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Inhibition of partially purified K^+/H^+-ATPase from guinea-pig isolated and enriched parietal cells by substituted benzimidazoles

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1 The cellular and subcellular distributions of adenosinetriphosphatases (ATPases) were examined in guinea-pig gastric mucosal cells. All cell types displayed Mg^{2+}-ATPase and bicarbonate (HCO_3^-)-stimulated ATPase activity. K^+-ATPase was located only in fractions derived from parietal cells.

2 Differential and density-gradient centrifugation of material prepared from parietal cells revealed that K^+-ATPase activity was located in a tubulo-vesicular membrane fraction. Enzyme activity was ten fold greater in this fraction than in a crude parietal cell homogenate.

3 The substituted benzimidazoles, omeprazole and picoprazole, inhibited K^+-ATPase (IC_{50} 1.8 ± 0.5 μmol l^{-1} and 3.1 ± 0.4 μmol l^{-1}, respectively). Detailed kinetic analysis indicated that these compounds were non-competitive and reversible inhibitors of the enzyme. In contrast cimetidine and verapamil were without effect on the enzyme.

4 The relevance of the inhibition of K^+-ATPase to the antisecretory activity of the benzimidazoles, in experimental animals and man, is discussed.

Introduction

The mechanism of HCl secretion in the gastric mucosa depends primarily on an adenosine triphosphate (ATP) hydrolyzing system (for review see: Forte, 1971). Since thiocyanate inhibits both acid secretion and a gastric bicarbonate-activated ATPase (Kasbekar & Durbin, 1965), this enzyme was initially regarded as the H^+ transporting enzyme. However, more detailed studies defined the HCO_3^--stimulated ATPase as a mitochondrial enzyme contaminating gastric microsomal fractions (Soumarmon *et al.*, 1974). Later a K^+-stimulated ATPase, localized in the lighter microsomal fraction of gastric mucosa (Ganser & Forte, 1973) was found to be related to H^+ secretion and to be capable of exchanging K^+ for H^+ (Sachs *et al.*, 1976). Recently, a new class of inhibitors of H^+ secretion, which are referred to as substituted benzimidazoles, has gained pharmacological interest. It was postulated that this class of compounds has a mechanism of action which is different from other known antisecretagogues (Sjöstrand *et al.*, 1978) and it has been shown that they interfere with the K^+/H^+-ATPase (Fellenius *et al.*, 1981). It was the purpose of the present study: (a) to describe the ATPase distribution of Mg^{2+}-dependent ATPases in different cell populations of guinea-pig gastric mucosa; (b) to elaborate a method for purification of K^+/H^+-ATPase from pietal cell enriched fractions and (c) to study the effect of the substituted benzimidazoles picoprazole and omeprazole, in comparison with other antisecretagogues, on K^+/H^+-ATPase and to elucidate the type and stability of their inhibitory action.

Methods

Cell preparation

The experiments were performed with guinea-pigs (260–340 g) of either sex. The procedure for the preparation of cell populations enriched with parietal cells or non-parietal cells was in principle that of Soll

(1978), with modifications, described in detail previously (Sewing *et al.*, 1983). Briefly, guinea-pig mucosal cells were isolated by collagenase and pronase digestion and were separated by zonal centrifugation with the Beckman elutriator system. The procedure resulted in a parietal cell-rich fraction consisting of approximately 75% parietal cells, and a non-parietal cell fraction consisting of 96% mucus and chief cells. The cells were tested for viability by the Trypan blue dye exclusion test and > 95% of cells in each preparation were found to be viable.

Purification of K^+/H^+-ATPase containing membranes

The different cell populations harvested from the elutriator were placed in a medium consisting of 0.25 mol l^{-1} sucrose containing 20 mmol l^{-1} Tris-HCl buffer (pH 7.4). Homogenization was performed by nitrogen cavitation in an Aristan Pressure Homogenizer (equilibrated for 40 min at 4°C; pressure : 3,000kPa). Subcellular fractions from a cell homogenate were obtained by differential centrifugation and defined as follows: 1,000 *g* for 8 min, cell debris and nuclear fraction; 20,000 *g* for 20 min, mitochondrial fraction; 100,000 *g* for 60 min, microsomes; the supernatant was the remaining fraction after the last centrifugation. For further purification crude microsomal fractions from parietal cells were layered over 22% and 35% (w/w) sucrose in 20 mmol l^{-1} Tris-HCl buffer (pH 7.4) and centrifuged in a Type 65 rotor at 50,000 r.p.m. (240,000 *g* at R_{max}) for 90 min. The appropriate fractions from differential and density gradient centrifugation were resuspended in 0.25 mol l^{-1} sucrose, 20 mmol l^{-1} Tris-HCl and stored at −80°C until use.

Enzyme assays

Basal Mg^{2+}-dependent ATPase activity was measured in 1.0 ml of the reaction medium consisting of 2 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} ATP, 5 μg protein and 50 mmol l^{-1} Tris-HCl buffer (pH 7.5). K^+-stimulated and HCO_3^--stimulated ATPase activity was defined as the activity in the basal medium in the presence of 10 mmol l^{-1} KCl or 20 mmol l^{-1} $NaHCO_3$, minus the activity in the basal medium. The 5'-AMPase assay was generally the same as that for Mg^{2+}-ATPase, the difference being that 2 mmol l^{-1} AMP was used as substrate and the assay contained 20 μg protein. The ATPase or AMPase reaction was started by the addition of the substrate, carried out at 37°C for 15 min and stopped with 1.0 ml ice-cold 20% trichloroacetic acid. Liberated inorganic phosphate from ATP or AMP was determined by the method of Sanui (1974). When the effects of different drugs and calcium on K^+-stimulated ATPase were examined, the compounds were preincubated with K^+/H^+-ATPase-containing membranes for 30 min at room temperature. Cytochrome c oxidase was measured by the method of Cooperstein & Lazarow (1951).

Washout experiments

Purified gastric vesicles (0.5 mg protein ml^{-1}) in 0.25 mol l^{-1} sucrose, 20 mmol l^{-1} Tris-HCl (pH 7.4) were preincubated with 5 μmol l^{-1} omeprazole at room temperature for 30 min. Subsequently 10 μl was transferred into the K^+/H^+-ATPase assay yielding a 100 fold dilution for omeprazole and protein.

Other determinations

Protein content was measured according to the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard. Results were statistically analysed using the *t* test for paired comparison.

Drugs

The following drugs were used: collagenase 125–230 u mg^{-1}, Na_2–ATP (Sigma, Munich), pronase E (70,000 p.u.k. g^{-1}) (Merck, Darmstadt), bovine serum albumin (Serva, Munich), verapamil (kindly supplied by Prof. Kretzschmar, Knoll AG, Ludwigshafen), cimetidine (kindly supplied by Dr M. E. Parsons, Smith Kline & French Ltd, Welwyn Garden City), omeprazole and picoprazole (kindly supplied by Dr E. Carlsson, Hässle, Mölndal, Sweden).

Results

Both cell populations contained Mg^{2+}-dependent and HCO_3^--stimulated ATPase, the activity being 5

Table 1 ATPase activity (μmol P_i mg^{-1} protein h^{-1}) in different cell types of guinea pig gastric mucosa

Cell type	*Basal ATPase*	*+ K^+*	*+ HCO_3^-*
Parietal Cells (75%)	69.0 ± 11.2	4.4* ± 1.2	31.5** ± 5.0
Non-parietal cells (< 4% PC)	13.9 ± 0.8	not detectable	9.2** ± 1.5

Values given are mean ± s.e.mean from 5 different enzyme preparations.
Asterisks denote differences from basal ATPase; $*P<0.05$, $**P<0.01$.

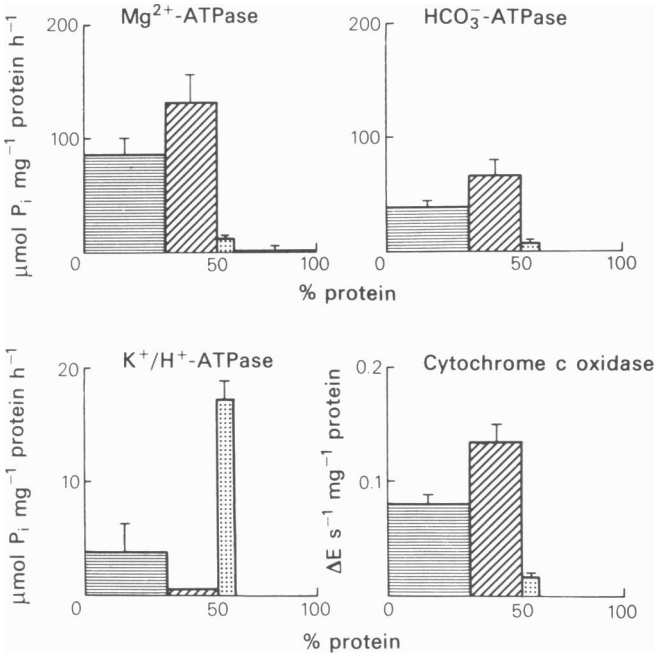

Figure 1 Distribution of enzyme activity (ordinates) and protein (abscissae) in subcellular fractions of guinea-pig parietal cell enriched populations. Values are means ± s.e.mean, $n = 5$. Horizontally hatched columns are cell debris (1,000g), diagonally hatched columns mitochondria (20,000g), stippled columns microsomes (100,000g) and solid column supernatant.

times and 3 times, respectively, higher in the parietal cell than in the non-parietal cell population. Only the parietal cell population contained measurable quantities of K^+-stimulated ATPase (Table 1). In parietal cells Mg^{2+}-dependent ATPase, HCO_3^--stimulated ATPase and the mitochondrial marker enzyme cytochrome c oxidase were distributed in identical proportions in the subcellular fractions (Figure 1), 47–49% in the cell debris, 48–52% in mitochondria and 1.7–2.0% in microsomes. Forty-eight % of the K^+/H^+-ATPase activity was found in the microsomal fraction. A large proportion of this enzyme was found in the low speed fraction, possibly due to whole cell contamination. In non-parietal cells, as in parietal cells, Mg^{2+}-dependent ATPase and HCO_3^--stimulated ATPase was distributed in a similar pattern to cytochrome c oxidase (Figure 2). The enzyme activities were about half of those found in the corresponding subcellular fractions of parietal cells. K^+/H^+-ATPase was not detectable in any subcellular fraction of non-parietal cells.

The crude microsomal fraction of parietal cells was purified by centrifugation on a discontinuous sucrose density gradient. This resolved the fraction into three bands (Table 2). Mg^{2+}-dependent ATPase and HCO_3^--stimulated ATPase accumulated in the denser region (35% sucrose) of the gradient together with cytochrome c oxidase activity. K^+/H^+-ATPase remained in the 22–35% interface region. Twenty-one % of HCO_3^--stimulated ATPase activity was recovered in this dense region and equal amounts of cytochrome c oxidase remained in this band. 5′-AMPase, an enzyme associated with plasma membranes, was also present in the interface band, but the concentration was higher in the upper 22% sucrose band. No ATPase activity was found in this light band and only trace amounts of cytochrome c oxidase were detectable. Thus, discontinuous density gradient centrifugation yielded a 10 fold purification of the K^+/H^+-ATPase activity which was virtually free of mitochondrial contamination. Fractions of the density gradient purified K^+/H^+-ATPase were used for subsequent studies.

Both substituted benzimidazoles, picoprazole and omeprazole, inhibited the K^+/H^+-ATPase activity in a concentration-dependent manner. The IC_{50} values were: omeprazole 1.8 ± 0.5 µmol l^{-1}, picoprazole 3.1 ± 0.4 µmol l^{-1}. The type of inhibition was studied in experiments in which the concentration of picoprazole was fixed at 1 µmol l^{-1} (the approximate IC_{50}

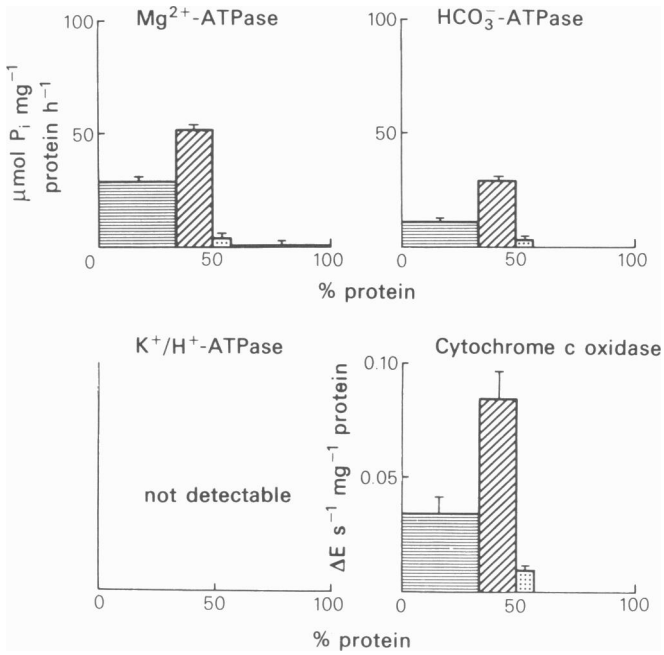

Figure 2 Distribution of enzyme activities (ordinates) and protein (abscissae) in subcellular fractions of guinea-pig cell populations depleted of parietal cells. Values are means ± s.e.mean, $n = 5$. Horizontally hatched columns are cell debris (1,000g), diagonally hatched columns mitochordria (20,000g), stippled columns microsomes (100,000g) and solid column supernatant.

of both compounds), and the KCl concentration was varied between 0.5–20 mmol l^{-1} (Figure 3). The K_a for K^+-stimulation was about 0.4 mmol l^{-1}. Neither of the inhibitors altered K_a, and a rise in K^+-concentration did not reverse the reduction of V_{max}. Transformation of the concentration-response curves according to Eadie (1952) and Hofstee (1952) indicated that the type of inhibition was non-competitive (Figure 4). In contrast, the classical inhibitor of Na^+/K^+-ATPase, ouabain (data not shown), the H_2-receptor antagonist cimetidine, and the calcium channel antagonist verapamil up to concentrations of 10^{-4} mol l^{-1} did not affect the enzyme (Figure 5). Tubulo-vesicular membranes (0.5 mg protein ml^{-1}) were exposed for 30 min to 5 µmol l^{-1} omeprazole. When 10 µl was transferred into the K^+-ATPase medium and subsequently assayed, the inhibition obtained was less than that in the presence

Table 2 Distribution of different enzymes of guinea-pig parietal cell microsomes separated on a 22,35% sucrose density gradient

Density gradient	*Mg^{2+}* [2 mmol l^{-1}]	*+ K^+* [10 mmol l^{-1}]	*+ HCO_3^-* [20 mmol l^{-1}]	*5'-AMPase*	*Cyt. c oxidase*
22% sucrose (Plasma membranes)		no enzyme activity detectable		1.05 ±0.05	0.48 ±0.12
22–35% interface (Tubulo-vesicles)	8.7 ± 1.9	40.4 ± 3.2	5.4 ± 1.0	0.46 ±0.04	0.72 ±0.24
35% sucrose (Mitochondria)	18.6 ± 4.5	9.3 ± 1.5	12.6 ± 3.3	0.12 ±0.02	1.80 ±0.42

Values given are mean + s.e.mean from 5 different enzyme preparations and the figures for ATPases and 5'-AMPase: µmol P_i mg^{-1} protein h^{-1}; cytochrome c oxidase: Δ E mg^{-1} protein min^{-1}.

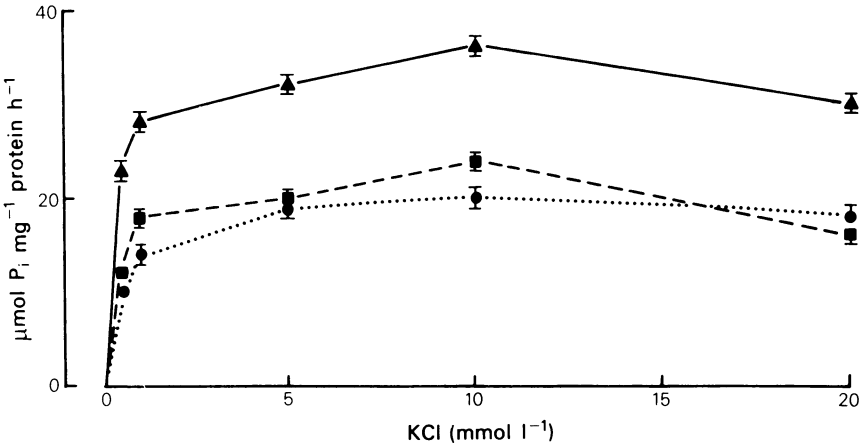

Figure 3 Activity of K^+/H^+-ATPase in the presence of various KCl concentrations. K^+/H^+-ATPase activity without inhibitors (▲), in the presence of omeprazole 1 µmol l^{-1} (●) or picoprazole 1 µmol l^{-1} (■). Picoprazole and omeprazole were dissolved in ethanol. Samples for K^+/H^+-ATPase assay contained 1% ethanol, which had no influence on K^+/H^+-ATPase activity. Values are mean ± s.e.mean, $n = 5$.

of 5 µmol l^{-1} in the normal K^+/H^+ -ATPase assay, indicating that the compound is readily and completely washed out (Table 3).

Discussion

Previous studies on K^+/H^+-ATPase were done with purified microsomal fractions obtained from whole gastric mucosa of frog (Ganser & Forte, 1973), rabbit (Ray & Forte, 1976) and pig (Forte *et al.*, 1975). The method presented here enabled the distribution of ATPase in different cell populations from guinea-pig gastric mucosa to be studied. Only the parietal cells contained K^+/H^+-ATPase activity. This is in accordance with results from experiments using indirect immunofluorescence staining techniques (Saccomani *et al.*, 1977). The HCO_3^--stimulated ATPase is pres-

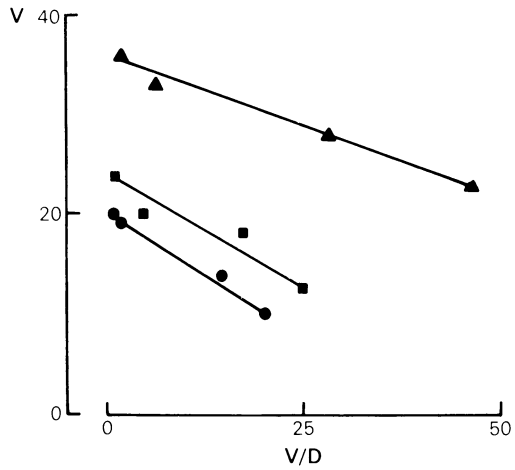

Figure 4 Eadie-Hofstee transformation of data from Figure 3. (▲) KCl alone, (●) KCl ± omeprazole 1 µmol l^{-1}, (■) KCl + picoprazole 1 µmol l^{-1}.

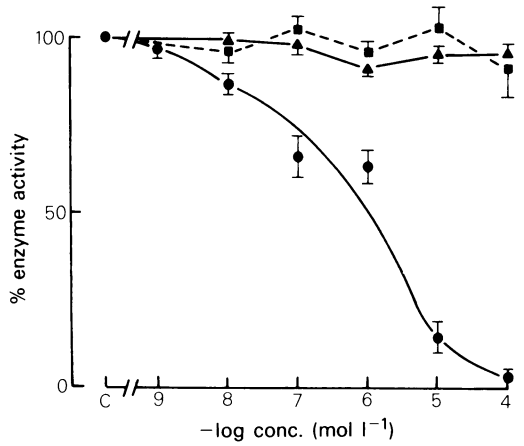

Figure 5 Effect of verapamil (■), cimetidine (▲) and omeprazole (●) on K^+/H^+-ATPase activity purified from guinea-pig parietal cells. Values are means ± s.e.mean, $n = 5$.

Table 3 K^+/H^+-ATPase activity in the presence of omeprazole under different preincubation conditions

		Omeprazole	
Control	*0.05 μmol l^{-1}* normal K^+/H^+-ATPase 5 μg ml^{-1} protein	*5 μmol l^{-1}* assay	*5 μmol l^{-1}* modified assay
100%	70.5 ± 3.4%	28.0 ± 3.8%	87.6 ± 5.8%

Values given are mean ± s.e.mean from 3 different enzyme preparations

*Incubation of 500 μg ml^{-1} purified tubulovesicular membranes with 5 μmol l^{-1} omeprazole for 30 min. This incubate was subsequently assayed in a 100 fold dilution for K^+/H^+-ATPase, in the standard medium which then contained 5 μg ml^{-1} protein and corresponded to 0.05 μmol l^{-1} omeprazole.

ent in both cell types. It was assumed that the HCO_3^--stimulated ATPase might be involved in H^+ and/or Cl^- transport in gastric membranes (Wiebelhaus *et al.*, 1971). Localization of HCO_3^--stimulated ATPase in non-pariental cells and cosedimentation with cytochrome c oxidase in crude and purified microsomal fractions from parietal cells indicated that this enzyme is of mitochondrial origin (see also Soumarmon *et al.*, 1974) and might have no transport function in gastric mucosal membranes. The pH optimum of 7.5 for obtaining K^+/H^+-ATPase activity from guinea-pig parietal cells is quite similar to that in frog (Ganser & Forte, 1973) and pig (Forte *et al.*, 1975). Substituted benzimidazoles have been shown to be potent inhibitors of gastric acid secretion *in vivo* (Olbe *et al.*,1979). The site of action of these compounds is the K^+/H^+-ATPase (Wallmark *et al.*, 1983). In our own experiments any inhibitory effect of picoprazole and omeprazole up to 10^{-4} mol l^{-1} on gastric Mg^{2+}-dependent ATPase activity was excluded (data not shown). Wallmark *et al.* (1983) have demonstrated that picoprazole does not inhibit renal Na^+/K^+-ATPase. These data underline the selectivity of substituted benzimidazoles. The inhibition of K^+/H^+-ATPase seen in this study is clearly not competitive but reversible, which is in agreement with the results from *in vitro* studies (Sewing *et al.*, 1983) where picoprazole and omeprazole were found to be non-competitive and reversible inhibitors of the uptake and accumulation of [^{14}C]-aminopyrine in guinea-pig isolated and enriched parietal cells.

These *in vitro* results partially agree with those from *in vivo* studies in man, where a single dose of omeprazole (Lind *et al.*, 1983) and picoprazole (Olbe *et al.*, 1982) causes a long acting (> 24 h), but progressively reversible inhibition of gastric acid secretion. Discrepancies in the time course of reversibility need to be solved. Sewing & Hannemann (1983) have shown, that in guinea-pig isolated and enriched parietal cells the calcium channel antagonists verapamil and gallopamil inhibit the accumulation of [^{14}C]-aminopyrine stimulated by histamine, dibutyryl-cyclic AMP and K^+. They concluded that the mechanism of action of this inhibition must be close to that for K^+/H^+-ATPase. In the purified K^+/H^+-ATPase enzyme system no inhibitory effect of calcium channel antagonists was found. Furthermore the suggestion that the inhibitory effects of verapamil and gallopamil have to be attributed to their action as calcium channel antagonists can be excluded since calcium itself acts as an inhibitor of K^+/H^+-ATPase (Figure 6).

Thus, we have shown that substituted benzimidazoles which inhibit H^+ secretion *in vivo* and *in vitro* are inhibitors of the H^+ transporting enzyme system, K^+/H^+-ATPase. Calcium channel antagonist possess a mechanism for inhibition of H^+ secretion different from that demonstrated for substituted benzimidazoles.

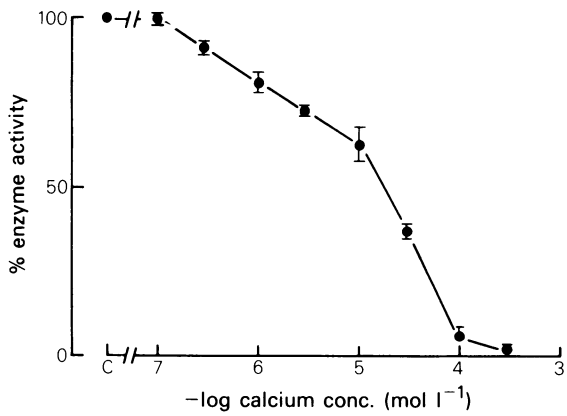

Figure 6 Effect of calcium on K^+/H^+-ATPase activity purified from guinea-pig parietal cells. Values are means ± s.e.mean, $n = 5$.

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