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SPECIFIC LABELLING OF GASTRIC H^+,K^+-ATPase BY OMEPRAZOLE

JAN FRYKLUND, KARIN GEDDA and BJÖRN WALLMARK
Hässle Gastrointestinal Research Laboratories, Department of Biology, S-431 83 Mölndal, Sweden

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Abstract—Acid secretion is conducted by the parietal cell of the gastric mucosa. The H^+,K^+-ATPase has been shown to be specifically located to this cell and during recent years been recognized as the gastric proton pump. Omeprazole, a known inhibitor of acid secretion, administered *in vivo* was found to bind specifically to the H^+,K^+-ATPase of the rabbit gastric mucosa. A stoichiometry of 2.1 mol radiolabel per mol phosphoenzyme was calculated at total inhibition of the H^+,K^+-ATPase enzyme activity. In isolated gastric glands prepared from omeprazole-treated animals, the secretagogue-induced increase in oxygen consumption, related to acid secretion, was inhibited to the same level as the H^+,K^+-ATPase activity. Both the degree of acid secretion inhibition induced by omeprazole and the amount of inhibitor bound to the H^+,K^+-ATPase were found to be dependent on the stimulation state of the parietal cell. Inhibition of secretion by the H_2-receptor blocker ranitidine prior to omeprazole treatment prevented both the inhibition of H^+,K^+-ATPase and oxygen consumption normally observed with omeprazole and, furthermore, reduced the binding levels of radiolabel to the enzyme. Inhibition of acid secretion by the H^+,K^+-ATPase inhibitor SCH 28080 totally prevented the binding of radiolabel to the H^+,K^+-ATPase. The inhibition by omeprazole could be fully reversed in gastric glands and H^+,K^+-ATPase isolated from omeprazole-treated animals by addition of β-mercaptoethanol. The major product formed during reactivation was the reduced form of omeprazole, compound H 168/22. Neutralization of the gastric glands *in vitro* with imidazole totally prevented the inhibitory action of omeprazole. These experiments demonstrate the necessity of acid for the inhibition of gastric acid secretion by omeprazole and the binding of the inhibitor to the H^+,K^+-ATPase, both *in vivo* and *in vitro*, and also the specificity of omeprazole for the H^+,K^+-ATPase.

The substituted benzimidazole, omeprazole,* represents a new class of potent, long-acting and specific inhibitors of gastric acid secretion [1–4]. Omeprazole is under extensive clinical evaluation and has shown significantly better healing results when compared to H_2-receptor antagonists in peptic ulcer patients [5–8], Zollinger–Ellison patients [9], and in patients with erosive reflux oesophagitis [10–12].

Omeprazole reduces acid secretion by a novel mechanism of action. The compound inhibits the proton pump of the gastric mucosa, the H^+,K^+-ATPase, situated in the secretory membrane of the parietal cell [4, 13]. In the rat, a strict relationship between inhibition of acid secretion and blockade of H^+,K^+-ATPase by omeprazole has been demonstrated [14]. The high specificity of omeprazole for inhibition of gastric acid secretion is suggested to depend mainly on three factors. Firstly, there is the localization of the H^+,K^+-ATPase in the apical membrane of the parietal cell, which separates the neutral cell cytosol from the very acidic secretory canaliculus ($pH < 1$). Secondly, there are the permeable weak-base properties of omeprazole, which enable the compound to concentrate in the acidic region of the parietal cell [3]. Thirdly, in the acidic environment at the canalicular face of the secretory membrane in the parietal cell an intramolecular rearrangement of omeprazole into an inhibitor of the H^+,K^+-ATPase is initiated by acid [15]. As a consequence, the degree of acid secretion inhibition is expected to be related to the stimulatory state of the parietal cell. The inhibitor reacts with SH groups within the H^+,K^+-ATPase, since binding of acid-transformed omeprazole is parallelled with modification of these groups. Furthermore, sulfhydryl-reducing agents can both prevent and reverse omeprazole inhibition induced under *in vitro* conditions. From such studies it has been possible to estimate the stoichiometry between binding and inhibition of the H^+,K^+-ATPase [16, 17].

The present study was undertaken to investigate: (1) the binding stoichiometry and specificity of omeprazole for the H^+,K^+-ATPase under these *in vivo* conditions; (2) the influence of the stimulatory state of the parietal cell on inhibition of acid secretion and H^+,K^+-ATPase by omeprazole.

* Abbreviations used: PEG, polyethylene glycol; AP, [dimethylamine-^{14}C]aminopyrine; dbcAMP, N^6,O^2-dibuturyl cyclic adenosine monophosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris; tris (hydroxymethyl)aminomethane; Na_2ATP, adenosinetriphosphate disodium salt; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; H^+,K^+-ATPase, magnesium-dependent, hydrogen ion-transporting, and potassium-stimulated adenosine triphosphatase (EC 3.6.1.3); omeprazole, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl]-sulfinyl]-1H-benzimidazole.

MATERIALS AND METHODS

Study design. Female rabbits of New Zealand white strain were given 10 μmol/kg omeprazole dissolved in polyethylene glycol 400 (PEG 400) as a subcutaneous injection in the neck. In some experi-

ments 0.75 mCi [^{3}H]omeprazole/kg was included in the dose. Control animals received PEG 400 as vehicle. The H_2-receptor blocker ranitidine was given in physiological saline and the H^+,K^+-ATPase inhibitor SCH 28080 was dissolved in PEG 400. Ranitidine and SCH 28080, 400 μmol/kg, respectively, were given 60 min prior to and 15 min after omeprazole or vehicle. Two hours after omeprazole administration gastric glands and H^+,K^+-ATPase were prepared from the gastric mucosa.

Gastric content pH. Stomach content pH was measured with a pH meter (Radiometer, Copenhagen) after dilution with distilled water.

Preparation of gastric glands. Gastric glands were prepared from the corpus region with minor modifications of the method described previously [18]. Briefly, after vascular perfusion of the stomach *in situ*, the corpus mucosa was separated from the underlying muscular layer. The mucosa was then minced with a pair of scissors and the tissue incubated with collagenase. Following digestion, the glands were washed and diluted to a concentration of about 10 mg gland dry weight/ml in the incubation medium (in mM: NaCl, 132.4; KCl, 5.5; Na_2HPO_4, 5.0; NaH_2PO_4, 1.0; $MgSO_4$, 1.2; $CaCl_2$, 1.0) pH 7.4 to which albumin 1.0 mg/ml and glucose 11 mM were added.

Oxygen consumption measurements in gastric glands. Oxygen uptake was monitored at 37° in a differential respirometer (Gilson). A 20% KOH solution on a filter paper was used as CO_2 absorber and placed in the central cup. One milliliter of the above gland suspension was added to 2 ml incubation medium containing test agents and equilibrated with the reference chambers for 30 min. When imidazole was used for neutralization of the glands, omeprazole was added from the sidearm after the initial 30-min equilibration period. Oxygen consumption was then recorded at 10-min intervals during the following 90-min period. The mean O_2 consumption during the 90-min period was calculated and expressed as μl O_2/mg gland dry wt·hr. The data were corrected for ambient temperature and atmospheric pressure.

Purification of H^+,K^+-ATPase. About 5 g of gastric mucosal tissue prepared after vascular perfusion as described above was homogenized in 10 vol. of 5 mM PIPES/Tris buffer pH 7.4 containing 0.25 M sucrose. The homogenization was performed in a Potter–Elvehjem homogenizer by 20 passes of a tight-fitting Teflon piston at 2400 rpm. The homogenate was fractionated by differential centrifugation. Nuclei and cell debris were spun down at 1000 *g* for 10 min, the mitochondria at 20,000 *g* for 20 min, and the crude microsomal fraction was finally obtained at 100,000 *g* for 60 min. The pellets were resuspended in 10, 5 and 3 ml, respectively, of 5 mM PIPES/Tris buffer (as above). The H^+,K^+-ATPase-containing vesicles were purified from the microsomal fraction by step gradient centrifugation. A volume of 2.5 ml of the crude microsomal suspension was loaded on a step gradient consisting of 7.5% Ficoll®, 30 and 60% sucrose (w/w) in 5 mM PIPES/Tris buffer, and centrifuged at 100,000 *g* for 60 min. One-milliliter fractions were collected from the three different interfaces and designated GI, GII and GIII, starting from the top of the gradient.

Assay of ATPase activities. The GI fraction was used for analyses of Mg^{2+}- and K^+-dependent ATPase activities. The incubation conditions were as follows: 5 μg samples of protein from the different subcellular fractions were incubated in 40 mM Tris buffer pH 7.4 containing 180 mM sucrose, 2 mM Na_2-ATP, 2 mM $MgCl_2$ with or without 10 mM KCl and gramicidin 5 μg/ml (total volume 1.1 ml). The hydrolysis of ATP was allowed to proceed for 10 min at 37°, whereafter the reaction was stopped by adding 1 ml of 3.6% ammonium molybdate in 12% perchloric acid. Hydrolysis of ATP was measured by determination of the release of inorganic phosphate (P_i) according to published procedure [19].

Incubation of H^+,K^+ATPase with β-mercaptoethanol. A sample of 100 μg protein/ml of the GI fraction was incubated with 100 mM β-mercaptoethanol in 40 mM Tris buffer pH 7.4 containing 180 mM sucrose for 30 min at 37°. The enzyme was then diluted to 2 μg protein/ml and 2 mM β-mercaptoethanol, whereafter the ATPase activity was assayed as above.

Phosphoenzyme levels in subcellular fractions. A 50 μg sample of the crude microsomal membrane fraction or the GI fraction were incubated for 15 sec at 22° in 40 mM PIPES/Tris pH 7.4 containing 2 mM Mg^{2+}, 5 μM [γ-^{32}P]ATP in the absence or presence of 100 mM K^+. Determination of the concentration of phosphoenzyme (EP) was as described elsewhere [20].

Determination of protein concentrations. Protein was determined according to the method of Bradford [21].

Electrophoresis of protein. Samples for electrophoresis were alkylated with 25 mM iodoacetamide for 15 min prior to solubilization in a cocktail consisting of 62 mM Tris–HCl pH 6.8, 10% glycerol (v/v), 2% SDS (w/v) and 0.001% (w/v) bromphenol blue. Ten to 30 μg samples of protein were electrophoresed at pH 8.3 on 1.5 mm SDS–PAGE slab gels in a discontinuous buffer system according to Laemmli [22]. The separating gel consisted of an acrylamide gradient ranging from 5 to 20% with a stacking gel of 4% at the top. The crosslinker concentration was 2.67%. Gels were run at constant current usually at 35 mA overnight. Staining and fixation were performed in 0.1% Coomassie blue R250 in 40% methanol 10% acetic acid in water for 1 hr followed by destaining in the same medium without Coomassie blue. H^+,K^+-ATPase isolated from hog stomachs was used as a standard in parallel with commercial molecular weight standards. Finally, the protein distribution in gels was quantified by a 2202 Ultrascan Laser densitometer (LKB) connected to a Varian 4270 integrator. The area under the 92 kDa peak was identified as the H^+,K^+-ATPase [23] and this area was expressed as a percentage of the area of the total protein in the sample loaded on the gel. Radiolabel incorporation into gel proteins was determinated after slicing the gel and extracting the radioactivity with Lumasolve/Lipoluma® 1:10 prior to counting in a liquid scintillation counter (LKB Rackbeta 1219).

Extraction and chromatography of omeprazole and its sulfide derivative. Fifty to 350 μl samples of the incubation medium or the membrane pellet were

extracted with 500 μl dichloromethane for 10 min. Following 10 min centrifugation, the aqueous phase was sucked off and 60–100 μl of the organic phase were injected into the chromatographic column. Analyses were performed on a high-performance liquid chromatograph (HPLC) to which an absorbance detector operating at 302 nm was fitted. The column support consisted of Lichrosorb S 160 (5 μm). The precolumn support was Brownlee GSS-013 (7 μm). The mobile phase was 2% methanol 98% dichloromethane (v/v).

The fractions containing [^{3}H] omeprazole and [^{3}H] H168/22 (the sulfide derivative) were collected and analyzed for radioactivity in a liquid scintillation counter. (For structures of omeprazole and H 168/22, see Ref. 24.)

Chemicals. Collagenase (type 1A), dbcAMP and Na_2ATP were obtained from Sigma Chemicals (St Louis, MO). [γ-^{32}P]ATP was purchased from Amersham International, U.K.

Omeprazole and [^{3}H]omeprazole were synthesized by AB Hässle (Mölndal, Sweden). The specific radioactivity was 3.6 mCi/mg (45.6 GBq/mmol) and the radiochemical purity >97%.

Other reagents were from the usual commercial sources and of the highest purity available.

Statistics. Values are mean values ± SEM. Numbers in parentheses indicate the number of experiments (i.e. number of rabbits). The normality distribution in each group was examined with the Wilk–Shapiro test. Comparisons between groups were done with either the Mann–Whitney U-test (non-parametrical samples) or the pooled variance *t*-test for normal distributed samples.

RESULTS

Gastric content pH

Both omeprazole 10 μmol/kg and ranitidine 400 μmol/kg × 2 inhibited acid secretion and, consequently, raised the pH of the gastric content. When omeprazole and ranitidine were given in combination, the pH increased further. This might indicate that neither omeprazole nor ranitidine at the dose levels used in this study completely inhibited acid secretion (Fig. 1).

Oxygen uptake in gastric glands

Gastric glands incubated in a respirometer exhibit a basal oxygen uptake which, to a large extent, is unrelated to acid secretion, since it is insensitive to inhibitors of acid secretion [24]. In contrast, when an acid secretagogue is added, the oxygen consumption is increased as a consequence of the increased ATP demand for the proton translocation by the H^+,K^+-ATPase [24, 25]. In this study, the oxygen consumption was stimulated by 1 mM db-cAMP. Administration of 10 μmol omeprazole/kg decreased the stimulated component of oxygen uptake by 67% in glands isolated from omeprazole-treated animals. In contrast, ranitidine treatment did not significantly decrease the db-cAMP-stimulated oxygen consumption. When the H_2-blocker was given prior to omeprazole administration, the inhibition of the stimulated oxygen uptake was significantly reduced as compared to the experiments when omeprazole was given alone (Fig. 1). Thus, the H_2-blocker largely prevented the omeprazole-induced inhibition.

H^+,K^+-*ATPase activity*

The ATPase activity was measured in the density gradient fraction (GI see above). The Mg^{2+}-stimulated ATPase activity was not influenced by any of the dose regimes (data not shown). However, the K^+-stimulated ATPase activity was decreased by 60% after treatment with 10 μmol omeprazole/kg (Fig. 1). Ranitidine did not influence the H^+,K^+-

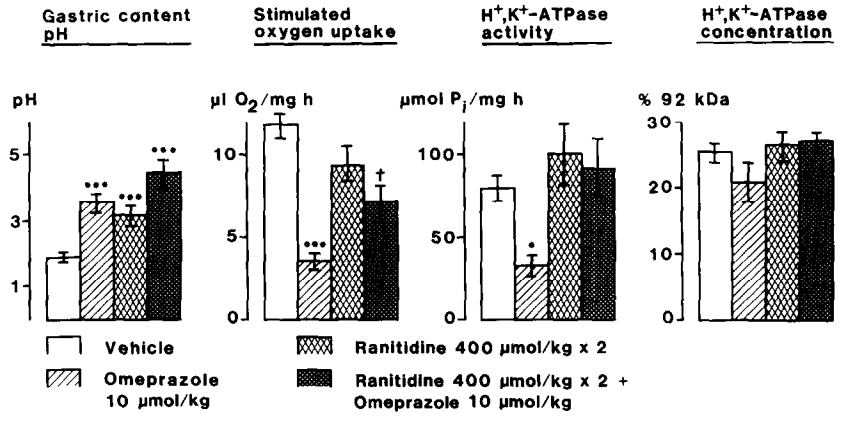

Fig. 1. Effect of omeprazole and ranitidine on gastric acid secretion parameters. Rabbits were given omeprazole 10 μmol/kg or ranitidine 400 μmol/kg as subcutaneous injections. Ranitidine was given 60 min prior to and 15 min after the omeprazole or vehicle (PEG 400) treatment. Two hours after omeprazole administration gastric glands and H^+,K^+-ATPase-containing vehicles (GI-fraction) were isolated from the gastric mucosa. The pH of the gastric content was measured. Oxygen uptake was estimated in the isolated glands. Stimulated oxygen uptake is the difference between basal and maximally stimulated oxygen uptake in the presence of 1 mM db-cAMP. The H^+,K^+-ATPase activity is the activity in the presence of Mg^{2+} plus K^+ minus the basal Mg^{2+}-stimulated ATPase activity. The H^+,K^+-ATPase concentration in the vesicle preparation was determined after SDS–PAGE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle. †$P < 0.05$ compared to omeprazole treated. Mean ± SEM (N = 4–16).

ATPase activity. Pretreatment with ranitidine prevented the omeprazole-induced reduction of the H^+,K^+-ATPase activity. These observations are in accordance with the oxygen consumption measurements.

Protein content and gastric mucosal H^+,K^+-ATPase concentration

No significant difference between the control group and the omeprazole-treated groups was found when the GI fraction was analyzed for H^+,K^+-ATPase concentration (Fig. 1). The same pattern was found in the microsomal fraction (not shown). These data suggest that the decrease in enzyme activity found after *in vivo* administration of omeprazole is not due to a decreased amount of H^+,K^+-ATPase in the fractions analyzed. Furthermore, when the protein concentration was estimated after fractionation into the microsomal and GI fractions, no significant difference between the groups was found. These findings indicate that no redistribution of H^+,K^+-ATPase between the fractions had occurred after administration of omeprazole.

Studies with neutralized gastric glands

In order to mimic the *in vivo* situation, where ranitidine was used to inhibit the acid secretion in the parietal cells, the permeable buffer imidazole was used. It has previously been suggested that the weak base imidazole dissipates stimulated aminopyrine accumulation in isolated gastric glands by acting as a permeable buffer [26] and, thus, neutralized the acid in the acidic compartments of the gastric gland. The fact that imidazole at 3 mM inhibited AP-uptake without decreasing the oxygen uptake shows that this is the case [24]. Thus, under acidic conditions, i.e. stimulation of acid secretion with 1 mM db-cAMP, omeprazole dose-dependently inhibited the stimulated oxygen uptake in gastric glands isolated from control rabbits, the IC_{50} values being $\sim 5.10^{-7}$ M. However, in the presence of 3 mM imidazole, the IC_{50} value was $>10^{-4}$ M (Fig. 2). These experiments demonstrate that acid transformation of omeprazole is necessary before it can exert an inhibitory effect on acid secretion.

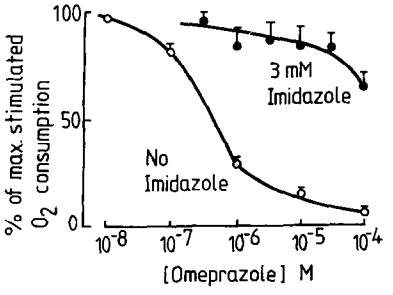

Fig. 2. Effect of imidazole neutralization on stimulated oxygen uptake in rabbit gastric glands. Oxygen consumption was stimulated from a basal uptake of 9.3 ± 0.7 to 24.9 ± 0.5 μl O_2/mg hr with 1 mM db-cAMP present. Stimulated minus basal uptake was set at 100%. Omeprazole was added after a 30-min equilibration period in the absence or presence of imidazole. Means ± SEM (N = 3–7).

Incorporation of radioactivity from [^{3}H]omeprazole into mucosal proteins. Stoichiometry of binding and phosphoenzyme levels

Following administration of radiolabelled omeprazole to the animals, a crude homogenate was prepared from the fundic mucosa and a density gradient fraction of this homogenate. The radioactivity distribution in these two fractions was analyzed by polyacrylamide gel electrophoresis and was confined to two regions: to the 92 kDa region (to which the standard hog H^+,K^+-ATPase also migrated) and in the front of the gel. The front radioactivity was washed out during the staining and destaining procedures and was presumably not protein-bound. This radioactivity was found to correspond to about 50% of the loaded radioactivity. The same amount of radioactivity was extractable from the sample with dichloromethane and identified after HPLC as the sulfide derivative of omeprazole. Figure 3 shows the protein distribution in the gels after electrophoresis of pooled samples from eight omeprazole-treated rabbits. After staining and a densitometer scan, the same gels were sliced, and the radioactivity in each slice determined. Purification of the initial fundic homogenate showed a concomitant purification of the 92 kDa protein in the GI fraction in which the radioactivity was located. These data demonstrate that omeprazole specifically binds to the proton pump of the gastric mucosa. After dichloromethane extraction of the sulfide derivative of omeprazole the amount of radiolabel bound to the H^+,K^+-ATPase was calculated and based on the quantity of 92 kDa protein present in the fractions used for the binding studies. The binding levels, expressed as nmol radiolabel/mg 92 kDa protein, are shown in Table 1. Omeprazole in a dose of 10 μmol/kg inhibited

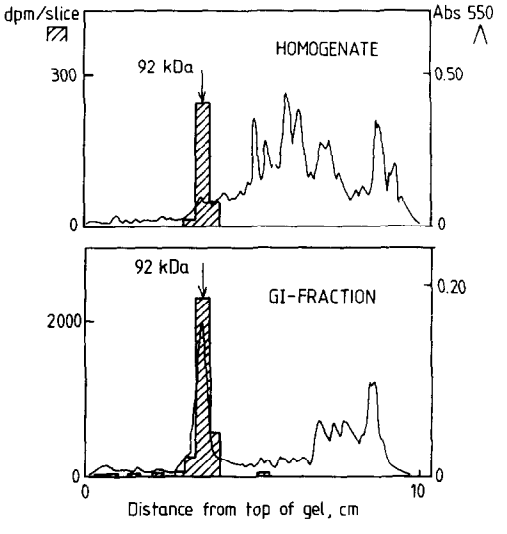

Fig. 3. Binding of radiolabel to gastric mucosal protein 2 hr after *in vivo* [^{3}H]omeprazole administration. Homogenate and H^+,K^+-ATPase-containing vesicles (GI-fractions) were loaded on the same gel and separated by SDS-PAGE. After electrophoresis the gel was stained and scanned for protein. Finally, the gel was sliced and the radioactivity in each slice was compared to the protein distribution in the gel (pooled material from 8 rabbits).

Table 1. Binding of radiolabel to H^+,K^+-ATPase in the GI-fraction 2 hr after [^{3}H]omeprazole treatment

Treatment	nmol Radiolabel/mg 92 kDa
Omeprazole, 10 μmol/kg	2.0 ± 0.18 (4)
Ranititidine, 400 μmol/kg × 2 Omeprazole, 10 μmol/kg	1.1 ± 0.18 (4)
SCH 28080, 400 μmol/kg × 2 Omeprazole, 10 μmol/kg	0.02 ± 0.007 (4)

0.75 mCi/kg [^{3}H]omeprazole was given in a total dose of 10 μmol/kg s.c. Ranitidine and SCH 28080 were administered 15 min prior to and 60 min after omeprazole. The incorporated radiolabel was calculated after dichloromethane extraction of the sulfide derivative of omeprazole and after estimation of the proportion of 92 kDa protein in each preparation. Mean ± SEM. Numbers in parentheses indicate the number of experiments

the enzyme activity by about 50%, which gives a stoichiometry of 2.0 nmol radiolabel/mg 92 kDa. Reduction of acid secretion by the H_2-blocker ranitidine decreased the binding levels to 1.1 nmol/mg 92 kDa. When acid production was inhibited prior to omeprazole administration by compound SCH 28080, a known H^+,K^+-ATPase inhibitor [27], the amount of bound radiolabelled inhibitor to the 92 kDa protein was close to zero 0.02 nmol/mg 92 kDa. Thus, inhibition of acid production in the parietal cell was paralleled by a decreased binding of radiolabelled inhibitor, generated from omeprazole to the proton pump under *in vivo* conditions.

In order to estimate the active site concentration of the H^+,K^+-ATPase, the GI-fraction from vehicle treated rabbits was phosphorylated with [γ-^{32}P]ATP. The maximal phosphoenzyme level was 0.47 ± 0.03 nmol P/mg protein. The concentration of 92 kDa protein in the same preparation was estimated by SDS–PAGE to be 25 ± 2% of total protein content, which gives a maximal phosphoenzyme concentration of 1.9 nmol 32 P/mg 92 kDa. Thus, extrapolation of binding data from 10 μmol/kg omeprazole, which gave about 50% inhibition of enzyme activity, to 100% inhibition results in a binding level of 2.1 nmol of bound inhibitor per nmol phosphoenzyme at complete inhibition.

Effect of mercaptans on omeprazole-inhibited glands and H^+,K^+-ATPase

The inhibitory effect of omeprazole in the isolated gastric gland preparation was found to be prevented when 10 mM β-mercaptoethanol or DTT was present before omeprazole was added. In a concentration of 20 mM, the mercaptan could also reverse already established inhibition [3], However, in the isolated H^+,K^+-ATPase preparation, low concentrations (10 μM) of β-mercaptoethanol were found to protect the enzyme when added before omeprazole, whereas high concentrations, 0.1 M, were required to partially reverse already established inhibition [17]. The effect of β-mercaptoethanol on gastric glands and H^+,K^+-ATPase exposed to omeprazole *in vivo* is shown in Fig. 4. β-Mercaptoethanol, in a concentration of 10 mM decreased the db-cAMP-stimulated oxygen uptake *per se*. When the mercaptan was added to glands which had been exposed to omeprazole *in vivo*, it was possible to restore the

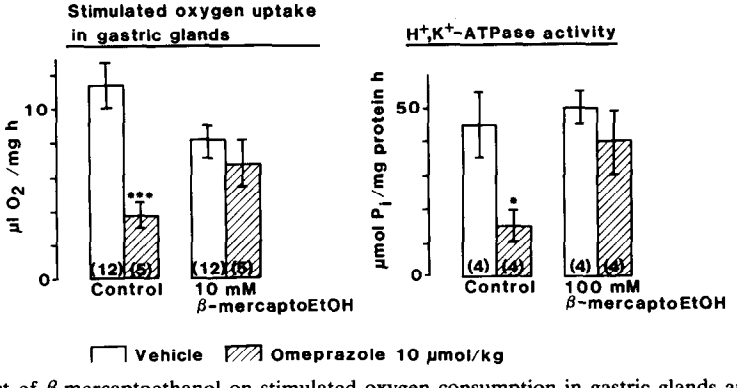

Fig. 4. Effect of β-mercaptoethanol on stimulated oxygen consumption in gastric glands and H^+,K^+-ATPase activity in vesicles (GI) isolated from rabbits treated with 10 μmol omeprazole/kg. Stimulated oxygen uptake is the difference between basal and maximally stimulated uptake in the presence of 1 mM db-cAMP. H^+,K^+-ATPase activity is the activity in the presence of Mg^{2+} plus K^+ minus the basal Mg^{2+}-stimulated ATPase activity. Means ± SEM. Numbers in parentheses indicate the number of experiments. *$P < 0.05$, ***$P < 0.001$ compared to vehicle.

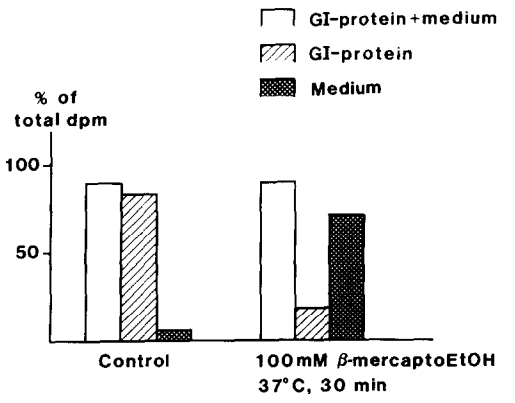

Fig. 5. Radioactivity distribution between the GI-protein and the medium. H^+,K^+-ATPase-containing vesicles were isolated from *in vivo* [^{3}H]omeprazole-treated rabbits. The GI-protein was incubated in the presence or absence of 100 mM β-mercaptoethanol. After 30 min at 37° the protein was spun down at 100,000 *g* for 2 hr, and the radioactivity in the supernatant and pellet determined. The supernatants were extracted with dichloromethane and analyzed by HPLC (see text). Pooled material from 8 rabbits.

oxygen uptake. Inhibition of the H^+,K^+-ATPase activity by about 50% with 10 μmol/kg omeprazole could be fully restored with 100 mM of the mercaptan, in agreement with earlier observations derived from *in vitro* effects of omeprazole [17].

The pooled GI-fractions obtained from eight omeprazole-treated rabbits were incubated with or without 100 mM β-mercaptoethanol. After 30 min of treatment at 37° the membrane protein was separated from medium by centrifugation at 100,000 *g* for 2 hr. The distribution of the radioactivity in the GI-membrane fraction protein and the supernatant medium following separation are shown in Fig. 5. Thus, the radioactivity was confined to the membrane fraction

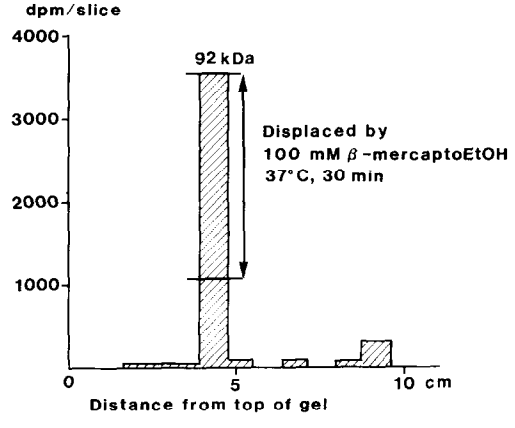

Fig. 6. Effect of β-mercaptoethanol treatment on 92 kDa-bound radiolabel. Microsomes isolated from [^{3}H]omeprazole-treated rabbits (10 μmol/kg) were incubated in the presence or absence of 100 mM β-mercaptoethanol at 37°. After 30 min the microsomes were spun down at 100,000 *g* for 2 hr, and the same amount of protein was loaded on the gel. After SDS–PAGE the gel was scanned for protein, sliced and counted in a liquid scintillation counter.

and displaced by β-mercaptoethanol. Dichloromethane extraction of the medium from these experiments and separation of the extracted compounds by HPLC showed ~90% of the radioactivity in the supernatant consisted of the sulfide derivative of omeprazole. Less than 4% of the radioactivity was omeprazole. Figure 6 shows the distribution of protein bound radioactivity in a microsomal fraction isolated from a rabbit treated with [^{3}H]omeprazole. The radioactivity in the 92 kDa protein band was displaced by incubation with 100 mM β-mercaptoethanol for 30 min, prior to SDS-PAGE. These data show that the reactivation of the H^+,K^+-ATPase activity observed following β-mercaptoethanol treatment of omeprazole inhibited enzyme is paralleled by a decreased binding of inhibitor to the 92 kDa protein and, furthermore, that the displaced inhibitor is the sulfide derivative of omeprazole.

DISCUSSION

Previous autoradiographic studies have revealed that the radioactivity following [^{3}H]omeprazole administration is bound to the secretory canaliculi and to the tubulovesicles of the parietal cell [28]. Furthermore, with the aid of immuno-histochemical techniques, it has previously been demonstrated that these cellular structures contain the H^+,K^+-ATPase [13]. The present study showed that the radioactivity in the gastric mucosa homogenate following [^{3}H]omeprazole administration was confined to peptides in the 92 kDa molecular weight region. Purification of the homogenate produced an enrichment of the 92 kDa protein, and since this protein was resolved from other protein on the SDS–PAGE, it could be demonstrated that it was the sole site of radioactivity. That this 92 kDa protein(s) holds the catalytic subunit of the gastric H^+,K^+-ATPase has been shown by phosphorylation experiments [23]. It can therefore be concluded that omeprazole, when given *in vivo*, selectively binds to the gastric H^+,K^+-ATPase.

It has been shown both in permeable and ion tight vesicle membrane H^+,K^+-ATPase preparations and in H^+,K^+-ATPase containing vesicles that there is a direct correlation between the degree of inhibition of H^+,K^+-ATPase activity and the amount of inhibitor bound to the preparation [16, 17, 29].

The binding data for omeprazole in the present study will give 2.1 mol inhibitor bound/mol phosphoenzyme at full inhibition of the H^+,K^+-ATPase activity. These data are in accordance with the *in vitro* binding studies of Lorentzon *et al.* [16] who found 2 mol inhibitor bound/mol phosphoenzyme in hog gastric vesicles, and Keeling *et al.* [29], who found 1.8 mol per phosphorylation site.

Omeprazole undergoes rearrangement into an inhibitor of the H^+,K^+-ATPase in acid and accordingly inhibits acid secretion. Thus, both the amount of protonated omeprazole that accumulates in the acidic compartments of the parietal cell, due to its weak base properties, and the rate at which the active inhibitor is formed from omeprazole will depend on the proton concentration in these compartments [3, 15]. In the present study, omeprazole admin-

istered *in vivo* inhibited both the H^+,K^+-ATPase activity, and the stimulated oxygen consumption in gastric glands by about 50% without affecting the concentration of the H^+,K^+-ATPase, pretreatment with ranitidine reduced acid secretion and largely prevented the inhibition of the H^+,K^+-ATPase and the stimulated oxygen consumption normally induced after omeprazole treatment. These experiments are in agreement with studies on gastric acid secretion in gastric fistula dogs, in which somatostatin or cimetidine infusion during omeprazole administration reduced and pentagastrin infusion increased the inhibitory effect of omeprazole [30]. The necessity of acid for the conversion of omeprazole was clearly shown by the fact that the compound failed to decrease the stimulated oxygen consumption, i.e. the H^+,K^+-ATPase activity, under conditions in which the secretory compartment of the parietal cell was neutralized by imidazole.

When acid secretion was decreased by ranitidine, the binding level of the inhibitor generated from omeprazole was reduced. However, when the acid secretion was inhibited by the H^+,K^+-ATPase inhibitor SCH 28080, whose enzyme-blocking mechanism differs from that of omeprazole [27], the inhibitor binding was close to zero. The difference in binding levels may reflect a difference in potency between the compounds. Thus, ranitidine, being less potent than SCH 28080, allows a higher degree of conversion of omeprazole and, subsequently more extensive binding to the H^+,K^+-ATPase. However, it cannot be excluded that omeprazole and SCH 28080 interact with the same binding site on the H^+,K^+-ATPase.

The inhibition of stimulated oxygen uptake and H^+,K^+-ATPase activity by omeprazole could be totally reversed by 10 and 100 mM β-mercaptoethanol, respectively. Reactivation of H^+,K^+-ATPase activity after omeprazole exposure *in vitro* has been demonstrated to release the reduced form of omeprazole, the sulfide derivative, into the medium. This results from the attack of the mercaptan on the disulfide bound between the inhibitor and the H^+,K^+-ATPase. The present study shows that the enzyme–inhibitor complex also after *in vivo* administration consists of a disulfide inhibitory complex, since β-mercaptoethanol released the reduced form of omeprazole into the medium resulting in reactivation of the H^+,K^+-ATPase.

The present data show that omeprazole specifically binds to the H^+,K^+-ATPase of the gastric mucosa. Both the amount of inhibitor bound and the degree of inhibition of acid secretion are dependent on the degree of activation of the parietal cell when omeprazole is administered.

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