,K^+*-ATPase with FITC and measurement of FITC fluorescence.* The microsomal fraction (0.75 mg/mL) was incubated in a solution containing 5 μM FITC, 2 mM EDTA and 100 mM Tris–HCl (pH 9.2) for 30 min at room temperature as described elsewhere [10, 19]. It was then eluted through a Sephadex G-50 column equilibrated with 250 mM sucrose and 40 mM Tris–HCl (pH 7.4). The FITC-labeled microsome solution was diluted to a protein concentration of approx. 100 μg/mL with a buffer containing 250 mM sucrose and 40 mM Tris–HCl (pH 7.4). FITC fluorescence was measured in a fluorescence spectrophotometer fitted with a magnetic stirrer and a water jacket at 23°. The excitation and emission wavelengths were 495 and 517 nm, respectively.

Treatment of the microsomal fraction with dithiothreitol. The microsomal fraction (10 μg of protein/mL) was incubated in a solution containing 10 mM dithiothreitol, 15 mM KCl and 40 mM Tris–HCl (pH 7.4) for 1 hr at room temperature. The measurement of the enzyme activity was then started by the addition of a very small amount of solution containing NADH, $MgCl_2$, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and MgATP necessary for the coupled-enzyme assay at 23°.

Protein determination. Protein was determined

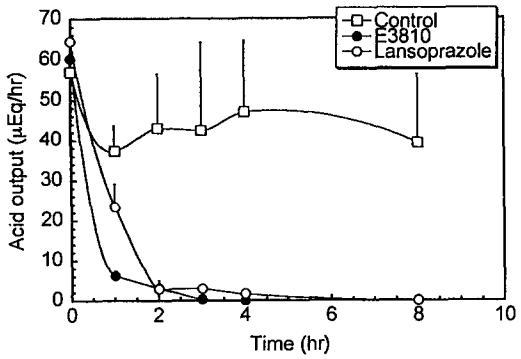

Fig. 2. Antisecretory effects of E3810 and lansoprazole on unstimulated acid secretion of gastric fistula rats. Rats were starved for 24 hr, and then inhibitors at the same dose of 30 mg/kg were given subcutaneously at $t = 0$. Values are means ± SE (N = 5).

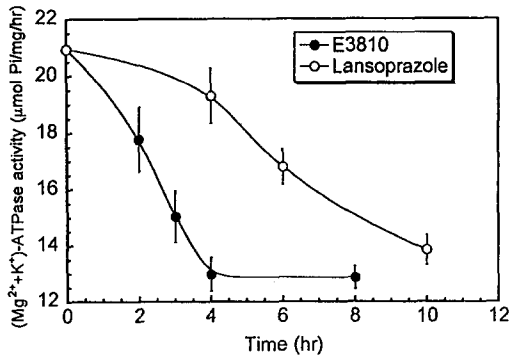

Fig. 3. Inhibitory effects of E3810 and lansoprazole on $(Mg^{2+} + K^+)$-ATPase activity of the rat microsomal fraction. The fractions were isolated from each rat at indicated times after the administration of E3810 or lansoprazole. The inhibitors were administered as described in the legend to Fig. 2. The total number of rats used in this experiment was 40. Each fraction was measured in duplicate. Values are means ± SE (N = 5).

according to the method of Lowry *et al.* [20], and bovine serum albumin was used as standard.

Statistical analysis. All values are expressed as means ± SE. The statistical significance of difference was determined by unpaired Student's *t*-test, and the difference was regarded as significant if $P < 0.05$.

RESULTS

Time course of inhibitions of acid secretion and H^+,K^+-ATPase activity in rats treated with E3810 or lansoprazole

Here, rats were starved for 24 hr and the inhibitor was then subcutaneously injected. Figure 2 shows that a single administration of E3810 or lansoprazole at the dose of 30 mg/kg inhibits the acid secretion of unstimulated rats with chronic fistulas. Within 2 hr after administration, both inhibitors almost completely inhibited the acid secretion, indicating that almost all acid pumps in the secretory canaliculi were inhibited.

Figure 3 shows that $(Mg^{2+} + K^+)$-ATPase activity of the microsomal fraction isolated from inhibitor-treated rats decreases with time after the single administration of E3810 or lansoprazole. K^+-stimulated ATPase activity (the pump enzyme activity) which is present in the microsomal fraction is associated with Mg^{2+}-stimulated ATPase activity. Mg^{2+} activity, measured by the Yoda and Hokin method, was 12.1 ± 1.2(4) μmol P_i/mg of protein/hr and not affected by treatment with E3810 or lansoprazole (Fig. 4). Omeprazole was also reported not to affect Mg^{2+}-ATPase activity in the rat microsomal fraction [21]. Furthermore, in the samples showing the lowest levels of $(Mg^{2+} + K^+)$-ATPase activities (4 and 8 hr, E3810-treated rats), K^+-ATPase activity was zero when measured following the method of Yoda and Hokin. These results show that the decrease in $(Mg^{2+} + K^+)$-ATPase activity shown in Fig. 3 is due to the decrease in K^+-ATPase activity. Figure 3 shows that it takes 2.5 hr for the half-maximal inhibition of the

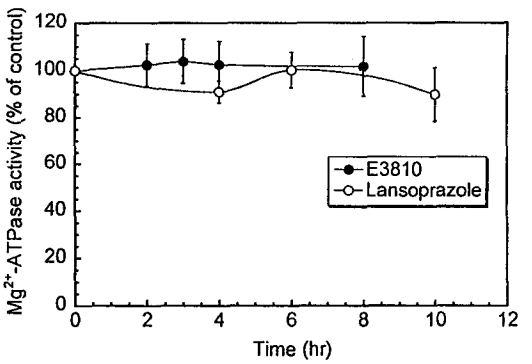

Fig. 4. Mg^{2+}-ATPase activity of the microsomal fractions. The control value was 12.1 ± 1.2 (N = 4) μmol P_i/mg/hr. These fractions were the same as those used for the experiments of Fig. 3. The total number of rats used in this experiment was 32. Values are means ± SE (N = 4).

microsomal pump in E3810-treated rats, and 6 hr in lansoprazole-treated rats.

We compared the results recorded in Figs 2 and 3. Two hours after the administration of E3810, K^+-ATPase activity was inhibited by approx. 40%, whereas the acid secretory rate was inhibited by approx. 94%. This is also the case for the lansoprazole-treated rats: K^+-ATPase activity 4 hr after administration was inhibited by only 20%, whereas acid secretion was inhibited by approx. 93%.

Recoveries of acid secretion and K^+-ATPase activity in rats treated with E3810 or lansoprazole.

To determine the effects of acid stimulation on the recovery of K^+-ATPase activity, the enzyme activities of two microsomal fractions isolated from

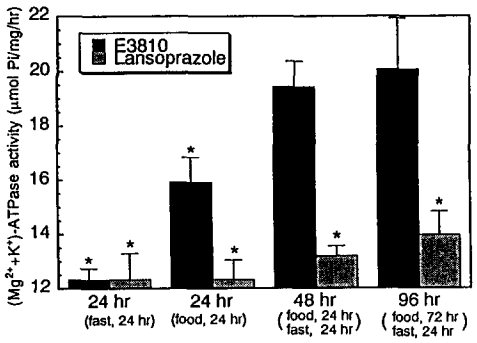

Fig. 5. Recovery of $(Mg^{2+} + K^+)$-ATPase activity following inhibitor treatment. After the administration of E3810 or lansoprazole (30 mg/kg, s.c.), rats were placed in cages for different periods following different food schedules as indicated. Then, the microsomal fractions were prepared from each rat, and ATPase activity was measured. N = 5. A statistically significant difference from control is denoted (*$P < 0.01$).

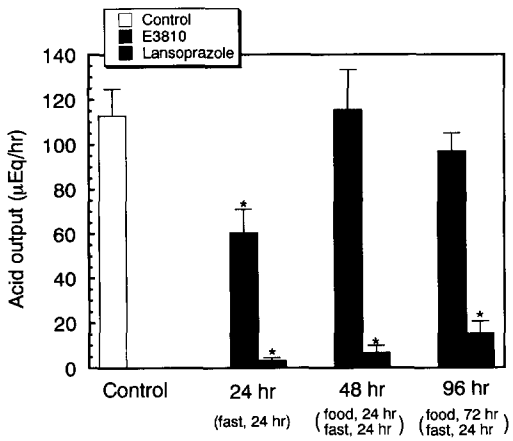

Fig. 6. Recovery of acid secretion. After a single administration of E3810 or lansoprazole (30 mg/kg, s.c.), rats were placed on different food schedules as indicated. Ligation of pylorus was started at the indicated times after the administration of inhibitor, and 3 hr later, the gastric juice was collected. N = 5–6. A significant difference from control is denoted (*$P < 0.01$).

rats fasted or food-stimulated for 24 hr after inhibitor administration were measured (Fig. 5). When E3810 was administered, K^+-ATPase activity in the food-stimulated rats recovered by approx. 50%, whereas almost no recovery was observed for fasted rats, indicating that food stimulation increased the re-activation of the pump enzyme activity present in the microsomal fraction. When lansoprazole was administered, no recovery was found for either fasted or food-stimulated rats.

Figure 5 also shows that K^+-ATPase activity recovers to the control level within 48 hr after administration of E3810, whereas it only recovers by approx. 20% even 96 hr after the administration of lansoprazole.

Figure 6 shows the recovery of the acid secretion

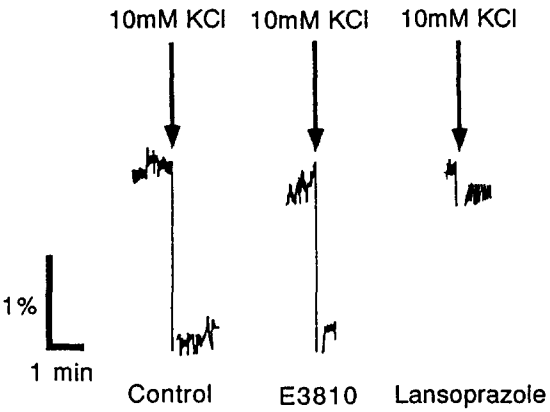

Fig. 7. K^+-induced fluorescence change. After drug administrations (both at 30 mg/kg, s.c.), rats were given food for 24 hr followed by 24-hr starvation for E3810, or were given food for 72 hr followed by 24-hr starvation for lansoprazole. The microsomal fractions were then prepared from each rat. A representative example from three experiments.

of pylorus-ligated rats after the single administration of the drugs. Here, the drugs (30 mg/kg) were subcutaneously injected in rats that were not fasted. Before the acid secretory test of pylorus ligation, rats were fasted for 24 hr. The acid secretory rate in E3810-treated rats recovered to a level of 54% of the control 24 hr after the administration of drug and completely to control level within 48 hr (24 hr free access to food followed by 24 hr fast). It recovered only by 20% in lansoprazole-treated rats even 96 hr after administration.

Effect of K^+ on the confirmational state of H^+,K^+-ATPase in the microsomal fractions

Figure 7 shows that the addition of K^+ to a solution containing the FITC-labeled control microsomal fraction, 250 mM sucrose and 40 mM Tris–HCl (pH 7.4) decreases FITC fluorescence, as previously found with hog gastric microsomes which contain the proton pump [10, 22, 23]. The decrease reflects the conformational change of the pump from the E_1H^+ to E_2K^+ form, where cation (H^+ or K^+) binds on the cytosolic surface of the pump at the E_1 form, and on the luminal surface at the E_2 form.

When the microsomal fraction isolated from rats that had 24 hr free access to food followed by 24 hr fast after the administration of E3810 was used, the extent of the K^+-induced decrease in fluorescence was the same as that of control. For the lansoprazole-treated fraction isolated 96 hr after the administration of lansoprazole (72 hr free access to food followed by 24 hr fast), the fluorescence decrease was approx. 18% of control.

Effects of dithiothreitol on K^+-ATPase activity

Figure 8 shows the effect of incubation of isolated microsomal fractions with 10 mM dithiothreitol for 1 hr at room temperature. The microsomal fractions were isolated from rats that were starved for 24 hr

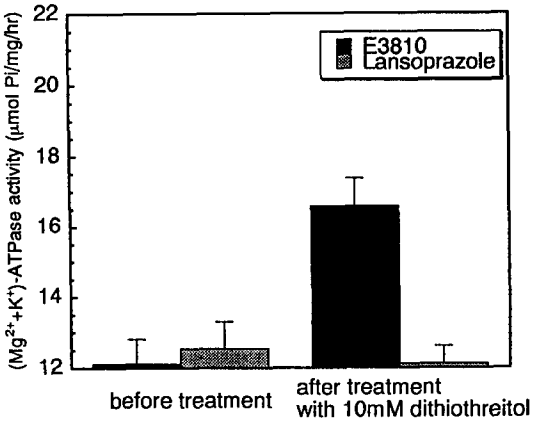

Fig. 8. Effects of dithiothreitol on (Mg^{2+} + K^+)-ATPase activity. The microsomal fraction was isolated from rats that were starved for 24 hr after the administration of E3810 or lansoprazole (30 mg/kg, s.c.). The fractions were then incubated in the presence of 10 mM dithiothreitol for 1 hr at room temperature. N = 5.

after a single subcutaneous injection of E3810 or lansoprazole. The incubation increased K^+-ATPase activity in the E3810-treated preparation, but not in the lansoprazole-treated one, indicating that the S–S cross-linking between the pump and the inhibitor was cleavable by dithiothreitol for E3810, but not for lansoprazole.

DISCUSSION

We found that after the administration of specific proton pump inhibitors in fasted rats, the initial inhibition of the pump occurs in the canalicular membrane, followed by slow inhibition of enzyme activity in the microsomal fraction (Figs 2 and 3). We consider that acid secretion *in vivo* reflects pump enzyme activity in the canalicular membrane. Furthermore, we used the light microsomal fraction (137,500 *g*) which was reported to consist of mostly tubulovesicles [14], and did not use a heavy vesicle fraction enriched in mitochondria and nuclei which was shown to contain the apical (canalicular) membrane vesicles [24]. The present finding, therefore, provides evidence that the inhibited pump in the canalicular membrane may be relocated in the tubulovesicular membrane. We excluded the possibility that the pumps in the tubulovesicles and the canalicular membranes are simultaneously inhibited or that the pump in the tubulovesicles is first inhibited, then relocated into the canalicular membrane. It has already been shown that tubulovesicles can not accumulate proton and that the inhibitor does not transform into the acid-activated form in tubulovesicles because of the low KCl conductance of the tubulovesicle membrane [25, 26]. The rate of relocation from the canalicular to tubulovesicle membrane in E3810-treated rats was 2.4-fold faster than that in lansoprazole-treated rats based on the half-inhibition time (2.5 vs 6 hr; Fig. 3). Thus, the internalization of the pump from the canalicular membrane depended on the inhibitor-pump complex. The molecular mechanism of internalization may be sensitive to the conformational flexibility of the pump which also depended on the inhibitor [10].

An interesting finding was that recovery of acid secretion occurred earlier than that of microsomal H^+,K^+-ATPase activity in the case of E3810 inhibition (Figs 5 and 6; comparison at 24 hr, fasted rats). This result could be explained by two different mechanisms: (1) the synthesized pump was transported via Golgi into the canalicular membrane without passing tubulovesicles, suggesting the presence of a previously unknown intracellular transport pathway; and (2) the selective reactivation of the inhibited pump in the canalicular membrane (due to higher glutathione concentration in canaliculi than inside tubulovesicles). In both cases, the recycling rate of the pump back to tubulovesicles must be taken into consideration: the recycling rate after complete inhibition of the pump in tubulovesicles may not be the same as the initial rate of 2.5 hr (possibly much slower) because the intracellular systems are alerted to produce new pumps and to degrade damaged (inhibited) ones, and the increased production and degradation of the pump may be balanced. Further study is necessary to clarify the exact mechanism.

The complete recovery of acid secretion after the administration of omeprazole in humans and dogs took 3 days [1, 2]. If recovery is assumed to be due only to *de novo* synthesis of the pump, the half-life of the proton pump protein is calculated to be 30–48 hr, so all of the pumps would be replaced every 72–96 hr [11]. Im *et al.* [27] found that the half-life of the pump was much longer (approx. 72 hr from the effect of cycloheximide on the pump enzyme activity in rat) and also suggested that omeprazole treatment increased the synthetic rate of the pump. The dose of 30 mg/kg in rats was near maximal for both E3810 [28] and lansoprazole [29]. When lansoprazole was administered at the dose of 30 mg/kg in the present study, the acid secretion and pump enzyme activity measured 96 hr after administration recovered by approx. 20%. This result may indicate that the half-life of the pump is greater than 96 hr if *de novo* synthesis of the proton pump after administration of lansoprazole was not inhibited.

On the other hand, acid secretion recovered completely within 2 days in E3810-treated rats. Recently we have suggested that the recovery of acid secretion after the administration of E3810 depends on *de novo* synthesis of the acid pump as well as on dissociation of the inhibitor from the acid pump by endogenous glutathione present in the secretory lumen [2]. The recovery of pump enzyme activity in the microsomal fraction was stimulated by food in E3810-treated rats but not in lansoprazole-treated rats (Fig. 5; 24 hr food vs 24 hr fast). The increased rate of recovery probably reflects the increased rate(s) of *de novo* synthesis, recycling between the canalicular and tubulovesicles and/or dissociation of the inhibitor from the pump.

A previous study reported that when lansoprazole (10 mg/kg) or omeprazole (30 mg/kg) was administered in histamine-stimulated rats via p.o., acid secretion recovered to approx. 56 or 100% level of control, respectively, 24 hr after administration [3, 29], indicating that the recovery of acid secretion in lansoprazole-treated rats was much slower than that in omeprazole-treated rats, as found here for lansoprazole versus E3810. The more rapid recovery found with lansoprazole (via p.o.) compared with the present result (s.c.) is apparently due to the smaller dose of the drug and the method of delivery: p.o. is a rather ineffective method because the drug decomposes in the acid gastric juice; for example, intraduodenal bioavailability of omeprazole in dogs was about 70%, whereas oral bioavailability was only 15% [30].

E3810 which specifically bound to the acid pump *in vivo* was dissociated from the pump by 1 hr treatment with 10 mM dithiothreitol, but lansoprazole could not be dissociated (Fig. 8). Omeprazole which specifically bound to the pump *in vitro* was dissociated by treatment with 100 mM 2-mercaptoethanol [4]. Furthermore, the conformational state shown in Fig. 7 suggests that lansoprazole still bound to most of the enzyme isolated 96 hr after administration of the drug because the conformational state of the lansoprazole-bound pump was partially sensitive to the addition of K^+ (Morii M and Takeguchi N, unpublished result). This result indicates that lansoprazole bound to the pump was not easily dissociated by endogenous gluthathione, which explains the slow recovery of acid secretion.

The amount of specific binding necessary for the complete inhibition of enzyme activity (unpublished observation by Morii M, Hayata Y and Takeguchi N) is the same as that of omeprazole [31]. The amount of specific binding for lansoprazole was reported to be three times that of omeprazole [32]. One characteristic difference between the chemical structures of E3810 and lansoprazole is the presence of the very lipophilic moiety of trifluorocarbon (CF_3) in the lansoprazole molecule (Fig. 1), and lansoprazole is more lipophilic than E3810 and omeprazole. From the present results on the reversibility of K^+-ATPase activity by membrane-impermeable dithiothreitol, it is suggested that the additional binding site(s) of lansoprazole is in the transmembrane domain(s) of the pump.

In conclusion, the pump is recycled continuously between the secretory canaliculi and the tubulovesicles, in addition to the well known fact that histamine stimulation induces the fusion of tubulovesicles with the apical membrane. It is not known whether this recycling occurs directly or indirectly via Golgi. Furthermore, the present and previous [27] findings suggest that the half life of the pump is approx. 72–96 hr or even greater. The time necessary for the complete recovery of acid secretion after maximal inhibition is not unique among these irreversible proton pump inhibitors but depends on the rates of *de novo* synthesis of the pump, dissociation of inhibitor from the pump and intracellular pump recycling. Some of these rates are affected differently by the inhibitors.

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