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Inhibition of $(H^+ + K^+)$-ATPase by omeprazole in isolated gastric vesicles requires proton transport

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Omeprazole was found to inhibit the $(H^+ + K^+)$-ATPase activity in isolated gastric vesicles only when acid was accumulated in the vesicle lumen. The ATPase activity was time- and dose-dependently inhibited in the presence of K^+ and valinomycin. Under conditions in which no pH-gradient was generated, i.e., in the presence of K^+ alone or NH_4^+, no effect of omeprazole was found. The degree of inhibition was directly correlated to the amount of inhibitor bound to the preparation. A stoichiometry of 2 mol radiolabelled inhibitor bound per mol phosphoenzyme was found on total inhibition of the K^+ plus valinomycin-stimulated activity. This inhibitory action of omeprazole on the ATPase activity could be fully reversed by addition of β-mercaptoethanol. The inhibition of the proton transport in the $(H^+ + K^+)$-ATPase-containing vesicles by omeprazole was also strictly correlated to the amount of bound inhibitor. The stoichiometry of binding at total inhibition of this reaction was found to be 1.4 mol per mol phosphoenzyme. The K^+-stimulated *p*-nitrophenylphosphatase activity was inhibited in parallel with the ATPase activity, whereas the phosphoenzyme levels were affected to a lesser extent by omeprazole. Gel electrophoresis of an omeprazole-inhibited vesicle preparation showed that the radiolabel was mainly found at 94 kDa, the molecular weight of the $(H^+ + K^+)$-ATPase catalytic subunit(s).

Introduction

The $(H^+ + K^+)$-ATPase of the gastric mucosa appears to be responsible for the large H^+ gradient generated by this tissue [1–5]. As such, it is a potential therapeutic target for compounds designed to inhibit acid secretion. A series of pyridylmethylsulfinylbenzimidazoles has been shown to inhibit selectively the isolated gastric $(H^+ + K^+)$-ATPase and thereby also acid secretion [6–9]. One of the compounds, omeprazole, is presently undergoing clinical trials as an antiulcer agent. So far, promising ulcer healing results have been obtained [10–12], and furthermore, severe hypersecretion can be controlled [13]. In the rat, a good correlation has been found between inhibition of $(H^+ + K^+)$-ATPase activity and the reduction in acid secretion following omeprazole treatment [14].

The mechanism of action of omeprazole has been studied in some detail. Omeprazole does not react with the $(H^+ + K^+)$-ATPase at neutral pH, but is activated in acid to form a cationic compound, which in turn is rearranged to form a reactive sulfenic acid or a sulfenamide (Scheme I).

Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid

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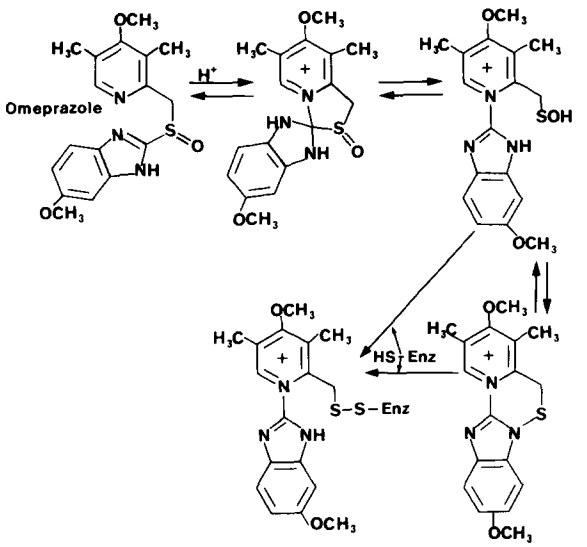

Scheme I. Mechanism of decomposition of omeprazole in acid. From P. Lindberg et al. [15].

Subsequently, these compounds react with SH groups of the $(H^+ + K^+)$-ATPase [8]. Sulfhydryl-group-reducing agents can both prevent and reverse the inhibitory action of omeprazole in rabbit gastric glands [16] and on the isolated $(H^+ + K^+)$-ATPase [8] showing that acid-induced transformation of omeprazole and subsequent interaction with the enzyme does involve modification of thiol groups.

Treatment of a lyophilized ATPase preparation at pH 6 with omeprazole provides one means of estimating the relationship between binding of the inhibitor to the preparation and inhibition of the enzyme activity. From such an approach, it has been found that in order to completely inhibit the ATPase activity a binding level of 10–30 nmol/mg protein was required [8,17] in a preparation that phosphorylates to a level of about 1 nmol/mg protein. Under those conditions, however, omeprazole would not be specific to the $(H^+ + K^+)$-ATPase and would also be able to react on both sides of the membrane. However, in the parietal cell, since acid activation occurs on the luminal face of the membrane, specificity could occur for the following reasons. Omeprazole is a weak base with a pK_a of 4 and would thus be significantly accumulated in acid spaces of a $pH < pK_a$. Only the parietal cell in mammals is known to develop such a low pH. The accumulation ratio would be a function of the pK_a, the pH gradient, and the permeability of the protonated species. Further, the transformation product of omeprazole following protonation carries a positive charge and may be less membrane-permeable than the parent compound and will possibly also tend to be trapped within the secretory canaliculus of the parietal cell following acid activation. This transformation product could react with available SH groups on peptides in the canalicular membrane or could diffuse across the membrane and react with SH groups in the cytosol of the parietal cell, probably reduced glutathione. This latter pathway is unlikely, given the charged nature of the intermediate. Electron microscopic autoradiography has shown labelling corresponding to the location of the $(H^+ + K^+)$-ATPase [18]. Thus the proton transport carried out by the $(H^+ + K^+)$-ATPase of the mammalian parietal cell results in selective modification of this enzyme by this type of acid-activated inhibitor.

To mimic the situation in the intact cell, inhibition of the $(H^+ + K^+)$-ATPase was performed under acid-transporting conditions in isolated vesicular preparations. In this system, the extravesicular, cytosolic face of the enzyme was maintained at neutral pH, while the intravesicular, luminal space was allowed to acidify. With this approach, it was possible to determine the stoichiometry of the reaction both with respect to ATPase activity and proton transport and the partial reactions of $(H^+ + K^+)$-ATPase that were affected (phosphorylation and K^+-activated *p*-nitrophenylphosphatase activity) and to determine the fraction of transport-competent enzyme in a vesicle preparation.

Experimental procedures

Materials

Gastric vesicles. Membrane vesicles containing the $(H^+ + K^+)$-ATPase were prepared from hog stomachs by differential and zonal density gradient centrifugation according to previously published methods [19]. The microsomal fraction was centrifuged through 0.25 M sucrose layered on a 7.5% (w/w) Ficoll 0.25 M sucrose step gradient. The material retained at the interface was frozen

in a final concentration of about 40% sucrose and used for the experiments. Freezing under these conditions resulted in partial loss of vesicle integrity but was required for experimental continuity.

Reagents. [γ-^{32}P]ATP was obtained from ICN, Irvine, CA. Acridine orange was purchased from Merck, F.R.G. Prepacked Sephadex G-25 columns (PD-10) were obtained from Pharmacia, Sweden. Both unlabelled and ^{3}H-labelled omeprazole were synthesized by AB Hässle, Mölndal, Sweden. All other reagents were of the highest purity available.

Methods

Inhibitory reaction conditions. Reaction mixtures were composed of 2 mM Hepes/NaOH (pH 7.4), 2 mM $MgCl_2 \pm 150$ mM KCl, ± 12–36 μg valinomycin, ± 100 mM NH_4Cl, omeprazole and ^{3}H-omeprazole in concentrations as indicated, 50 or 100 μg protein and 4 mM ATP in a final volume of 1 ml. Incubation was performed at 37°C for up to 15 min, although when 100 μg protein was used the reaction was terminated after 5 min.

Determination of binding levels. This procedure was used for the ATPase activity and proton-transport experiments (see Figs. 2b and 5). The inhibitory reaction was terminated by dissipation of the pH gradient by addition of 1 μg of the H^+,K^+exchange ionophore nigericin/50 μg protein. Thereby further decomposition of omeprazole into the active compound was prevented. Separation of the membrane protein from excess omeprazole was achieved by gel filtration over Sephadex G-25 (prepacked PD-10 columns, Pharmacia, Sweden). The columns were washed with 25 ml H_2O and equilibrated with 25 ml 2 mM Pipes/Tris (pH 7.4). The first 2.8 ml of the eluate were discarded, while the following 2 ml containing the bulk of the protein were used for measurement of bound radiolabel. The free omeprazole did not elute until 3 ml after the 2 ml that contained the vesicles. An aliquot was added to 10 ml cytoscint (West Chem, San Diego, CA) and counted in a scintillation counter, and the rest of the eluate was used for protein measurements.

Removal of substrate and ions from inhibited protein. This procedure was used when any kind of reaction was to be assayed after the inhibitory reaction, i.e., for the *p*-nitrophenylphosphatase activity, the determination of phosphoenzyme levels, the reversal of inhibition by β-mercaptoethanol and for some of the ATPase activity experiments. Passing the protein over PD-10 columns did not remove all ATP and KCl. Addition of 4 ml Sephadex G-75 on top of the prepacked column resolved protein from essentially all the ATP and KCl used in the inhibitory reaction. The columns were washed and equilibrated as described above. A sample of 1 ml was added and the first 3.5 ml of eluate were discarded. The next 2 ml were eluted in 4×0.5 ml fractions. An aliquot was taken from each fraction and protein was determined. The two fractions containing the highest protein concentrations were pooled and used for assays and measurement of bound radiolabel.

ATPase activity. The ATPase activity was measured at 37°C under the same conditions as the inhibitory reaction (Figs. 1 and 2a). When the activity was measured after the protein had been passed over a Sephadex column, 6–10 μg protein and 2 mM MgATP were used. The release of inorganic phosphate was determined as described by Yoda and Hokin [20].

p-Nitrophenylphosphatase activity. The reaction mixture contained 50 mM Tris-acetate (pH 7.4), 6 mM $MgCl_2$, ± 20 mM KCl, 6–10 μg protein and 6 mM *p*-nitrophenylphosphate in a final volume of 1 ml. After 20 min incubation at 37°C, the reaction was stopped by addition of 1 ml 1 M NaOH. The release of *p*-nitrophenol was measured at 405 nm according to Torriani [21].

Reversal of omeprazole-induced inhibition. The enzyme was inhibited as described above in the presence of 10 μM omeprazole. After incubation for 5 min at 37°C, the inhibitory reaction was stopped by addition of 1 μg nigericin/50 μg protein plus CDTA to a final concentration of 9 mM. β-mercaptoethanol was added to a final concentration of 100 mM, and after 15 min the sample was passed over a Sephadex column. The eluate was assayed for NH_4^+-stimulated ATPase activity.

Determination of phosphoenzyme levels. The assay medium consisted of 50 μM [γ-^{32}P]ATP, 2 mM $MgCl_2$, 2 mM Hepes/NaOH (pH 7.4) and 20–30 μg protein in a final volume of 1 ml. The enzyme was phosphorylated for 30 s at 22°C and the amount of phosphoenzyme was determined as previously described [22].

Proton transport. The incubation mixture contained 2 mM Hepes/NaOH (pH 7.4), 2 mM $MgCl_2$, 150 mM KCl, 50 μg protein with or without omeprazole in a final volume of 1 ml. Valinomycin, 12 μg, was added 1 min before the reaction was started by the addition of 4 mM ATP (final concentration). Incubation was performed at room temperature. After varying times of up to 10 min, acridine orange was added to a final concentration of 10 μM, and the accumulation of the dye in the vesicles was monitored in an Aminco DW-2 dual-beam spectrophotometer set at 491 nm and 547 nm. The absorbance was adjusted to a specific level, and after 3 min 1 μg nigericin was added and the absorbance change due to dissipation of the pH-gradient was recorded. When binding levels were to be determined, the content of the cuvette was passed over a Sephadex column 5 minutes after the nigericin addition, and binding was measured as described above.

In the experiment presented in Fig. 4, the incubation medium was slightly different, containing 25 μg vesicles, 1 mM Pipes/Tris (pH 7), 150 mM KCl, 1 mM glutathione and 2 mM MgATP.

Gel electrophoresis of labelled ($H^+ + K^+$)-ATPase. 3 mg protein were labelled in the presence of 5 μM omeprazole under the inhibitory conditions described above, in the presence of K^+ alone or K^+ plus valinomycin. After 5 min incubation at 37°C, the inhibitory reaction was stopped by 12-fold dilution of the samples into ice-cold 2 mM Pipes-Tris (pH 7.4) and centrifugation at 100000 × *g* for 1 h. The resulting pellets were resuspended in 2 mM Pipes/Tris (pH 7.4) and centrifuged twice more. The final pellets were resuspended in 20 mm Tris-HCl (pH 7.4) and the protein content was determined. 200 μg protein was diluted into 2 mM Pipes/Tris (pH 7.4) and recentrifuged as described above. The pellets were resuspended in 150 μl 2 mm Pipes/Tris (pH 7.4). Iodoacetamide in a final concentration of 25 mM was added to 120 μl sample and left at room temperature for 20 min to alkylate unreacted SH groups on the enzyme. This, to a large extent, prevented loss of bound compound during the solubilization of the protein with SDS. 1% SDS, 0.002% bromophenol blue (final concentrations) and solid sucrose were added. The sample was divided and loaded onto two tube-gels (length, 3 inches; diameter, 0.25 inches). Gel electrophoresis was run according to Laemmli [23]. Of the two gels, one was used for staining and scanning the protein content and the other was used for slicing and for counting radioactivity. The slices were dissolved in 30% H_2O_2 at 80°C overnight.

Protein determination. Protein was determined by the Bio-Rad microassay procedure using gammaglobulin as standard. This method of determination of proteins in gastric vesicles is comparable to the method of Lowry et al. [24] using bovine serum albumin as standard.

Results

Inhibition of ATPase activity

Valinomycin, a K^+ ionophore, allows entry of K^+ along with Cl^- into the interior of the inside-out vesicles, i.e., to the luminal side of the enzyme where the K^+ stimulatory site is located [25]. This allows a pH gradient to be formed in the presence of MgATP. When omeprazole is added to such valinomycin-treated vesicles, it is expected to accumulate in the acidic interior, due to its weak base properties, leading to the generation of the acid-activated inhibitor(s) [16].

NH_4^+ is able to activate the ($H^+ + K^+$)-ATPase, as do Tl^+,K^+ and Rb^+ [19]. However, in contrast to K^+, it does not require modification of membrane permeability to activate the ($H^+ + K^+$)-ATPase maximally. This is because NH_3 is able to cross the vesicle membrane and form NH_4^+ in the vesicle interior, after which the NH_4^+ is then able to activate the pump. The extrusion of NH_4^+ by the ($H^+ + K^+$)-ATPase also uncouples ($H^+ + K^+$)-ATPase activity from the formation of a proton gradient, and thus allows measurement of ATPase activity in the absence of ionophores and of pH gradients in intact vesicles.

Fig. 1 shows the effect of omeprazole on the ATPase activity in the presence of K^+ alone, valinomycin and K^+ and in the presence of NH_4^+. As expected from the above, the NH_4^+-stimulated activity was not inhibited by omeprazole, as formation of a pH-gradient did not occur. Equally, the activity in the presence of KCl alone was not inhibited, showing that the ionophore-independent stimulation of the ($H^+ + K^+$)-ATPase activity by K^+ was due to vesicles in the preparation

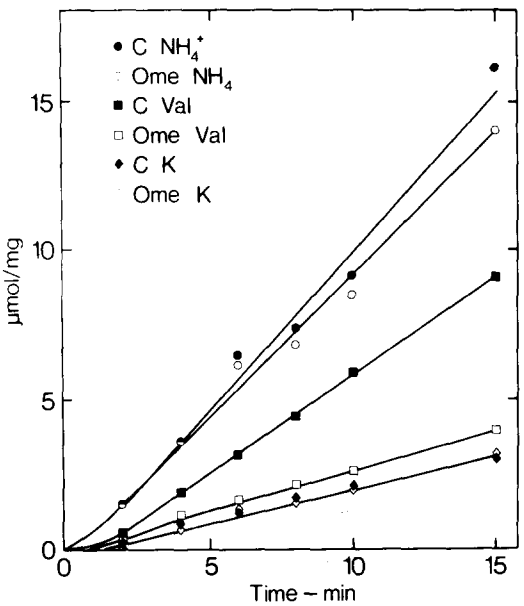

Fig. 1. Time-course of inorganic phosphate release by ($H^+ + K^+$)-ATPase-containing vesicles. The membrane protein (50 µg) was incubated at 37°C with 100 mM NH_4Cl (●); 150 mM KCl and 2 µg valinomycin (■); 150 mm KCl (◆) in the presence of 4 mM ATP, 2 mM $MgCl_2$ and 2 mM Hepes NaOH (pH 7.4). Open symbols indicate the presence of 20 µM omeprazole.

that were incapable of forming pH gradients, but were leaky to K^+. In contrast to the above, the valinomycin-stimulated ATPase activity was fully inhibited down to the basal K^+-stimulated activity in the presence of omeprazole, as would be predicted on the basis of the formation of an acidic pH in the vesicle interior only under these experimental conditions.

Determination of the fraction of transport-competent enzyme

The availability of an acid space-dependent covalent inhibitor allows a direct measurement of the fraction of transporting ($H^+ + K^+$)-ATPase in any given preparation. This information is also important when the stoichiometry of omeprazole binding is to be compared to the quantity of phosphoenzyme generated by [γ-^{32}P]ATP, since only the fraction of enzyme that is transport-competent is significant in this stoichiometric comparison.

This fraction can be determined directly by comparing NH_4^+ activation of uninhibited and inhibited enzyme, following removal of omeprazole. As shown in Table I, column A, the NH_4^+ stimulation of the enzyme was identical after a variety of preincubation conditions, except when both valinomycin and omeprazole were present. With this condition of preincubation, only 48% of the NH_4^+-stimulation activity was recovered, showing that 52% of the ATPase activity was present in transport-competent vesicles.

Also shown in Table I is the effect of identical primary incubation conditions on the *p*-nitrophenylphosphatase partial reaction of the ($H^+ + K^+$)-ATPase. This reaction is ionophore independent, since no transport is obtained with *p*-nitrophenylphosphate as substrate. Since omeprazole is as effective in the inhibition of *p*-nitrophenylphosphatase as in that of ATPase (see below), the residual *p*-nitrophenylphosphatase activity will reflect uninhibited ATPase molecules. The residual *p*-nitrophenylphosphatase activity was unaffected by omeprazole except when the preincubation conditions allowed vesicular acidification, namely in the presence of valinomycin.

TABLE I

NH_4^+-STIMULATED ATPase ACTIVITY AND K^+-STIMULATED *p*-NITROPHENYLPHOSPHATASE ACTIVITY AFTER OMEPRAZOLE TREATMENT

The ($H^+ + K^+$)-ATPase-containing vesicles (100 µg) were preincubated with or without 5 µM omeprazole in the presence of 4 mM ATP, 2 mM $MgCl_2$, 2 mM Hepes/NaOH (pH 7.4), 150 mM KCl with or without 36 µg valinomycin or with 100 mM NH_4Cl for 5 min at 37°C. The reaction was terminated by filtration over Sephadex G50+G75 and the eluate was reassayed for remaining NH_4^+-stimulated ATPase activity and K^+-stimulated *p*-nitrophenylphosphatase activity.

Preincubation conditions	A NH_4^+-stimulated ATPase activity (µmol/mg per h)	B K^+-stimulated *p*-nitrophenylphosphatase activity (µmol/mg per h)
K^+	52.1	35.1
K^+ + valinomycin	53.9 100%	35.2 100%
NH_4^+	57.9	30.9
K^+ + 5 µM omeprazole	50.2	32.3
K^+ + valinomycin + 5 µM omeprazole	25.7 48%	16.4 47%
NH_4^+ + 5 µM omeprazole	53.3	32.7

Here, 53% of the *p*-nitrophenylphosphatase activity was abolished by the omeprazole, which is directly comparable to the inhibition of the NH_4^+-stimulated activity following preincubation.

The fraction of transporting vesicles can also be calculated by comparing the K^+-stimulated (in the absence of valinomycin) and NH_4^+-stimulated activities. At 150 mM K^+, there is partial inhibition of $(H^+ + K^+)$-ATPase activity [22], and this inhibition is apparently not found with 100 mM NH_4^+. Since the K^+ stimulation in the absence of ionophore is due to leaky vesicles, namely with 150 mM K^+ present on both sides of the ATPase, the effect of the two concentrations of NH_4^+ and K^+ must first be compared in lyophilized material, and the degree of K^+ inhibition then used to correct the basal K^+-stimulated level. The ATPase activity in the presence of 100 mM NH_4^+ was 50.5 μmol/mg per h and in the presence of 150 mM KCl 26.5 μmol per h. Thus, in the presence of this concentration of K^+, the ATPase was inhibited by 47%. Using Fig. 1 as an example, a value of 65% transport-competent ATPase could be calculated. Using these three approaches, the fraction of transport-competent ATPase ranged from 44 to 65% in the preparations used in these experiments.

Correlation between inhibition of ATPase activity and binding of radiolabelled inhibitor generated from ^3H-omeprazole

The K^+-valinomycin-stimulated activity was decreased as a function of the incubation time and the concentration of omeprazole (Fig. 2a). At low concentrations, of omeprazole, the inhibition was slower than at high concentrations and the degree of inhibition did not reach the same levels as those found at high omeprazole concentrations. Under the same conditions, omeprazole was progressively incorporated into the preparation, in parallel with the inactivation of the ATPase activity, Fig. 2b. Some binding occurred, most notably at high omeprazole concentrations, under non-inhibiting conditions, i.e., in the presence of K^+ alone. Since this binding did not affect the activity (e.g., Fig. 1), it was considered nonspecific and has been subtracted in Fig. 2b. In Fig. 3, the remaining ATPase activity is plotted against the corresponding binding level. The degree of inhibition obtained was strictly correlated to the level of

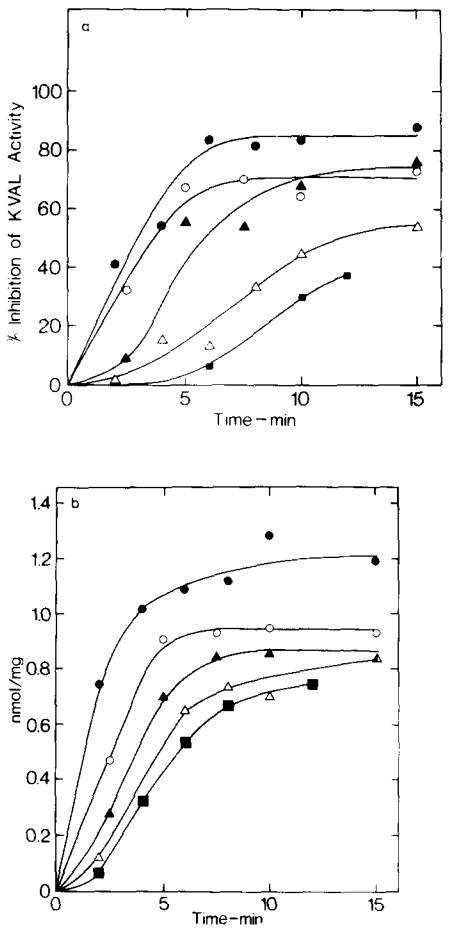

Fig. 2. Time and concentration dependence of omeprazole-induced inhibition of and binding to the $(H^+ + K^+)$-ATPase. The vesicles (50 μg) were incubated with 2 μM (■); 2.5 μM (△); 5 μM (▲); 10 μM (○) and 20 μM (●) omeprazole in the presence of 2 mM Hepes/NaOH (pH 7.4), 4 mM ATP, 2 mM $MgCl_2$ and 150 mM KCl with or without 12 μg valinomycin at 37°C for the times indicated. (a) Inhibition of K^+ plus valinomycin-stimulated ATPase activity; (b) binding levels. The results obtained in the presence of K^+ alone are subtracted from the results in the presence of K^+ plus valinomycin. For the ATPase activity, the K^+-stimulated part was 40% of the K^+ plus valinomycin-stimulated activity. In the presence of increasing concentrations of omeprazole, the K^+ plus valinomycin-stimulated activity was increasingly inhibited, while the K^+-stimulated part was unaffected. The binding levels in the presence of K^+ alone increased from 10%–40% of the levels obtained with K^+ plus valinomycin using 2–20 μM omeprazole.

specifically bound radiolabel, regardless of the concentration of omeprazole and incubation time used. The intercept obtained on the abscissa indi-

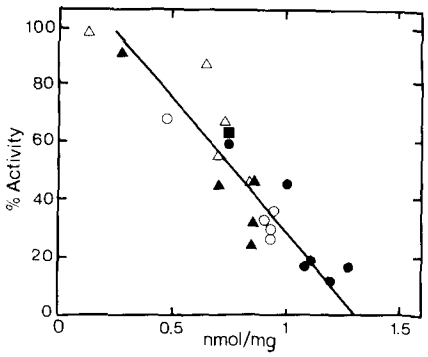

Fig. 3. Correlation between inhibition of ATPase activity and binding levels. Data were taken from Fig. 2. The line was fitted to the data by means of linear regression analysis ($r = 0.905$).

cates that 1.3 nmol radiolabel bound per mg of protein was needed to inhibit the K^+-valinomycin-stimulated activity by 100% in this preparation. However, since the binding levels reflected only specific binding to the acid-accumulating vesicles and since it was only these vesicles that were inhibited, a correction had to be made when the binding of omeprazole was to be related to the quantity of phosphoenzyme. The particular preparation used for Fig. 3 contained 65% acid-stimulating vesicles. After this figure was taken into consideration, the intercept obtained was changed to 2 nmol/mg. The steady-state level of phosphoenzyme in this particular preparation was 1 nmol/mg, giving a stoichiometry of 2 mol radiolabel bound per mol phosphoenzyme for full inhibition of enzyme activity.

Inhibition of partial reactions of the ($H^+ + K^+$)-ATPase

The effect of omeprazole on two of the partial reactions of the ($H^+ + K^+$)-ATPase, phosphoenzyme formation and K^+-stimulated *p*-nitrophenylphosphatase activity, was studied after first inhibiting the enzyme under transporting conditions and subsequently reassaying the reaction of interest. As shown in Table II, the phosphoenzyme levels were not affected to the same extent as the ATPase activity and inhibition of phosphoenzyme formation could therefore not explain all the loss of ATPase activity. In addition to catalyzing the hydrolysis of ATP, it has been shown that the ($H^+ + K^+$)-ATPase is able to hydrolyze *p*-nitrophenylphosphate [19]. This *p*-nitrophenyl phosphatase activity is referred to as a partial reaction, since no ion transport occurs. An example of the effect of omeprazole on this activity is presented in Table IB. Under conditions where the ATPase activity of the acid accumulating vesicles were fully inhibited, the *p*-nitrophenylphosphatase activity was also totally blocked.

TABLE II

COMPARISON OF THE EFFECT OF OMEPRAZOLE ON ATPase ACTIVITY AND PHOSPHOENZYME FORMATION

The vesicles (100 μg) were preincubated with or without 20 μM omeprazole in the presence of 2 mM $MgCl_2$, 4 mM ATP, 2 mM Hepes/NaOH (pH 7.4) and 150 mM KCl or 150 mM KCl plus 36 μg valinomycin for 5 min at 37°C. Separation of the vesicles from the preincubation medium was achieved by gel filtration. Part of the eluate was used for determination of remaining NH_4^+-stimulated ATPase activity and part of it was used for determination of the phosphoenzyme level.

Preincubation conditions	NH_4^+-stimulated ATPase activity (μmol/mg per h)	Steady-state level of phosphoenzyme (nmol/mg)
K^+	35.7	0.60
K^+ + valinomycin	44.4 100%	0.60 100%
K^+ + 20 μM omeprazole	37.2	0.62
K^+ + valinomycin + 20 μM omeprazole	18.5 42%	0.51 85%

Inhibition of proton transport

The use of acridine orange uptake is a particularly convenient means of determining proton transport in vesicles. Initial experiments showed that the dye interacted with omeprazole, preventing inhibition (data not shown). Therefore, the conditions chosen involved preincubation of the vesicles in the presence of ATP, K^+, valinomycin with varying concentrations of omeprazole followed by addition of 10 μM acridine orange. To estimate the amount of proton uptake achieved, the absorbance change obtained upon addition of the H^+,K^+ exchange ionophore, nigericin, was recorded. This protocol is shown in Fig. 4, where it can be seen that there was an omeprazole dose-dependent loss in the nigericin-dependent absorbance change. Carrying out this assay using

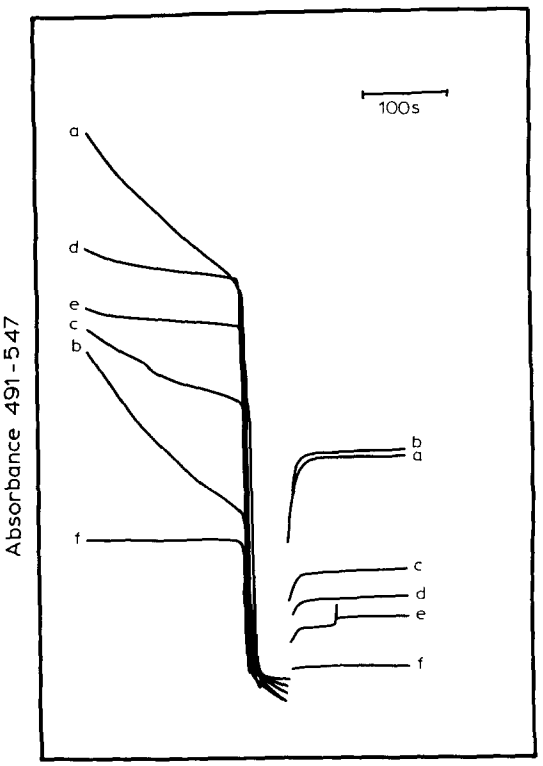

Fig. 4. Effect of omeprazole on proton transport. Gastric $(H^+ + K^+)$-ATPase vesicles (25 μg) were incubated with 2 mM MgATP, 150 mM KCl, 1 mM Pipes/Tris (pH 7.0), 1 mM glutathione and 5 μg valinomycin for 10 min. Acridine orange was added to a final concentration of 10 μM and the absorbance change was followed for 3 min, whereafter the absorbance was adjusted to the same level and 0.15 μg nigericin was added. Control, no omeprazole (a); 1 μM (b); 3 μM (c); 5 μM (d); 10 μM (e) and 100 μM (f) omeprazole.

radioactive omeprazole allowed the determination of the stoichiometry of binding as related to proton transport. In Fig. 5, it can be seen that a linear correlation was obtained between binding and inhibition of proton transport. The value of 0.95 nmol radiolabel bound per mg protein at 100% inhibition (1.4 nmol/mg, when corrected for the fraction of acid-accumulating vesicles in the preparation) was smaller than that found for inhibition of the valinomycin component of the ATPase activity.

Reversal of omeprazole inhibition

Sulfhydryl-containing compounds, such as dithiothreitol, β-mercaptoehtanol and glutathione,

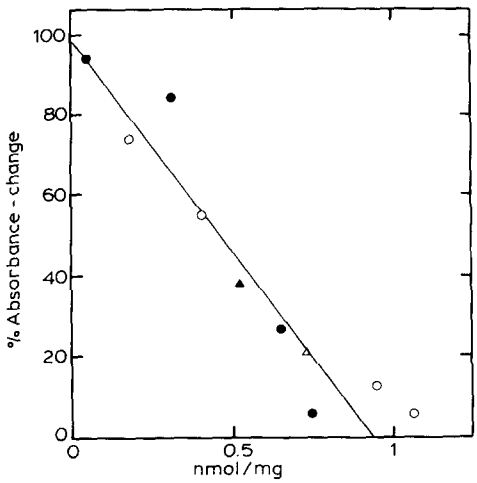

Fig. 5. Correlation between inhibition of proton transport and binding levels. 15 μM ^{3}H-omeprazole incubated with the vesicles for 2, 4, 6 and 8 min (○); 20 μM ^{3}H-omeprazole for 2, 3, 5 and 8 min (●). In addition, the effect of 1 mM glutathione in the medium was investigated at 37°C using 5 μM omeprazole without (△) and with (▲) glutathione.

are known to be able to reverse omeprazole inhibition of the $(H^+ + K^+)$-ATPase in broken membranes, as well as protect against inhibition [8, 16]. In this preparation, too, high concentrations of β-mercaptoethanol were able to completely re-

TABLE III

REVERSAL OF INHIBITION AND BINDING OF OMEPRAZOLE BY β-MERCAPTOETHANOL

The enzyme (100 μg) was preincubated with or without 100 μM omeprazole in the presence of 4 mM ATP, 2 mM $MgCl_2$, 2 mM Hepes/NaOH (pH 7.4), 150 mM KCl and 36 μg valinomycin. After incubation for 5 min at 37°C, the reaction was stopped by addition of 2 μg nigericin and 10 mM CDTA. β-mercaptoethanol was added to a final concentration of 100 mM to one of the omeprazole-treated samples. The samples were then kept at 37°C for 15 min, whereafter they were passed over Sephadex columns. The eluates were reassayed for remaining NH_4^+-stimulated ATPase activity and for levels of bound radiolabel.

Preincubation conditions	NH_4^+-stimulated ATP activity (μmol/mg per h)	Binding levels (nmol radiolabel/mg protein)
No omeprazole	35.6	-
Omeprazole	15.8	1.02
Omeprazole + β-mercaptoethanol	43.0	0.11

verse the inhibited enzyme, Table III. The bound radiolabel was displaced in parallel with recovery of the enzyme activity. In contrast, a concentration of glutathione of 1 mM, which has been previously shown to protect lyophilized ($H^+ + K^+$)-ATPase against omeprazole inhibition [16] minimally affected omeprazole inhibition in intact vesicles (Figs. 4 and 5).

Peptide labelling by omeprazole

Since gastric vesicles are obtained in varying degrees of purity, it was important to determine the location of the bound radiolabel following omeprazole-induced inhibition, in terms of the labelled peptides. Thus, following labelling of the vesicle preparation under acid-transporting conditions, gel electrophoresis was carried out. Fig. 6 shows the protein and radioactivity distribution pattern obtained. The majority of the label was found in the region of 94 kDa, the molecular weight of the ($H^+ + K^+$)-ATPase. The radioactivity found above this region is presumably due to undissociated enzyme, since β-mercaptoethanol could not be used. The radioactivity at the dye front probably represents inhibitor that was dissociated from the enzyme during the solubilization procedure. Thus, it can be concluded that only the ($H^+ + K^+$)-ATPase subunit(s) is (are) labelled out of the various peptide bands found in this preparation.

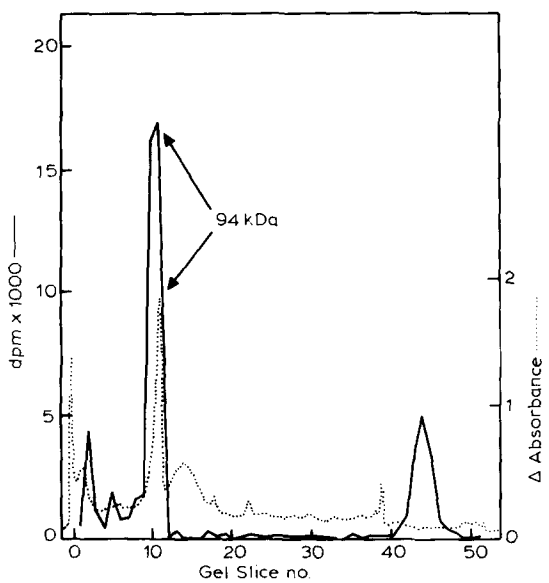

Fig. 6. SDS gel profile of a ($H^+ + K^+$)-ATPase vesicle preparation following omeprazolepinduced inhibition. The vesicles were incubated with 5 μM ^{3}H-omeprazole. Gel electrophoresis was performed according to Laemmli [23] in a 10% acrylamide gel. Dotted line: profile of Commassie blue staining. Solid line: dpm × 1000.

Discussion

As outlined above, there are several properties of omeprazole suggesting that the reactive species accumulate on the luminal side of the parietal cell membrane. The inhibition of the enzyme would thus be due to a reaction between the inhibitor and luminally accessible cysteine residues.

Other in vivo studies have shown much higher levels of bound inhibitor at 100% inhibition of ATPase activity than those reported in the present study [8,17]. In these studies, permeable membrane preparations have been used in which omeprazole has been incubated at pH 6. Under these conditions, the drug would react on either side of the membrane with both luminal and cytosolic SH-groups of the enzyme. In this study, the in vivo situation has been mimiced, since (a) the pH of the medium was buffered to pH 7.4, which prevented interaction at the cytosolic face of the enzyme, and (b) binding and inhibition were dependent on acidification of the vesicle lumen. Only when acid accumulation was allowed to occur in the vesicular preparation, was there evidence for an effect of omeprazole.

A linear correlation between the quantity of inhibitor bound and the degree of inhibition was found, which gave a stoichiometry of 2 mol inhibitor bound per mol phosphoenzyme at 100% inhibition of ATPase activity. Discrepancies have been reported between the maximal phosphorylation levels obtained and binding capacities of labels that were specific to the ATP site. Thus, in preparations where more than 80% of the peptides are located at 94 kDa, which corresponds to the molecular weight of the phosphorylated subunit, phosphorylation from ATP and FITC-labelling did not exceed 1.5 nmol/mg protein [26]. However, vanadate binding to the enzyme has been reported to reach 3 nmol/mg protein, and it has been claimed that phosphorylation from 8-azido-ATP and also from inorganic phosphate reached

this level [27–29]. It would appear that half of the enzyme is phosphorylated from ATP in the steady state. Given that vanadate binding or phosphorylation from 8-azido-ATP or inorganic phosphate is a correct measure of the number of catalytic subunits, binding of one inhibitor molecule per catalytic subunit would seem to be required for inactivation of the enzyme.

The inhibition of the enzyme activity was fully reversed by β-mercaptoethanol. However, proton transport was inhibited even when glutathione was present in the medium in a concentration sufficient for protection. These results indicate that β-mercaptoethanol penetrates the membrane and reacts in the vesicle lumen, providing further evidence that the inhibitory reaction by omeprazole involved luminally accessible thiol groups.

Along with inhibition of the ATPase activity, it was observed that the *p*-nitrophenylphosphatase activity of the preparation was coherently inhibited, giving further evidence that this reaction is indeed a partial raction of the $(H^+ + K^+)$-ATPase. However, inhibition of the phosphoenzyme formation did not correlate with inhibition of the ATPase activity. Also, in lyophilized membrane preparations of the $(H^+ + K^+)$-ATPase, a discrepancy between inhibition of ATPase activity and phosphoenzyme formation has been observed [8].

The stoichiometry of inhibitor binding at full inhibition of proton trnasport was found to be 1.4 mol inhibitor, bound per mol phosphoenzyme. That this value is slightly lower than that found for ATPase activity might be explained by the greater sensitivity of the H^+ transport measurement to ATPase activity inhibition, since there is invariably a significant leak of protons in these preparations. Therefore, it would not be necessary to inhibit 100% of the ATPase activity to abolish a measurable pH-gradient. Furthermore, in earlier work where the dye signal was calibrated [30], it was noted that the probe was insensitive to pH gradients of less than 1 unit; hence a 10-fold residual H^+ gradient would not be detected.

Since gel electrophoresis revealed that the vast majority of covalently bound drug was in the 94 kDa region of the gel, i.e., whatever the composition of the membranes it was only the catalytic subunit of the $(H^+ + K^+)$-ATPase that reacted, the stoichiometry calculated above did not have to be corrected by subtraction of counts associated with other peptides.

The action of omeprazole on this in vitro system therefore seems to be very similar to its action in vivo, since the data above show that omeprazole can be used selectively to inhibit the gastric $(H^+ + K^+)$-ATPase, the reaction being dependent on an adequately low luminal pH. Consequently, under these conditions, this type of compound is an interesting probe of the reaction pathway and structure of this enzyme. Finally, it might be pointed out that the structure of the activated compound and its luminal side reactivity would argue for the presence of a cysteine residue that reacts either in the luminal sector or in the hydrophobic membrane spanning sector of the $(H^+ + K^+)$-ATPase.

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