INHIBITION OF GASTRIC (H$^+$ + K$^+$)-ATPase BY THE SUBSTITUTED BENZIMIDAZOLE, PICOPRAZOLE

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The substituted benzimidazole, picoprazole, inhibited the gastric (H$^+$ + K$^+$)-ATPase in a concentration- and time-dependent manner. Half-maximal inhibition of the (H$^+$ + K$^+$)-ATPase activity was obtained at about $2 \cdot 10^{-6}$ M under standard conditions. In addition to the inhibition of ATPase activity, parallel inhibition of phosphoenzyme formation and the proton transport activity were achieved. Radiolabelled picoprazole was found to bind to 100 kDa peptide; this peptide was shown by phosphorylation experiments to contain the catalytic centre of the (H$^+$ + K$^+$)-ATPase. Studies on the (Na$^+$ + K$^+$)-ATPase indicated that this enzyme was unaffected by picoprazole. From the data presented and from other pharmacological studies, it is proposed that this compound inhibits acid secretion at the level of the parietal cell by its ability to inhibit the gastric proton pump, the (H$^+$ + K$^+$)-ATPase.

Introduction

The substituted benzimidazoles inhibit gastric acid secretion both in vivo and in vitro [1–3]. Furthermore, in isolated gastric gland preparations [4] these inhibitors have been shown to inhibit acid production monitored as accumulation of a weak base, aminopyrine [5,6]. In this glandular system and in isolated guinea pig mucosa, the

substituted benzimidazoles inhibited not only basal secretion, but also secretion induced by the secretatogues histamine and dibutyryl-cAMP. Inhibition was found to be noncompetitive [1,6]. Furthermore, aminopyrine accumulation stimulated by high K$^+$ concentration was also antagonized by the compounds [6]. Based on these data, a mechanism of action peripheral to cell receptor sites and probably located within the parietal cell seemed likely [5,6]. During recent years, an (H$^+$ + K$^+$)-ATPase has been purified from the gastric mucosa of various species [7–9] and has been localized to the secretory surface of the parietal cell [10]. The addition of ATP to isolated vesicles containing the enzyme results in H$^+$ uptake into the intravesicular space in exchange for intravesicular K$^+$ [11,12]. The transport process is apparently electroneutral [13]. Entry of KCl into the vesicle is rate-limiting for proton translocation. The (H$^+$ + K$^+$)-ATPase thus plays an important role in H$^+$ secretion across the membranes of the secretory canaliculus of the

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Abbreviations: (H$^+$ + K$^+$)-ATPase, Proton plus potassium-stimulated ATP phosphohydrolase; (Na$^+$ K$^+$)-ATPase, Sodium plus potassium ion transport ATP phosphohydrolase (EC 3.6.1.3); Pipes, piperazine-*N,N'*-bis-(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; dibutyryl-cAMP, N^6,O^{21}-dibutyryladenosine 3':5'-cyclic monophosphate.

parietal cell [13,14]. It seemed possible, therefore, that the substituted benzimidazoles affect acid secretion by acting directly on the $(H^+ + K^+)$-ATPase. This paper presents data for one substituted benzimidazole, picoprazole, and describes its inhibitory action on the $(H^+ + K^+)$-ATPase in more detail.

Material and Methods

Materials. Gastric $(H^+ + K^+)$-ATPase was prepared from hog stomachs as described by Saccomani et al. [15]. In brief, membrane vesicles containing the $(H^+ + K^+)$-ATPase were prepared by differential and zonal density gradient centrifugation. The microsomal fraction was centrifuged through a 0.25 M sucrose/0.25 M sucrose-8% (w/w) Ficoll ® step gradient in a Kontron TZT 48 zonal rotor (Basel, Switzerland) for 4 h at 47000 rpm. The material retained at the interface was used for the experiments.

For transport experiments, the vesicles were equilibrated in a mixture of 150 mM KCl, 2 mM MgCl₂ and 2 mM Pipes adjusted to pH 6.7 with Tris base. After equilibration, the vesicles were stored at 4°C until use on the same day. All subsequent mixing of the sample solutions was done near isotonicity of the equilibrated sample. For experiments in which free access of ions to either face of the enzyme was required, the vesicle preparation was lyophilized and stored at -80°C until resuspension. The $(Na^+ + K^+)$-ATPase preparation was obtained as described by Jørgensen [16].

Reagents. $[\gamma\text{-}^{32}P]$ATP was obtained from the Radiochemical Centre, Amersham, U.K. ATP was obtained from Sigma, U.S.A. Acridine orange was obtained from Merck, Darmstadt, F.R.G. Picoprazole, (H 149/94), methyl 6-methyl-2-[[(3-methyl-2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole-5-carboxylate and $[^{14}C]$picoprazole were synthetized by the Chemical Department, AB Hässle, Mölndal, Sweden (Fig. 1). Picoprazole was dissolved in methanol. The final methanol concentration did not exceed 1% which alone had no effect on any of the activities measured.

Enzyme assays. K^+-stimulated ATPase activity of the $(H^+ + K^+)$-ATPase is designated K^+-ATPase activity. $(Na^+ + K^+)$-stimulated ATPase
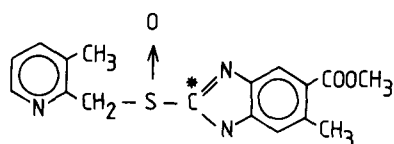
Picoprazole (H 149/94)

Fig. 1. Structural formula of picoprazole. * indicates the position of the ^{14}C label.

activity of the $(Na^+ + K^+)$-ATPase is designated $(Na^+ + K^+)$-ATPase activity. K^+-stimulated *p*-nitrophenylphosphatase activity of the $(H^+ + K^+)$-ATPase and $(Na^+ + K^+)$-ATPase are designated *p*-nitrophenylphosphatase $((H^+ + K^+)$-ATPase) and *p*-nitrophenylphosphatase $((Na^+ + K^+)$-ATPase), respectively. It should be noted that *p*-nitrophenylphosphatase $((Na^+ + K^+)$-ATPase) was assayed in the absence of Na^+.

ATPase activity. This was measured by the release of inorganic phosphate, which was assayed according to the method of Fiske and SubbaRow [17].

K^+-ATPase activity. The assay medium consisted of 2 mM MgCl₂, 75 mM Pipes-Tris buffer (pH 7.4), 2 mM Na₂ATP, with or without 10 mM KCl in a total volume of 2 ml. The assay was incubated for 15 min at 37°C. The reaction was then stopped by the addition of 1 ml 10% trichloroacetic acid. A maximum of 10% of the substrate was hydrolyzed over the incubation period, giving linear reaction rates. In lyophilized material and in the absence of K^+, reaction rates were about 10 μmol P_i/mg protein per h. In the presence of both Mg^{2+} and K^+, reaction rates were about 100 μmol P_i/mg protein per h. For all inhibition experiments, reaction rates in the presence of Mg^{2+} and K^+ but in the absence of inhibitor were set to 100%.

$(Na^+ + K^+)$-ATPase activity was assayed in a medium consisting of 5 mM Pipes-Tris buffer (pH 7.4), 2 mM MgCl₂, 2 mM Na₂ATP with or without 10 mM KCl plus 100 mM NaCl in a total volume of 2 ml, and incubated for 3 min at 37°C. The reaction was stopped by the addition of 1 ml 10% trichloroacetic acid. In the absence of K^+ and Na^+, reaction rates were about 20 μmol P_i/mg protein per h. In the presence of Na^+ and K^+,

reaction rates were about 400 μmol P_i/mg protein per h. For the inhibition experiments reaction rates in the presence of Mg^{2+}, Na^+ and K^+ but in the absence of inhibitor were set to 100%.

p-Nitrophenylphosphatase activity. This was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate [18].

p-Nitrophenylphosphatase $((H^+ + K^+)$-ATPase) activity was assayed in a medium consisting of 20 mM imidazole-acetic acid buffer (pH 7.5), 3 mM $MgCl_2$, 3 mM Tris-*p*-nitrophenyl phosphate with or without 10 mM KCl in a total volume of 0.5 ml. The assay was incubated for 3 min at 37°C. The reaction was stopped by the addition of 25 μl 50% trichloroacetic acid. In the absence of K^+, reaction rates were about 4 μmol *p*-nitrophenol/mg protein per h. In the presence of both Mg^+ and K^+, reaction rates were about 75 μmol *p*-nitrophenol/mg protein per h. This reaction rate was given a value of 100%.

p-Nitrophenylphosphatase $((Na^+ + K^+)$-ATPase) activity. This was assayed as described for *p*-nitrophenylphosphatase $(H^+ + K^+$-ATPase). In the absence of K^+, reaction rates were about 7 μmol *p*-nitrophenol/mg protein per h. In the presence of both Mg^+ and K^+, reaction rates were about 160 μmol *p*-nitrophenol/mg protein per h, which was taken as the 100% value.

Phosphorylation of the gastric membranes. $(H^+ + K^+)$-ATPase was preincubated for 30 min at 37°C. The medium contained 10^{-6}, 10^{-5} and 10^{-4} M picoprazole, 40 mM Tris-HCl (pH 7.4) and 20 μg of membrane protein. The phosphorylation reaction was then measured by the addition of 2 mM $MgCl_2$ and 5 μM [γ-^{32}P]ATP for 15 s, at 22°C. The reaction was stopped by the addition of 1 ml ice-cold 10% $HClO_4$ containing 5 mM ATP and 40 mM Na_2PO_4. The precipitated protein was collected on a 4 μm Millipore® type SSWP filter. The filter was washed with 70 ml of 5% $HClO_4$ containing 10 mM Na_2PO_4. The Cerenkov radiation of the filter was measured in a scintillation counter.

SDS-polyacrylamide gel-electrophoresis. Membranes undergoing SDS-polyacrylamide gel electrophoresis were either first phosphorylated by means of [γ-^{32}P]ATP or incubated with [^{14}C]picoprazole. The phosphorylation procedure was carried out in a medium consisting of 200 μg/ml

gastric membranes, 2 mM $MgCl_2$ and 5 μM [γ-^{32}P]ATP in the presence or absence of 0.1 M KCl. The final volume was 200 μl. The phosphorylation reaction was stopped with 1% SDS to solubilize the membranes. About 40 μg protein was applied to a density gradient polyacrylamide gel with a starting density of 4% and a final density of 30%. Gels were run in a 0.1 M Tris-borate buffer (pH 6.0) in 0.1% SDS for 2.5 h. Protein was stained with Coomassie blue and scanned in a gel densitometer at 550 nm. Gels run with ^{32}P-labelled membranes were sectioned manually immediately after electrophoresis and each slice was counted.

For binding studies with ^{14}C-picoprazole, gastric membranes (10 μg/ml) were incubated at 37°C in 2 mM Pipes-Tris (pH 7.4) with 10^{-4} M [^{14}C]picoprazole in a total volume of 20 ml for 2 h and then centrifuged for 1 h at $100\,000 \times g$. The resulting pellet was resuspended in 1% SDS and about 40 μg protein applied to each gel. Gels were then treated as described for the phosphorylation experiments.

Proton transport experiments. H^+ transport rates and the extent of pH gradient formation were monitored in an Aminco DW2 spectrophotometer (Americal Instrument Company, U.S.A.) in the dual beam model set at 490 and 535 nm [19]. Vesicles were preequilibrated at a concentration of 40 μg/ml in 2 mM Pipes-Tris, 150 mM KCl and 2 mM $MgCl_2$ at pH 6.7 for 3 h at 22°C. The equilibrated sample was mixed with 10^{-4} M picoprazole and after various incubation times, a sample was withdrawn and placed in a cuvette. 20 μM Acridine orange was added and a baseline recorded, after which 0.6 mM Na_2ATP was added to the incubation and the proton transport rate assayed.

Protein determination. Protein was measured by the method of Lowry et al. [20].

Results

Kinetics of the inactivation of K^+-ATPase activity

The time course for the inhibition of K^+-ATPase activity is shown in Fig. 2A. The inhibition was found to be both time- and concentration-dependent, since the reduction in enzyme activity increased progressively with increased
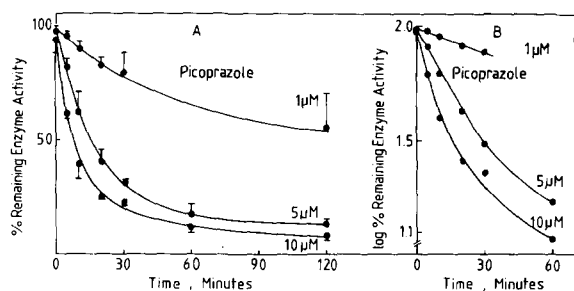
Fig. 2. A. Time course for inactivation of $(H^+ + K^+)$-ATPase. Membrane suspensions (10 μg of protein/ml) were incubated in a medium consisting of 5 mM Pipes-Tris (pH 7.4) at concentrations of picoprazole as indicated. After incubation times, indicated at the abscissa, samples were assayed for K^+-ATPase activity. The K^+-stimulated ATPase activity in the absence of picoprazole was given a value of 100%. Each time point is the mean of four separate determinations. The bars indicate the standard error of the mean (S.E.) for each time point. B. Semilogarithmic point of the data in Fig. 2A.

incubation time and inhibitor concentrations. When the data from Fig. 2A were replotted semi-logarithmically (Fig. 2B), the reaction of pico-prazole with the enzyme was linear over the first 75% of the total reaction. However, for the remaining 25% a deviation from a straight-line relationship between the logarithm of the remaining enzyme activity and time was obtained. From the linear portions of the curves in Fig. 2B, half-lives,
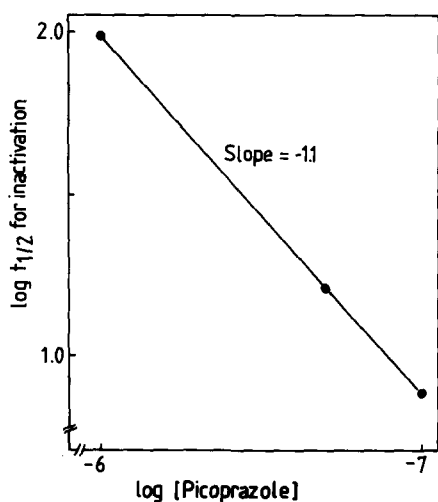

Fig. 3. Double logarithmic plot of the $t_{1/2}$ of inactivation against picoprazole concentrations. The $t_{1/2}$ was calculated from the linear part of Fig. 2B.
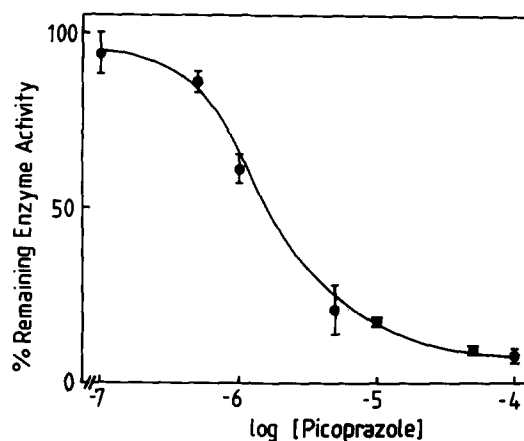

Fig. 4. Concentration dependence for inhibition of $(H^+ + K^+)$-ATPase by picoprazole. Gastric membranes (10 μg of protein/ml) were incubated for 30 min in 5 mM Pipes-Tris (pH 7.4) with picoprazole at concentrations indicated. After incubation, samples were assayed for K^+-ATPase activity. The enzyme activity in the absence of picoprazole was given a value of 100%. Each time point is the mean of two separate determinations.

$t_{1/2}$, were calculated for the inhibition reaction at each inhibitor concentration. These values were then plotted in a double logarithmic plot against the picoprazole concentrations (Fig. 3). This plot was linear with a slope close to one. This allows the conclusion that inhibition follows pseudo-first-order kinetics [21], at least over the first 75%. The concentration-effect relationship is shown in Fig. 4. Increasing concentrations of picoprazole resulted in progressive inhibition of the K^+-ATPase activity. Half-maximal inhibition occurred at about $2 \cdot 10^{-6}$ M.

pH dependence of inhibition

From the results in Fig. 5, it was found that enzyme inhibition increased progressively as the proton concentration was increased. Despite the greater inhibition at lower pH, however, a pH of 7.4 was taken as standard for most subsequent experiments, since this was the optimal pH for the enzyme activity [22].

Binding of picoprazole to $(H^+ + K^+)$-ATPase

The binding of picoprazole to $(H^+ + K^+)$-ATPase was analyzed by SDS-polyacrylamide gel electrophoresis. A major peak was evident at about
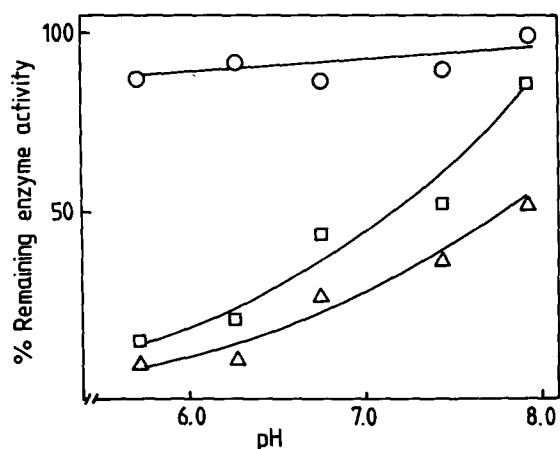
Fig. 5. Effects of pH on the inactivation of $(H^+ + K^+)$-ATPase. Membrane suspension (10 μg of protein/ml) was incubated in 2 mM Pipes-Tris buffer at pH 5.7, 6.3, 6.8, 7.4 and 7.8 for 30 min, with picoprazole at concentrations of 1 μM ($\bigcirc$———$\bigcirc$); 5 μM ($\square$———$\square$) and 10 μM ($\triangle$———$\triangle$). After incubation, samples were adjusted to pH 7.4 by the addition of 75 mM Pipes-Tris buffer and K^+-ATPase activity was assayed. K^+-ATPase activity in the absence of picoprazole was given a value of 100%.

100 kDa (Fig. 6B). This peak was also found to contain large amounts of radioactivity. However, large quantities of radioactivity that did not correspond to any protein peak were retained at the end of the gel. This was probably due to binding of [^{14}C]picoprazole to lipid components, as the low molecular weight region of the gel stained with Sudan black, a marker for lipids (data not shown). In order to identify the protein containing the catalytic peptide of the $(H^+ + K^+)$-ATPase, we studied the incorporation of ^{32}P from AT^{33}P [23]. The distribution of ^{32}P after phosphorylation of the membrane preparation is shown in Fig. 6A. The 100 kDa peptide was found to contain the ^{32}P-label (open bars) and, furthermore, when the experiment was performed in the presence of K^+, the label of the 100 kDa peptide was lost (shaded bars). It can therefore be concluded that both the ^{14}C- and ^{32}P-labels are confined to the same peptide.
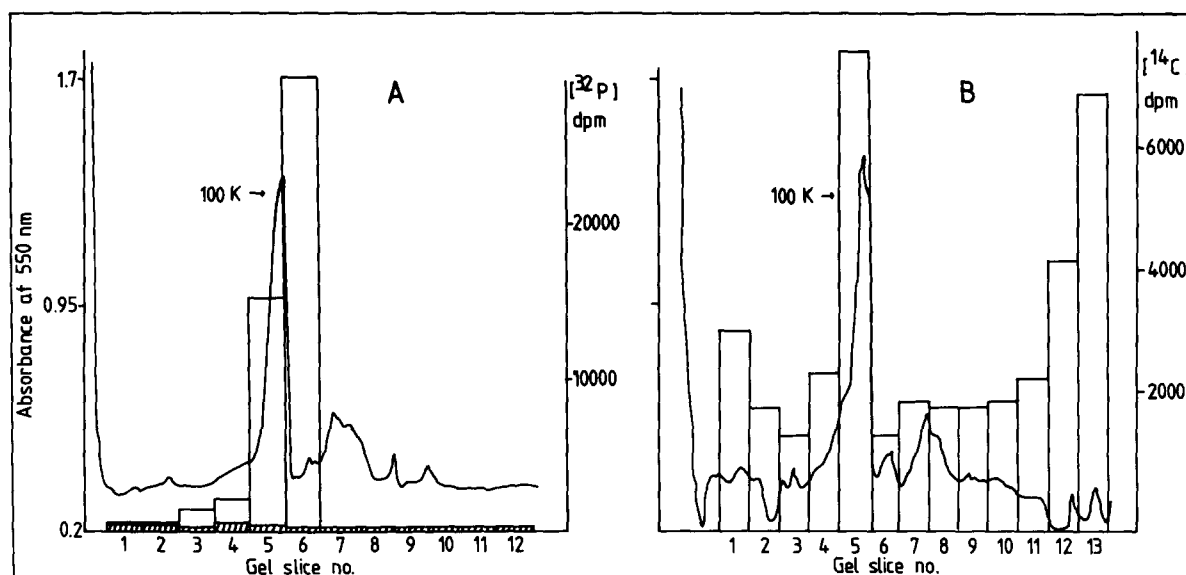

Fig. 6. A. Binding of [γ-^{32}P]ATP to $(H^+ + K^+)$-ATPase. Gastric membranes (200 μg of protein/ml) were incubated and phosphorylated as described in Methods. Open bars show the distribution of the ^{32}P-label in the presence of MgAT^{32}P and shaded bars in the presence of MgAT^{32}P plus 100 mM KCl. Gels were prepared and run as detailed in the Methods section. 100 K indicates the position of the 100 kDa peptide. B. Binding of [^{14}C]picoprazole to $(H^+ + K^+)$-ATPase. Gastric membranes (10 μg of protein/ml) were incubated with 10^{-4} M [^{14}C]picoprazole, after which the incubate was centrifuged at 100 000 $\times$ g for 60 min and solubilized in 1% sodium dodecyl sulphate. Open bars show the distribution of [^{14}C]picoprazole. 100 K indicates the position of the 100 kDa peptide.

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