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BY 1023/SK&F 96022: BIOCHEMISTRY OF A NOVEL $(H^+ + K^+)$-ATPase INHIBITOR

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Abstract—The mechanism by which the substituted benzimidazole sulphoxide BY 1023/SK&F 96022 inhibited the $(H^+ + K^+)$-ATPase, the enzyme responsible for hydrogen ion secretion in the stomach, was studied in a variety of *in vitro* preparations. In gastric preparations that were capable of active hydrogen ion transport with consequent lumenal acidification, BY 1023/SK&F 96022 inhibited with high potency and in a time-dependent manner consistent with the acid-induced conversion of the parent benzimidazole sulphoxide to a covalent inhibitor (cyclic sulphenamide). The following IC_{50} values were obtained for the inhibition of aminopyrine accumulation: intact gastric glands stimulated with 1 mM dibutyryl cAMP, 1.0 μM; permeabilized gastric glands stimulated with 5 mM ATP, 0.42 μM; intact gastric vesicles stimulated with 150 mM KCl, 9 μM valinomycin and 2 mM MgATP, 3.5 μM. In a preparation that could not generate pH gradients, lyophilized gastric vesicles at pH 7.4, BY 1023/SK&F 96022 inhibited K^+-stimulated ATPase activity with relatively low potency, 70 μM, indicating its good chemical stability at neutral pH. As assessed by ATPase inhibition, this stability was three times greater than that of omeprazole. Inhibition by BY 1023/SK&F 96022 was not reversed by dilution in either permeabilized gastric glands or intact gastric vesicles. Inhibition could, however, be completely reversed by subsequent incubation with 20 mM β-mercaptoethanol (intact gastric glands) or 100 mM dithiothreitol (intact gastric vesicles) suggesting a disulphide link between inhibitor and enzyme. The concentration of glutathione needed to protect against inhibition by BY 1023/SK&F 96022 was 10,000 times higher in intact, compared with lyophilized, gastric vesicles indicating an interaction with the lumenal (extracellular) face of the $(H^+ + K^+)$-ATPase. BY 1023/SK&F 96022 and omeprazole were also found to inhibit acidification in purified kidney lysosomes with IC_{50} values of 194 and 75 μM, respectively. Protection by 10 μM glutathione suggested that this did not result from intralysosomal activation of these inhibitors. Thus, BY 1023/SK&F 96022 has the combined properties of good chemical stability at neutral pH and effective conversion to the cyclic sulphenamide at acidic pH. In this way the activation to the cyclic sulphenamide may be optimally restricted to the parietal cell canaliculus.

The benzimidazole sulphoxides, such as omeprazole, are chemically transformed (activated), in a pH dependent manner, into cyclic sulphenamides (Fig. 1) which are then able to react covalently with thiol groups [1–3]. Such a reaction, with cysteine residues on the gastric $(H^+ + K^+)$-ATPase, is believed to be the mechanism by which these compounds inhibit acid secretion [4–6]. The acid-enhanced activation of inhibitor *in vivo* is expected to occur predominantly in the acidic canaliculus of the stimulated parietal cell. Since the cyclic sulphenamide is cationic, it may not easily cross the apical membrane, and should interact preferentially with cysteine groups on the lumenal surface of the $(H^+ + K^+)$-ATPase.

In order to study the biochemistry of this inhibitory process *in vitro*, it is important to mimic physiological conditions, particularly with regard to the acidic pH on the lumenal face of the $(H^+ + K^+)$-ATPase. The mechanism by which omeprazole inhibits the $(H^+ + K^+)$-ATPase has been shown to vary depending upon the presence or absence of lumenal acid [7, 8].

BY 1023/SK&F 96022 (Fig. 1) is a benzimidazole sulphoxide selected for its combined properties of good chemical stability at neutral pH and effective conversion to the cyclic sulphenamide at acidic pH [9]. In this way the activation to the cyclic sulphenamide may be optimally restricted to the parietal cell canaliculus. In this paper we show that, under conditions *in vitro* that mimic those *in vivo*, BY 1023/SK&F 96022 is an irreversible inhibitor that covalently reacts with thiol groups on the $(H^+ + K^+)$-ATPase that are accessible from the lumenal surface of this ion pump.

MATERIALS AND METHODS

Preparation of intact gastric glands. Intact gastric glands were prepared from anaesthetized New Zealand rabbits by high pressure perfusion of the stomach followed by collagenase digestion of pieces of fundic mucosa as previously described [10].

Aminopyrine accumulation in intact gastric glands. Intact gastric glands were suspended in Krebs–Henseleit solution containing 2 mg/mL rabbit serum albumin, 2 mg/mL glucose and 0.125 μM [dimethylamine-^{14}C]aminopyrine, pH 7.4. Glands were stimulated with 1 mM dibutyryl cAMP or 60 mM KCl. BY 1023/SK&F 96022 was included at the start of the incubation. After 30 min (or the period of time indicated) at 37°, the glands were sedimented by rapid

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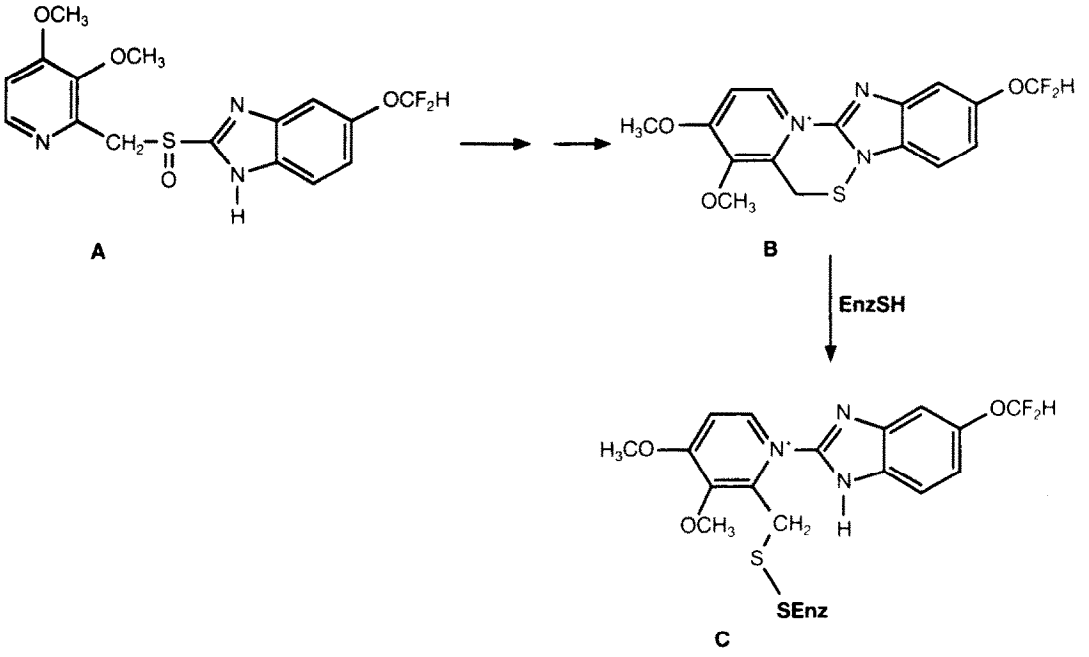

Fig. 1. Structure of BY 1023/SK&F 96022 (A). The activation to the cyclic sulphenamide (B) and subsequent reaction with a cysteine residue on the $(H^+ + K^+)$-ATPase (C) are also shown.

centrifugation and the aminopyrine accumulation ratio was determined from the distribution of radioactivity between the gland and supernatant as previously described [11].

Aminopyrine accumulation in permeabilized gastric glands. Intact gastric glands were incubated for 5 min at 37° in buffer containing 50 mM Hepes,* 6 mM phosphate, 1.2 mM $MgSO_4$ and 60 mM KCl, pH 7.4. They were subsequently permeabilized by the addition of 20 μg/mL digitonin for a further 5 min after which they were diluted 10-fold into the same buffer containing 5 mM ATP and 0.125 μM [dimethylamine-^{14}C]aminopyrine. The aminopyrine accumulation ratio was measured after 30 min as described above.

Preparation of gastric vesicles. Intact and lyophilized gastric vesicles containing the $(H^+ + K^+)$-ATPase were prepared from pig gastric mucosa by differential and density gradient centrifugation as described previously [6, 8]. Vesicles were stored at −70° for up to 3 months. Dilution of enzyme prior to use was either in 0.25 mM sucrose for intact vesicles or 10 mM PIPES/Tris pH 7.0 for lyophilized vesicles. In order to stimulate ATPase activity, K^+ must have free access to the lumenal (intravesicular) face of the $(H^+ + K^+)$-ATPase, thus the proportion of intact vesicles in each preparation could be determined by the dependence of the K^+-stimulated ATPase activity on the H^+/K^+ ionophore nigericin. With intact vesicles, 84% (N = 2 preparations) of activity required ionophore. In lyophilized vesicle preparations only 12% (N = 2 preparations) of total K^+-stimulated ATPase activity was dependent on the presence of nigericin, thus demonstrating the increased permeability of the vesicle membrane.

Aminopyrine accumulation in vesicles. Aminopyrine accumulation ratio in intact gastric vesicles (25 μg protein/mL) was determined in the presence of 10 mM PIPES/Tris buffer, pH 7.0, 2 mM MgATP, 150 mM KCl, 9 μM valinomycin, 3 μM [dimethylamine-^{14}C]aminopyrine (100–120 mCi/mmole, Amersham) and 0.1 mg/mL bovine serum albumin. After incubation at room temperature for 30 min, 3 mL of ice-cold washing solution (150 mM KCl containing 10 mM PIPES/Tris buffer, pH 7.0) was added, and the vesicles harvested by rapid filtration on Whatman GF/B filters. Each filter was washed with two further 3-mL aliquots of ice-cold washing solution, after which the radioactivity that had accumulated into the vesicles was counted using 10 mL Picofluor-15 scintillant. In order to calculate an aminopyrine accumulation ratio (the ratio of the aminopyrine concentration inside the vesicle to that outside), it was assumed that the intravesicular volume was 2 μL/mg protein [12].

ATPase assays. K^+-stimulated ATPase activity of gastric vesicles (0–6 μg protein/assay) was determined at 37° by the formation of inorganic phosphate [13] from 2 mM ATP in the presence of 2 mM $MgSO_4$ and 10 mM PIPES/Tris buffer, pH 7.0. Total assay volume was 1 mL. ATPase activity was stimulated with 1–10 mM KCl (lyophilized vesicles) or 10 mM KCl and 6 μM nigericin (intact vesicles, osmotically maintained with 280 mM sucrose). Where acidification of the vesicle lumen was required (intact

* Abbreviations used: AO, acridine orange; PIPES, piperazine-*N*,*N*′-bis-2-ethane-sulphonic acid; Tris, Tris(hydroxymethyl)-methylamine; MOPS, 3-[*N*-morpholino]propanesulphonic acid; CDTA, trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxethylpiperazine-*N*′-2-ethanesulphonic acid.

vesicles), ATPase activity was stimulated with 150 mM KCl and 9 μM valinomycin. Assays were started by the addition of vesicles and were continued for up to 30 min. Assay blanks were determined in the absence of vesicles and were used to correct data. In some cases data were expressed as International Enzyme Units (1 μmole product formed per min) per mg protein (I.U./mg).

Effect of DTT on BY 1023/SK&F 96022 inhibited vesicles. Intact vesicles were incubated with BY 1023/SK&F 96022 and 10 μM glutathione under acidifying conditions (150 mM KCl, 9 μM valinomycin) at pH 7.4. After 30 min the inhibition was stopped by the addition of 10 mM CDTA and 6 μM nigericin. Vesicles were then incubated at 37° for up to 30 min in the presence or absence of 100 mM dithiothreitol (DTT). The DTT was then removed by the gel filtration of 1 mL of sample using pre-washed Pharmacia Sephadex PD-10 columns. Columns were pre-washed using 25 mL water, 20 mL sucrose buffer (0.3 M sucrose, 2 mM PIPES/Tris buffer pH 7.4) containing 0.1% (w/v) bovine serum albumin and 15 mL sucrose buffer. After loading, columns were washed with 2 mL sucrose buffer and the vesicles were eluted in 1 mL sucrose buffer. The ATPase activity in the eluate was determined by incubation with 10 mM KCl at pH 7.4 as described above. Since nigericin was found to co-elute with vesicles it was not included in the assay.

Purification of rat kidney lysosomes. Lysosomes were prepared from the kidney cortices of male Wistar and Sprague–Dawley rats (350–450 g) using the method of Harikumar and Reeves [14]. Lysosome purification was monitored by assaying for acid phosphatase activity [15]. Latency, which was determined by the increase in activity in the presence of 0.1% Triton X-100, was routinely found to be greater than 70%.

Inhibition of lysosomal acidification. Lysosomal acidification was determined by quenching of the fluorescence signal of acridine orange (AO) [14]. Freshly prepared lysosomes (70–220 μg protein/mL) were incubated at 37° under conditions allowing acidification (0.3 M sucrose, 8 mM potassium gluconate, 8 mM KCl, 1 mM $MgSO_4$, 2 mM ATP, 2 μM valinomycin and 50 mM MOPS/Tris pH 7.4). Acidification was initiated by the addition of 1 mM $MgSO_4$. In some experiments 0.5 μM AO was added to the lysosomes at the start of the incubation and changes in fluorescence were monitored continuously. For incubations containing BY 1023/SK&F 96022 or omeprazole, acidification was allowed for 30 min after which 0.5 μM AO was added. Once a stable AO signal was obtained the extent of acidification was determined by the reversal of the AO quench upon the addition of 6 μM nigericin to dissipate the pH gradient. The later addition of AO ensured that the development of the pH gradient by the lysosome was not attentuated by the accumulation of AO, thus optimizing the conditions for intra-lysosomal activation of the benzimidazole sulphoxide inhibitors. Fluorescence measurements were made in a Perkin-Elmer LS-5 fluorimeter, the excitation and emission wavelengths used were 493 and 546 nm, respectively.

Protein assays. Protein was determined according to Lowry *et al.* [16] using bovine serum albumin as a standard.

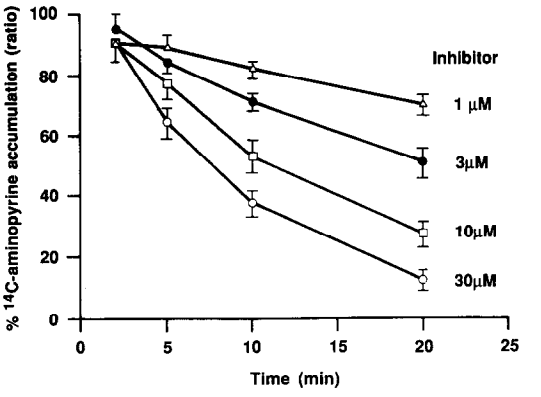

Fig. 2. Inhibition by BY 1023/SK&F 96022 of aminopyrine accumulation in intact gastric glands. Intact gastric glands, stimulated with 60 mM KCl, were incubated as described. BY 1023/SK&F 96022 was included at the concentrations shown. At the times indicated the aminopyrine accumulation ratio was determined as described in Materials and Methods. Values shown are the percentages of uninhibited controls (means and SE) from four separate experiments, where the control aminopyrine accumulation ratio was in the range 50 to 150.

Chemicals. Ionophores were dissolved in methanol. BY 1023/SK&F 96022 and omeprazole were dissolved in dimethylsulphoxide. These solvents, at the maximum concentrations used, were shown not to affect either ATPase activity or hydrogen ion transport by intact vesicles or lysosomes.

Data analysis. Inhibition curves were analyzed by the computer algorithm ALLFIT [17].

RESULTS

Effect of BY 1023/SK&F 96022 on gastric glands

In intact gastric glands, BY 1023/SK&F 96022 inhibited KCl-stimulated aminopyrine accumulation (an index of acidification within the gland) in a time dependent and concentration dependent manner (Fig. 2). Following incubation for 30 min with dibutyryl cAMP-stimulated gastric glands, BY 1023/SK&F 96022 inhibited aminopyrine accumulation with an IC_{50} of 1.0 μM (95% confidence limits 0.27–3.8 μM, N = 6). Under these conditions omeprazole inhibited with an IC_{50} of 0.7 μM (95% confidence limits 0.14–3.3, N = 5). The addition of 20 mM β-mercaptoethanol to gastric glands previously inhibited with BY 1023/SK&F 96022 resulted in a time dependent recovery of activity (Fig. 3). The addition of 20 mM β-mercaptoethanol to uninhibited gastric glands had no effect on the control aminopyrine accumulation ratio.

In digitonin-permeabilized glands, BY 1023/SK&F 96022 inhibited ATP-stimulated aminopyrine accumulated with an IC_{50} of 0.42 μM (95% confidence limits 0.16–1.07 μM, N = 6). Reversibility was assessed by permeabilizing intact gastric glands after inhibition by BY 1023/SK&F 96022 and then diluting the inhibitor 10-fold into fresh medium. The

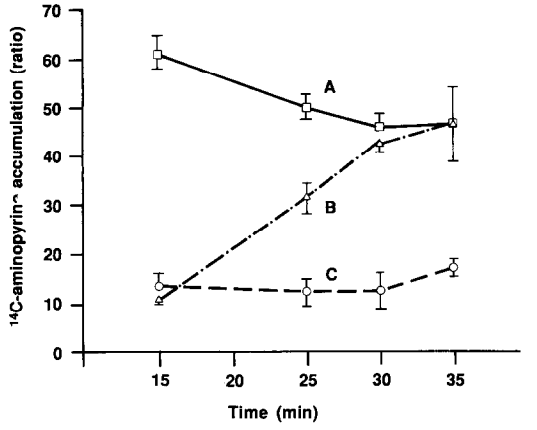

Fig. 3. Reversal of inhibition in intact gastric glands by β-mercaptoethanol. Intact gastric glands were incubated under stimulated conditions (1 mM dibutyryl cAMP) in the presence (○) and absence (□) of 30 μM BY 1023/SK&F 96022. After 15 min, 20 mM β-mercaptoethanol was added to inhibited glands (△). Aminopyrine accumulation was determined at the times indicated. Values shown are means and SE (N = 3 to 5 experiments).

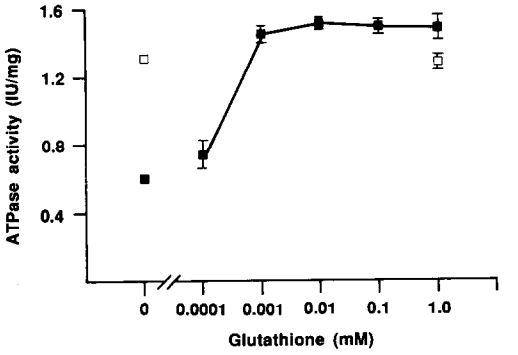

Fig. 4. Protection by glutathione of the inhibition by BY 1023/SK&F 96022 in lyophilized vesicles. Lyophilized vesicles (60 μg protein/mL) were pre-incubated in 10 mM PIPES/Tris pH 7.0, in the presence (■) or absence (□) of 120 μM BY 1023/SK&F 96022 and a range of glutathione concentrations. After 30 min at 37° a 10-fold dilution was performed and the residual ATPase activity was determined after incubations for 15 min in the presence of 11 mM PIPES/Tris pH 7.0, 2 mM MgATP and 1 mM KCl. Data were corrected for values obtained in the absence of enzyme. Values shown are the means ± range of duplicate determinations.

Table 1. Inhibitory potencies of BY 1023/SK&F 96022 and omeprazole at different pH values

Compound	IC_{50} (μM)	
	pH 6.1	pH 7.4
BY 1023/SK&F 96022	18 ± 3	70 ± 1
Omeprazole	4.9 ± 0.6	25 ± 5

Lyophilized vesicles (30 μg protein/mL) were pre-incubated at either pH 6.1 or 7.4 or 30 min after which a five-fold dilution was performed and residual ATPase activity was determined as described in Materials and Methods. The IC_{50} values are the means ± SE of three (BY 1023/SK&F 96022) or five (omeprazole) experiments and refer to the concentration of inhibitor in the preincubation.

degree of inhibition produced by 10 and 30 μM BY 1023/SK&F 96022, 15 min after the start of the initial incubation (and 5 min after the inhibitor concentration had been reduced by dilution to 1 and 3 μM), was 48% and 67%, respectively. By comparison with data in Fig. 2 at 10 min, this degree of inhibition more closely followed that produced by the concentrations of inhibitor before the dilution step. Thus, in permeabilized gastric glands, the inhibitory effect of BY 1023/SK&F 96022 could not be reversed by dilution.

Effect of BY 1023/SK&F 96022 on gastric vesicles. Direct effects of BY 1023/SK&F 96022 on the $(H^+ + K^+)$-ATPase were studied in purified gastric vesicle preparations in the presence and absence of pH gradients across the vesicle membrane.

Preincubation of lyophilized vesicles (where no pH gradient can be generated) with a range of concentrations of BY 1023/SK&F 96022 for 30 min at pH 7.4 and 37° resulted in a weak inhibition of ATPase activity reflecting the low rate of activation to the cyclic sulphenamide at neutral pH (Table 1). This inhibition was completely protected against by low (1 μM) concentrations of glutathione (Fig. 4). Under equivalent conditions, omeprazole inhibited more strongly probably as a result of its poorer stability at neutral pH (Table 1). Preincubation of lyophilized vesicles with BY 1023/SK&F 96022 or omeprazole at pH 6.1 resulted in a three-fold increase in potency relative to that at pH 7.4. Thus both compounds reacted more readily with the $(H^+ + K^+)$-ATPase at acidic pH values where the conversion to the active cyclic sulphenamide could occur more rapidly.

In intact, acid-accumulating vesicles, where the activation to the cyclic sulphenamide was favoured by the low pH, the potency of BY 1023/SK&F 96022 was much greater with an IC_{50} for the inhibition of aminopyrine accumulation of 3.5 ± 0.2 μM (mean ± SE, N = 3 experiments, Fig. 5). Concentrations of glutathione as high as 1 mM were unable to protect aminopyrine accumulation under these conditions, indicating that inhibition occurred lumenally and was not due to the activation of BY 1023/SK&F 96022 on the accessible, outside (cytosolic) face of the gastric vesicle. However, higher (10 mM) concentrations of glutathione gave complete protection (Fig. 6). This may have been due to a slow penetration of glutathione into the vesicle interior at these higher concentrations.

The reversibility of inhibition by BY 1023/SK&F 96022 was assessed in intact vesicles. Intact vesicles (60 μg protein/mL) were incubated with 80 μM BY 1023/SK&F 96022 in the presence and absence of hydrogen ion transport (Fig. 7). Gluthatione was included to prevent inhibition from any inhibitor activated on the cytosolic face of the vesicle. Under conditions favouring lumenal acidification. BY 1023/SK&F 96022 caused a time dependent inhibition of ATPase activity. Following a 20-fold dilution of the

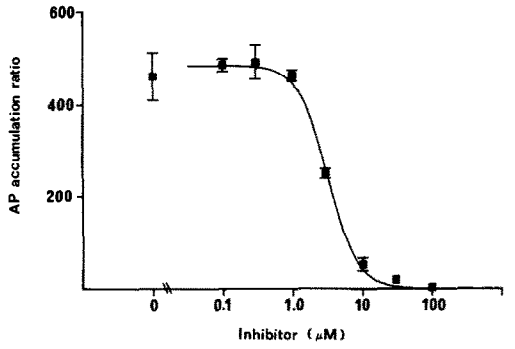

Fig. 5. Inhibition by BY 1023/SK&F 96022 of aminopyrine accumulation in intact vesicles. The aminopyrine accumulation ratio was determined as described. The data were analysed using ALLFIT with the minimum of the inhibition curve constrained to an aminopyrine accumulation ratio of 1 (i.e. no accumulation). The IC_{50} from this representative experiment was 3.2 μM. Values shown are the means ± range of duplicate determinations.

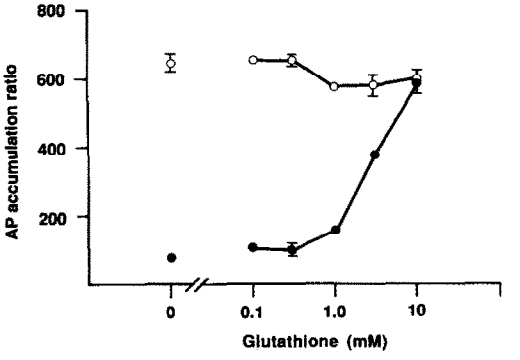

Fig. 6. Protection by GSH of the inhibition by BY 1023/SK&F 96022 of aminopyrine accumulation by intact vesicles. The aminopyrine accumulation ratio was determined in the presence (●) or absence (○) of 10 μM BY 1023/SK&F 96022 and varying concentrations of GSH. Values shown are the means ± range of duplicate determinations.

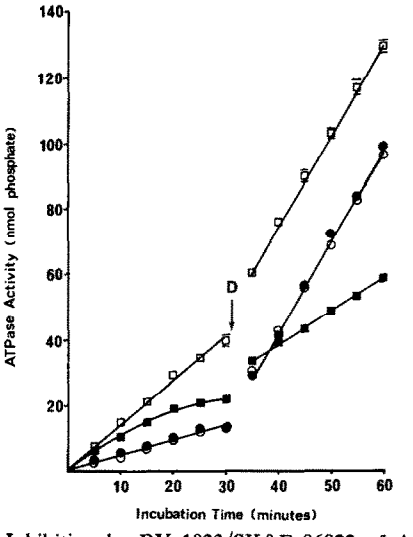

Fig. 7. Inhibition by BY 1023/SK&F 96022 of ATPase activity of intact vesicles. Intact vesicles (60 μg protein/mL) were incubated at 37° with 150 mM KCl, 2 mM MgATP, 10 μM glutathione and 10 mM PIPES/Tris pH 7.0, in the presence (□) or absence (○) of valinomycin, with (solid symbol) or without (open symbol) 80 μM BY 1023/SK&F 96022. After 30 min a 20-fold dilution of enzyme was performed (D) with final conditions: 10 mM KCl, 2 mM MgATP, 280 mM sucrose, 10 mM PIPES/Tris pH 7.0 and 6 μM nigericin. At various times, samples (equivalent to 3 μg protein) were taken and the P_i cumulatively released from ATP was determined. Data at each time point were corrected for P_i released in the absence of enzyme. Values shown are the means ± range of duplicate determinations. The slopes of the lines after dilution were used to calculate residual ATPase activity.

inhibitor and the inclusion of nigericin to dissipate the pH gradient, K^+-stimulated ATPase activity remained inhibited by 67 ± 3% (mean ± range, N = 2), relative to similarly treated vesicles in the absence of inhibitor. If valinomycin was omitted from the initial incubation of BY 1023/SK&F 96022 with vesicles (such that luminal acidification could not occur). ATPase activity after dilution was not inhibited relative to control (101 ± 4% of control, mean ± range, N = 2). Thus inhibition of the $(H^+ + K^+)$-ATPase required luminal acidification and was not reversible by dilution.

If inhibition by BY 1023/SK&F 96022 was the result of covalent modification of a cysteine residue on the enzyme to form a mixed disulphide, then reaction with thiol containing compounds could result in a restoration of ATPase activity. Intact gastric vesicles were therefore inhibited with BY 1023/SK&F 96022 under conditions favouring intravesicular acidification after which residual hydrogen ion pumping was stopped by the addition of CDTA and the pH gradients were dissipated with nigericin. The inhibited enzyme was then incubated with and without 100 mM dithiothreitol. K^+-stimulated ATPase activity was then measured after the dithiothreitol had been removed by gel filtration. Surprisingly, the degree of inhibition obtained with 100 μM BY 1023/SK&F 96022 in these experiments was only 28% compared with 67% obtained with 80 μM BY 1023/SK&F 96022 in the absence of treatment with CDTA and gel filtration. Control experiments showed that this was not due to a time dependent reversal of inhibition in the absence of dithiothreitol. This effect appeared to result from the gel filtration although the mechanism remains unclear. Nevertheless, under these conditions 100 mM dithiothreitol was found to restore ATPase activity completely (Table 2).

Effect by BY 1023/SK&F 96022 on lysosomal acidification

Since the benzimidazole sulphoxides are acid activated inhibitors of thiol containing enzymes, there exists the possibility that they could exert effects in acidic compartments other than the parietal cell canaliculus. One such compartment is that of the lysosome, although the degree of acidification

Table 2. Reversal of BY 1023/SK&F 96022 inhibited ATPase activity by dithiothreitol in intact vesicles

Preincubation	Incubation	% K^+-ATPase activity
Control		100
BY 1023/SK&F 96022		72 ± 1
BY 1023/SK&F 96022	DTT	103 ± 4

Intact vesicles (60 μg protein/mL) were pre-incubated at 37° with 150 mM KCl, 10 mM PIPES/Tris pH 7.4, 2 mM MgATP, 10 μM glutathione and 9 μM valinomycin in the presence or absence of 100 μM BY 1023/SK&F 96022. After 30 min samples were treated with 10 mM CDTA, 6 μM nigericin with and without 100 mM DTT and incubated for a further 30 min. The vesicles were then passed down a pre-equilibrated PD-10 column to remove the dithiothreitol and CDTA and were assayed for ATPase activity with 10 mM KCl, 280 mM sucrose, 10 mM PIPES/Tris pH 7.4 and 2 mM MgATP. Data were corrected for incubations in the absence of enzyme. Values are means ± SE of three experiments.

Table 3. Inhibition of lysosomal acidification by BY 1023/SK&F 96022 and omeprazole

Compound	IC_{50} (μM)
Omeprazole	75 ± 26
BY 1023/SK&F 96022	194 ± 90

Lysosomes were incubated with a range of inhibitor concentrations for 30 min under the conditions described in Materials and Methods. Acridine orange (0.5 μM) was added and the change in the fluorescence signal on the addition of 6 μM nigericin was determined. The change in fluorescence was converted to % inhibition and the data analysed to obtain the IC_{50} values. Each value is the mean ± SE of six experiments.

achieved is much lower that that associated with the gastric $(H^+ + K^+)$-ATPase. The effects of BY 1023/SK&F 96022 and omeprazole were assessed in purified preparations of lysosomes derived from rat kidney.

Following initiation of acidification by Mg^{2+}, the steady-state level of lysosomal acidification was stable for greater than 30 min, as determined by quenching of the fluorescent dye acridine orange (AO). When *N*-ethylmaleimide (50 μM), a covalent inhibitor of the lysosomal ATPase, was added during steady-state AO quench, a time dependent and complete inhibition of acidification was observed which was prevented by the presence of 10 mM DTT (data not shown).

BY 1023/SK&F 96022 or omeprazole were incubated with lysosomes for 30 min under conditions permitting acidification but in the absence of AO. When AO was added it was found that both compounds had inhibited the quench in a dose dependent manner (Table 3). The potency of inhibition for BY 1023/SK&F 96022 was 2.6-fold weaker than that found for omeprazole. The presence of 10 μM glutathione reduced the inhibition of acidification by 500 μM BY 1023/SK&F 96022 from 85 ± 7% to 12 ± 12% (mean, range, N = 2 experiments).

DISCUSSION

The benzimidazole sulphoxide omeprazole has been shown to inhibit gastric acid secretion by covalent interaction with the gastric $(H^+ + K^+)$-ATPase [1–6]. BY 1023/SK&F 96022, a novel benzimidazole sulphoxide, inhibited pH gradient formation in intact gastric glands stimulated by dibutyryl cAMP or KCl. Thus, like omeprazole, this compound inhibited acid transport at a point distal to receptor activation and second messenger generation. Consistent with this, direct inhibition of the gastric $(H^+ + K^+)$-ATPase by BY 1023/SK&F 96022 was observed in purified preparations of gastric vesicles.

Inhibition by benzimidazole sulphoxides is a complex process in which the parent compound is first converted, in an acid-activated reaction, to a cyclic sulphenamide. This can then react covalently with essential cysteine residues on the $(H^+ + K^+)$-ATPase [1, 2]. However, the manner in which the benzimidazole sulphoxide, omeprazole, inhibits the $(H^+ + K^+)$-ATPase *in vitro* has been shown to be highly dependent upon the conditions of the experiment [8]. Thus, in lyophilized gastric vesicles, which are unable to generate a pH gradient across the vesicle membrane, activation to the cyclic sulphenamide occurs equally on both sides of the vesicle membrane and inhibition results from reaction with multiple cysteine residues on both the cytosolic and lumenal faces of the $(H^+ + K^+)$-ATPase. In contrast, in intact vesicles, where active hydrogen ion transport by the $(H^+ + K^+)$-ATPase has acidified the vesicle interior, activation to the cyclic sulphenamide occurs predominantly on the lumenal face of the pump. Under these circumstances inhibition results from a reaction with much fewer, lumenally accessible cysteine residues. In this paper, we report experiments using gastric vesicles, designed to investigate the mechanism by which BY 1023/SK&F 96022 inhibits the $(H^+ + K^+)$-ATPase. In order to mimic the conditions found *in vivo* as closely as possible, these experiments have been performed under conditions of active hydrogen ion pumping and intravesicular acidification.

The importance of lumenal acidification can be seen when the potency of BY 1023/SK&F 96022 is compared in the presence and absence of active transport. Thus, BY 1023/SK&F 96022 inhibited aminopyrine accumulation in lumenally acidified intact vesicles with a potency 20 times greater than

that for the inhibition of ATPase activity in lyophilized vesicles at pH 7.4, suggesting that, under acidifying conditions, BY 1023/SK&F 96022 interacted preferentially with the lumenal face of the $(H^+ + K^+)$-ATPase. Such a conclusion was supported by the differential ability of the membrane impermeant glutathione to protect against inhibition. In lyophilized vesicles at pH 7.0, concentrations of glutathione as low as 1 μM were able to protect against inhibition induced by 120 μM BY 1023/SK&F 96022. This is presumably because under these conditions only a small fraction of parent inhibitor was converted to the active cyclic sulphenamide, which preferentially reacted with free glutathione rather than cysteine residues on the $(H^+ + K^+)$-ATPase. In contrast, in intact vesicles, a 100-fold excess of glutathione over BY 1023/SK&F 96022 failed to prevent inhibition of acidification as measured by aminopyrine accumulation. This suggested that inhibition was not due to activation of BY 1023/SK&F 96022 outside the vesicle nor to intravesicularly activated BY 1023/SK&F 96022 diffusing out of the vesicle and reacting with the cytosolic side of the $(H^+ + K^+)$-ATPase. However, higher concentrations of glutathione (10 mM), were able to protect against inhibition probably as a result of slow membrane penetration of a proportion of the glutathione into the vesicle interior.

The effectiveness of BY 1023/SK&F 96022 as an inhibitor in intact hydrogen ion transporting vesicles appeared to depend upon the method used to assess $(H^+ + K^+)$-ATPase activity. Thus, aminopyrine accumulation was completely inhibited at concentrations of BY 1023/SK&F 96022 in excess of 10 μM. In contrast, incubation with 80 μM BY 1023/SK&F 96022 inhibited ATPase activity by only 67%. Increasingly the concentration of BY 1023/SK&F 96022 did not result in greater inhibition (data not shown). This suggests that not all vesicles in this preparation have the same membrane permeability to hydrogen ions. Those vesicles with the lowest membrane permeability to hydrogen ions would be capable of generating the greatest pH gradient and should therefore be preferentially detected by the aminopyrine accumulation technique. It is also in these vesicles that the activation of BY 1023/SK&F 96022 should occur most readily leading to the inhibition of the $(H^+ + K^+)$-ATPase. In contrast, ATPase activity would measure all vesicles irrespective of their abilities to generate pH gradients. Therefore, that fraction of ATPase activity inhibited by BY 1023/SK&F 96022 under conditions favouring hydrogen ion accumulation may represent the proportion of vesicles in the preparation that are "tight" to hydrogen ions.

As expected for a time dependent inhibitor, dilution of BY 1023/SK&F 96022 did not lead to a recovery of activity measured either as aminopyrine accumulation in permeabilized gastric glands or as ATPase activity in intact gastric vesicles. This is consistent with covalent interaction between BY 1023/SK&F 96022 and the $(H^+ + K^+)$-ATPase.

Despite the lack of reversal by dilution, incubation with high concentrations of thiol-containing compounds after inhibition with BY 1023/SK&F 96022 did result in a restoration of activity both in intact vesicles (dithiothreitol) and in intact gastric glands (β-mercaptoethanol). This suggested that the cyclic sulphenamide, generated from BY 1023/SK&F 96022, reacted to form a mixed disulphide with cysteine residues on the $(H^+ + K^+)$-ATPase. Subsequent reaction between other thiols and the disulphide could then release the inhibitor from the $(H^+ + K^+)$-ATPase.

Like omeprazole, therefore, BY 1023/SK&F 96022 inhibited the gastric $(H^+ + K^+)$-ATPase irreversibly from the lumenal face of the membrane, probably following the formation of mixed disulphides with cysteine residues accessible from this side of the protein. Once activated, cyclic sulphenamides are non-selective sulphydryl agents [1–3, 6]. The selectivity of benzimidazole sulphoxides depends, therefore, upon restricting their activation to those conditions of high acidity found in the parietal cell. Selectivity may be optimized by reducing the rate of chemical activation at neutral pH values while retaining sufficient rates of activation under acidic conditions compatible with effective inhibition of acid secretion. BY 1023/SK&F 96022 has been selected, in part, for these properties, as discussed below.

Although the mechanisms of inhibition of the $(H^+ + K^+)$-ATPase by benzimidazole sulphoxides in lyophilized vesicle preparations differs significantly from that which occurs *in vivo*, the rate of inactivation of ATPase activity under these conditions may be used as an indirect measure of the chemical rate of activation of the benzimidazole sulphoxide to the corresponding cyclic sulphenamide. In this way, the increase in potency of BY 1023/SK&F 96022 when incubated with lyophilized gastric vesicles at pH 6.1 compared with pH 7.4 demonstrated the increased conversion to the active cyclic sulphenamide under conditions of greater acidity. At both pH values, the potency of BY 1023/SK&F 96022 was lower than that that of omeprazole by a factor of approximately three-fold. This is consistent with the greater chemical stability of BY 1023/SK&F 96022 at these pH values. Under strongly acid conditions around pH 1, the half-lives of omeprazole and BY 1023/SK&F 96022 are both very short, being 2 and 5 min, respectively [18, *]. Thus, although BY 1023/SK&F 96022 still activates less rapidly than omeprazole at pH 1.0, the rapid rate of activation is less likely to limit the inhibition of acid secretion *in vivo*. Consistent with this, the potencies of omeprazole and BY 1023/SK&F 96022 were very similar in the intact gastric gland preparation stimulated with dibutyryl cAMP.

Given that benzimidazole sulphoxides are acid activated sulphydryl agents it is possible they could exert effects at sites other than the parietal cell of the gastric mucosa, particularly in acidic compartments where the glutathione concentration may be insufficient to react with any cyclic sulphenamide generated. Such a compartment could be the lysosome. Indeed both BY 1023/SK&F 96022 and omeprazole

* Sturm E, Byk Gulden Pharmaceuticals, unpublished results.

inhibited acidification in rat kidney lysosomes, indicating their ability to interact with this different class of H^+-ATPase. However, the high concentrations required for inhibition and the protection by low concentrations of glutathione suggested that inhibition resulted from the generation of the cyclic sulphenamide at pH 7.0 on the outside (cytosolic) face of the lysosome. Thus, unlike the gastric ($H^+ + K^+$)-ATPase, the lysosomal H^+-ATPase does not appear to have any essential cysteine residues that can be blocked by benzimidazole sulphoxides activated within the lysosome. Whether intra-lysosomal activation of either of these benzimidazole sulphoxides had effects on any of the many lysosomal enzymes was not investigated in this study. However, BY 1023/SK&F 96022 inhibited lysosomal acidification with a potency three-fold lower than omeprazole, again indicating its improved stability at neutral pH values.

In summary, BY 1023/SK&F 96022 inhibited acidification in the parietal cell through an interaction with the ($H^+ + K^+$)-ATPase. Inhibition followed the activation of the parent compound to a cyclic sulphenamide on the lumenal (acidic) face of the membrane. This inhibition was not reversible by dilution but activity could be restored by thiol containing compounds, suggesting the formation of a mixed sulphide with cysteine residues accessible from the lumenal face of the pump. The rate of chemical activation of BY 1023/SK&F 96022 as a function of pH, confers good selectivity upon this compound and suggests that it is well suited for the treatment of acid related disease in the gastrointestinal tract.

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