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SCH 28080 IS A LUMENALLY ACTING, K^+-SITE INHIBITOR OF THE GASTRIC ($H^+ + K^+$)-ATPase

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Abstract—SCH 28080 (2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile) is an effective inhibitor of acid secretion *in vivo* and is a reversible, K^+-competitive inhibitor of the gastric ($H^+ + K^+$)-ATPase *in vitro*. The actions of SCH 28080 have been studied on gastric vesicle preparations containing the ($H^+ + K^+$)-ATPase. At pH 7, inhibition was competitive with respect to K^+ for both ATPase ($K_i = 24$ nM) and pNPPase ($K_i = 275$ nM) activities.

A close analogue of SCH 28080 (methylated in the 1-N position), that was not expected to cross membranes freely, inhibited ATPase and pNPPase activity less effectively in intact vesicle preparations, where the lumenal (extracellular) face of the membrane was not directly accessible. This suggested that SCH 28080 inhibited both enzyme activities at a lumenal site on the enzyme.

Being a protonatable weak base ($pK_a = 5.6$), SCH 28080 would be expected to accumulate on the lumenal, acidic side of the parietal cell membrane in its protonated form. The potency of SCH 28080, relative to that of the "non-protonatable" analogue, increased at low pH, commensurate with the proportion of SCH 28080 in the protonated form. Thus the accumulating protonated form was the active inhibitory species.

SCH 28080 (50 nM) blocked the rapid, K^+-stimulated dephosphorylation of the catalytic phosphoenzyme intermediate of the ($H^+ + K^+$)-ATPase at room temperature. At 4°, higher concentrations of the inhibitor were required, suggesting that the rate of inhibitor binding was slow at low temperatures.

SCH 28080 (2-methyl-8-(phenylmethoxy)imidazo-[1,2-*a*]pyridine-3-acetonitrile) has been reported to be an effective inhibitor of acid secretion *in vivo* [1]. The observation that, in the isolated guinea-pig gastric mucosa, SCH 28080 inhibited acid secretion stimulated by dibutyryl cAMP plus theophilline [2], suggested that its site of inhibition was distal to receptor activation. Subsequent work has indicated that this compound is a potent inhibitor of the gastric ($H^+ + K^+$)-ATPase in membrane preparations derived from the rabbit [3].

The mechanism whereby SCH 28080 inhibits the ($H^+ + K^+$)-ATPase differs importantly from that of the substituted benzimidazole sulphoxides, such as omeprazole. Omeprazole is an irreversible inhibitor of the ($H^+ + K^+$)-ATPase, which acts following an acid-catalysed conversion into a relatively non-selective sulphydryl agent [4, 5]. SCH 28080 has been reported to inhibit the gastric ($H^+ + K^+$)-ATPase in a freely reversible manner [6] that is kinetically competitive with respect to the K^+-activation of ATPase activity [6–8]. Since K^+ activates ATPase activity by interaction from the lumenal side of the membrane containing the ($H^+ + K^+$)-ATPase [9], it might be inferred that SCH 28080 also interacts from the lumenal side. This has important implications for the mechanism of inhibition *in vivo* since SCH 28080 is a protonatable weak base ($pK_a = 5.6$) and therefore will be substantially protonated under the acid conditions in the stomach. In contrast, when studied *in vitro* at neutral pH, less than 5% of the inhibitor will be protonated. It is important, therefore, to determine how protonation affects the ability of this compound to inhibit the ($H^+ + K^+$)-ATPase.

The kinetic cycle of the ($H^+ + K^+$)-ATPase is complex [10] and involves the enzyme alternating between forms (E_1, E_2) in which the cationic sites face first the cytoplasm (E_1) and then the lumen (E_2). In the first part of the catalytic cycle, ATP reacts with the E_1 form of the enzyme to form the catalytic phosphoenzyme intermediate, E_1–P. E_1–P converts to E_2–P, whose dephosphorylation is catalysed by lumenal K^+ to yield $E_2 \cdot K^+$. The conversion of $E_2 \cdot K^+$ to $E_1 \cdot K^+$ and the subsequent loss of K^+ from the cytosolic face returns the enzyme to the start of the cycle. The ($H^+ + K^+$)-ATPase is also able to catalyse the hydrolysis of *p*-nitrophenyl phosphate (pNPP). This reaction, which is also stimulated by K^+, is considered to be analogous to the K^+-stimulated dephosphorylation of E_2–P in the ATPase reaction [11]. However, an important difference between the pNPPase and ATPase activities of this enzyme is that the pNPPase activity is stimulated by K^+ on the cytosolic side of the membrane.

The exact point at which SCH 28080 interacts with this cycle is not yet clear. It has been suggested that SCH 28080 binds to an E_2 form of the enzyme since the inhibition of ATPase activity is competitive with respect to lumenally binding K^+ [8]. Extending this argument it might be expected that SCH 28080 bound to the E_2–P form of the enzyme as does K^+. However, in the same study, it was reported that SCH 28080 did not block the rapid dephospho-

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rylation of the phosphoenzyme intermediate by K^+. It was therefore concluded that an E_2 dephosphoform was involved.

In this paper we have compared the effects of SCH 28080 on the ATPase and pNPPase activities of the $(H^+ + K^+)$-ATPase with those of a close *N*-methylated analogue (Fig. 1). This analogue possesses a permanent positive charge by virtue of its quaternary nitrogen. As such it is a valuable probe for assessing which side of the membrane-spanning $(H^+ + K^+)$-ATPase binds the "K^+-site" inhibitors and for determining the effects of protonation on the action of SCH 28080. In addition we report on our data for the effects of SCH 28080 on the catalytic phosphoenzyme intermediate of the $(H^+ + K^+)$-ATPase.

MATERIALS AND METHODS

Preparation of gastric vesicles. Gastric vesicles were prepared from pig fundic mucosa as previously described [4, 12]. Briefly, tissue was homogenised in isotonic medium and a microsomal fraction obtained by differential centrifugation. This material was separated on a discontinuous density gradient and that fraction at the interface between the 0.25 M sucrose and 0.25 M sucrose plus 9% Ficoll layers was taken. The interface fraction, comprising intact gastric vesicles, was diluted in an equal volume of 60% (w/v) sucrose and was stored at −70°. Where free access to the lumenal face of the enzyme was required, the interface fraction was spun down in hypotonic medium and was freeze-dried overnight. These lyophilised gastric vesicles were resuspended in 10 mM Tris/Cl buffer, pH 7.0 and were stored at −70°. Following lyophilisation, K^+ was able to stimulate ATPase activity independently of the presence of the K^+/H^+-ionophore nigericin, thus demonstrating the increased permeability in the vesicle membrane.

Determination of enzyme activities. ATPase activity was determined at 37° in the presence of 10 mM Pipes/Tris buffer, pH 7.0, 2 mM $MgSO_4$, 2 mM ATP, 0–10 mM KCl. In some cases, usually when intact vesicles were used, tonicity was maintained with 0.3 M sucrose and K^+ was allowed access to the vesicle interior with 6 μM nigericin. For the pH studies, 10 mM Pipes/Tris buffer was used over the range pH 6.1 to 7.0 and 10 mM Mops/Tris buffer at pH 7.4. The measured pH at the end of incubation was used in the subsequent data analysis. At the end of the incubation (0–30 min) the inorganic phosphate hydrolysed from ATP was determined spectrophotometrically [13].

pNPPase activity was determined at 37° in the presence of 10 mM Pipes/Tris buffer, pH 7.0, 5 mM $MgSO_4$, 5 mM pNPP, 0–10 mM KCl. The incubation was stopped by the addition of 0.5 ml 0.5 M NaOH and the pNP hydrolysed was determined spectrophotometrically at 410 nm.

In both cases, K^+-stimulated enzyme activity was calculated after subtraction of the corresponding enzyme rate in the absence of KCl. Data are expressed as IU/mg protein, where 1 IU corresponds to a rate of product formation of 1 μmol/min. Control experiments were performed to ensure that, at each pH used, enzyme activities in lyophilised vesicles showed linear time courses in the presence and absence of the inhibitors used.

Determination of phosphoenzyme intermediate. $(H^+ + K^+)$-ATPase preparation (100 μg protein/ml) was incubated at room temperature in the presence of 10 mM Pipes/Tris buffer, pH 7.0, 2 mM $MgCl_2$ and 5 μM [γ-32P]ATP. After a defined period, normally 15 sec, the reaction was quenched with 1 ml ice-cold stopping solution (10% (w/v) perchloric acid, 5 mM non-labelled ATP and 40 mM Na_2HPO_4) and was filtered through a pre-soaked Whatman GF/B filter. The filter was washed 5 times with 10 ml ice-cold washing solution (5% (w/v) perchloric acid and 10 mM NaH_2PO_4) and was transferred to a liquid scintillation vial to which was added 10 ml Picofluor-15. Phosphoenzyme levels were corrected for background filter-associated radioactivity by subtracting the values obtained in the absence of enzyme sample.

Assessment of reversibility of inhibition. Intact gastric vesicles (100 μg protein/ml) were pre-incubated at 37° in 10 mM Mops/Tris buffer, pH 7.4, containing 0.3 M sucrose in the presence and absence of 100 μM SCH 28080. After various times (0–20 min) aliquots containing 10 μg vesicles were taken and assayed for residual ATPase activity in the presence of 10 mM KCl and 5 μg/ml nigericin as described. At the end of the pre-incubation the remaining intact vesicles were dialysed overnight at 4° against 2000 vol. of assay buffer and were assayed for ATPase activity as described above.

Analysis of kinetic data. K^+ activation data, in the presence and absence of inhibitors, were fitted to rectangular hyperbolae, using the ALLFIT programme [14]. The effects of inhibitors on the apparent V_{max} and K_m values were assessed and if competitive inhibition was indicated, the data were fitted to the equation for this inhibition using an iterative least squares regression [15].

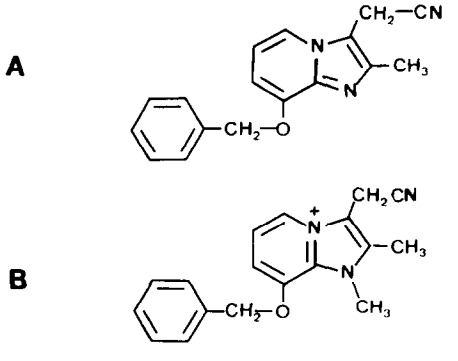

Fig. 1. The structures of SCH 28080 (A) and its *N*-methylated analogue (B).

RESULTS

Effect on ATPase activity

The inhibition of K^+-stimulated ATPase activity by SCH 28080 was reversible in nature as shown by the fact that assay time courses were linear in its presence, no time dependence was observed in the onset of inhibition at 37° and full enzyme activity

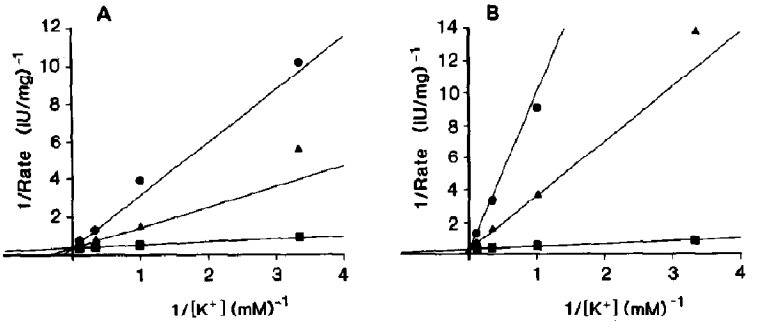

Fig. 2. The inhibition of K^+-stimulated ATPase activity by SCH 28080 and its *N*-methylated analogue. K^+-stimulated ATPase activity was determined with 3 μg/ml lyophilised vesicles at pH 7 as described in the text (a) in the presence of 0 (■), 0.3 (▲) and 1.0 (●) μM SCH 28080 and (b) in the presence of 0 (■), 0.3 (▲) and 1.0 (●) μM *N*-methylated SCH 28080. Values shown are single determinations of enzyme activity from representative experiments.

was restored once SCH 28080 had been removed by dialysis (data not shown).

In the absence of K^+, only 38% of basal ATPase activity was inhibited by SCH 28080. The IC_{50} value for this inhibition was 72 nM. The SCH 28080 insensitive portion of basal ATPase activity was not inhibited by 100 μM ouabain and was only reduced by a further 19% by 10 μg/ml oligomycin (data not shown). This suggested that the gastric vesicle preparation was not contaminated with significant quantities of ($Na^+ + K^+$)-ATPase or mitochondrial ATPase activities.

The inhibition of K^+-ATPase activity at neutral pH by both SCH 28080 and its quaternary analogue, was kinetically competitive with respect to K^+ (Fig. 2a, b). Furthermore, using the graphical method of Yonetani and Theorell [16], evidence was obtained that both inhibitors bound to the enzyme in a mutually exclusive manner (Fig. 3).

In order to investigate from which side of the vesicle membrane inhibition of ATPase activity occurred, the IC_{50} values for the *N*-methylated analogue of SCH 28080 were compared using intact and lyophilised vesicle preparations. ATPase activity was determined at pH 7.0 and was stimulated with 10 mM KCl. In the case of intact vesicles, K^+ was allowed into the vesicle lumen by the inclusion of 6 μM nigericin. IC_{50} values so obtained were 15 ± 3 μM (range N = 2) for the intact preparation and 0.33 ± 0.04 (range N = 2) for the lyophilised preparation. Thus this positively charged compound, which might not be expected readily to cross biological membranes was 45 times more potent when it had free access to the lumenal face of the enzyme.

Over the pH range 6.1–7.4, the inhibition of ATPase activity by both SCH 28080 and by its *N*-methylated analogue remained competitive with respect to K^+. Table 1 shows the calculated values of the K_m for K^+ and the K_i for each inhibitor over this pH range. The kinetic constants for the two species with fixed charge, K^+ and the analogue of SCH 28080, increased as the pH decreased. This suggested a pH-induced change to the enzyme which affected its ability to interact with these species. In order to remove this effect from the analysis of SCH 28080, the affinity of this compound was expressed relative to that of the *N*-methylated analogue. The relative affinity of SCH 28080 increased with decreasing pH in a manner that parallelled the

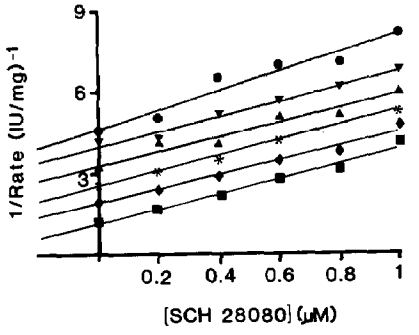

Fig. 3. The interaction between SCH 28080 and its *N*-methylated analogue at the inhibitory K^+-site. K^+-stimulated ATPase activity was determined with 6 μg/ml lyophilised vesicles at pH 7 as described in the text in the presence of combinations of SCH 28080 and its *N*-methylated analogue. Data are plotted after the method of Ref. 16 and show the effect of SCH 28080 on enzymatic rate at 0 (■), 0.2 (◆), 0.4 (*), 0.6 (▲), 0.8 (▼) and 1.0 (●) μM *N*-methylated analogue. Parallel lines indicate mutually exclusive binding of the two inhibitors. Values shown are from single determinations of enzyme activity.

Table 1. The effect of pH on the inhibition of K^+-stimulated ATPase activity by SCH 28080 (Sch) and by its *N*-methylated analogue (Me-Sch)

pH	K^+ K_m (mM)	Sch K_i (nM)	Me-Sch K_i (nM)
6.11	1.75 ± 0.23	20 ± 6	49 ± 1
6.53	1.00 ± 0.14	25 ± 5	29 ± 2
6.94	0.54 ± 0.04	24 ± 5	15 ± 2
7.35	0.26 ± 0.01	66 ± 5	14 ± 1

At each pH the K_m for K^+ and the K_i for the inhibitor were calculated as described. Data are expressed as K_m (mean ± SEM; N = 4 experiments) and K_i (mean ± range; N = 2 experiments).

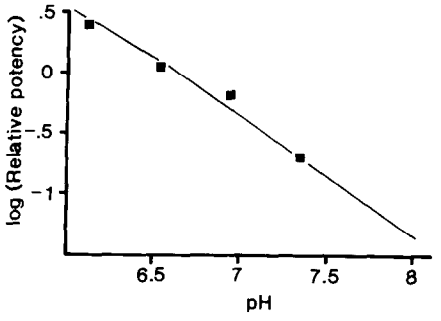

Fig. 4. The effect of pH on the potency of SCH 28080. The K_i values for the K^+-competitive inhibition of ATPase activity were determined for SCH 28080 and its *N*-methylated analogue over the pH range 6.1–7.4. The relative potency was defined as K_i (analogue) divided by K_i (SCH 28080). Ths solid line drawn shows the predicted change in relative potency for a compound where pK_a = 5.6, assuming that only the protonated form was active.

proportion of SCH 28080 in the protonated form (Fig. 4).

Effect on pNPPase activity

The inhibition of pNPPase activity by SCH 28080 was competitive with respect to K^+ at pH 7.0 (Fig. 5), but with a lower affinity than that for the ATPase reaction at the same pH (K_i = 275 ± 5 nM; range, N = 2 experiments). In lyophilised vesicle preparations, the *N*-methylated analogue of SCH 28080 was also competitive with respect to K^+ (K_i = 174 ± 15 nM; range, N = 2 experiments).

To investigate from which side of the membrane the inhibition of pNPPase activity occurred, assay time courses were determined in both intact and lyophilised vesicles in the presence and absence of the *N*-methylated analogue of SCH 28080. In lyophilised vesicles this compound showed a constant inhibition over a 20 min time course. However in intact vesicles the same concentration of this compound was much less effective and showed a time dependent inhibition suggesting a lumenal site of action (Fig. 6).

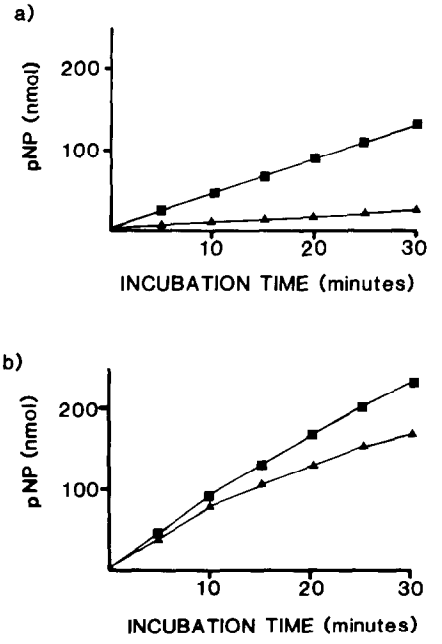

Fig. 6. Effect of the *N*-methylated analogue of SCH 28080 on the time course of pNPPase activity. (a) The time course of pNPPase hydrolysis was determined at 37° using 10 μg/ml lyophilised vesicles, 0.28 M sucrose, 5 mM $MgSO_4$, 10 mM KCl, 5 mM pNPP at pH 7.5 in the presence (▲) and absence (■) of 20 μM *N*-methylated SCH 28080. (b) Identical conditions to those above except that 10 μg/ml intact vesicles were used.

Effect on phosphoenzyme levels

SCH 28080 reduced the level of phosphoenzyme intermediate, formed in 15 sec at room temperature, with an IC_{50} value of 0.53 μM. A portion of the phosphoenzyme intermediate appeared to be refractory to inhibition (Fig. 7). When phosphoenzyme intermediate, pre-formed at room temperature, was chased with 0.5 mM KCl and an excess of non-radioactively labelled ATP (2 mM), a rapid dephosphorylation was observed (Fig. 8). The presence of 50 nM SCH 28080 slowed the rate of dephosphorylation if the inhibitor was allowed to

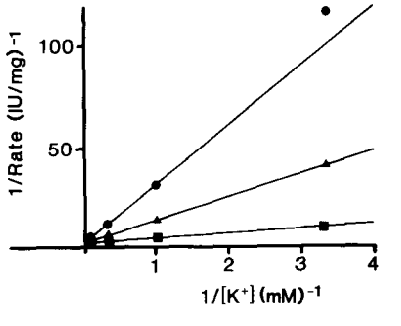

Fig. 5. The inhibition of K^+-stimulated pNPPase activity by SCH 28080 K^+-stimulated pNPPase activity was determined with 6 μg/ml lyophilised vesicles at pH 7 as described in the text and the presence of 0 (■), 1 (▲) and 3 (●) μM SCH 28080. Values shown are means ± range (N = 2) of enzyme activity from a representative experiment.

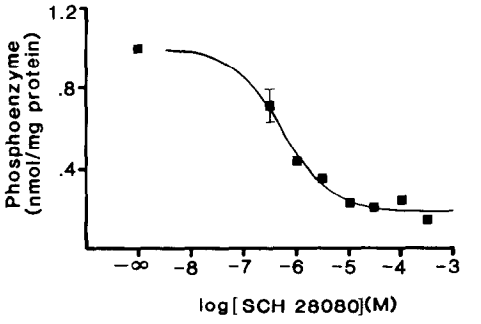

Fig. 7. Lyophilised gastric vesicles (100 μg/ml) were incubated at room temperature and pH 7.0 for 15 sec in the presence of 5 μM [γ-^{32}P]ATP and SCH 28080 as indicated. The acid stable phosphoenzyme was then determined as described in the text. Values shown are means ± range (N = 2).

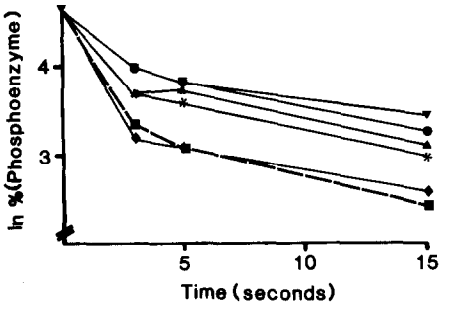

Fig. 8. Lyophilised gastric vesicles (100 μg/ml) were incubated at room temperature and pH 7.0 for 15 sec in the presence of 5 μM [γ-^{32}P]ATP. The pre-formed phosphoenzyme was then incubated with 50 nM SCH 28080 for the following times 0 (◆), 15 (*), 30 (▲), 45 (▼) and 60 (●) seconds. Finally dephosphorylation was initiated by the addition of 0.5 mM KCl and 2 mM non-labelled ATP. At the times indicated the residual phosphoenzyme was determined as described in the text. In control tubes the dephosphorylation was performed in the absence of SCH 28080 (■). Values shown are from single determinations.

pre-equilibrate with the enzyme. No effect of SCH 28080 on this dephosphorylation was detected if the SCH 28080 was added to a K^+ chase experiment at the same time as the K^+. When phosphoenzyme was preformed at 4°, 100 μM SCH 28080 was found to block the dephosphorylation induced by 0.5 mM KCl (Fig. 9).

DISCUSSION

SCH 28080 has been shown to be an effective inhibitor of acid secretion *in vivo* [1]. This inhibition does not appear to depend on the nature of the secretagogue used and suggests a site of action distal to the acid-activating receptors on the parietal cell [2]. This conclusion is consistent with the highly potent inhibition of the gastric $(H^+ + K^+)$-ATPase by SCH 28080 *in vitro* [3, 7].

The inhibition of K^+-stimulated ATPase activity by SCH 28080 has been reported to be both freely reversible and competitive with respect to the activating cation, K^+ [6–8]. Our data would support these findings. SCH 28080 therefore belongs to the class of "K-site" inhibitors of the $(H^+ + K^+)$-ATPase which also includes nolinium bromide [17] and a number of hydrophobic amines [18]. Interestingly, SCH 28080 showed a higher affinity for the enzyme in the inhibition of ATPase activity as compared with pNPPase activity. Since pNPP hydrolysis is not believed to occur via a phosphoenzyme intermediate, this might reflect a higher affinity of SCH 28080 for the phosphorylated intermediate of the ATPase reaction.

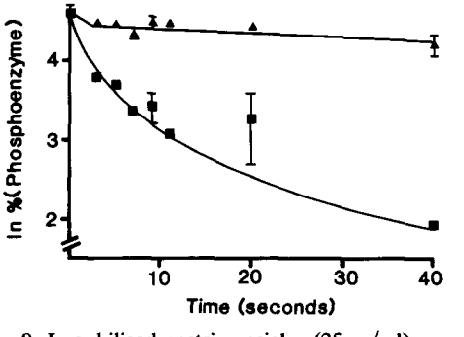

Fig. 9. Lyophilised gastric vesicles (25 μg/ml) were incubated at 4° and pH 7.0 for 15 sec in the presence of 5 μM [γ-^{32}P]ATP. Dephosphorylation was initiated by the addition of 0.5 mM KCl and 2 mM non-labelled ATP in the presence (▲) and absence (■) of 100 μM SCH 28080. At the times indicated the residual phosphoenzyme was determined as described in the text. Values shown are means ± range (N = 2).

We were not able to use SCH 28080 directly to show from which side of the membrane the inhibition of enzyme activity occurred because of its high membrane permeability. However, the *N*-methylated analogue of SCH 28080, by virtue of its charged state, would not be expected to cross membranes freely. We have assumed that these two inhibitors interact with the same site on the enzyme, based upon their close structural similiarity and their mutually competitive binding. The *N*-methylated analogue of SCH 28080 was less effective as an inhibitor of ATPase and pNPPase activities in intact (compared with lyophilised) preparations of the enzyme suggesting that membrane penetration to the vesicle lumen (equivalent to the extracellular space in the intact tissue) was required for its actions. By analogy, SCH 28080 inhibited both ATPase and pNPPase activities from a lumenal site.

It should be noted, however, that competitive interaction between two ligands does not necessarily guarantee a common site of action, as, in the case of pNPPase activity, lumenally acting inhibitor was kinetically competitive with respect to cytosolic K^+.

Since SCH 28080 is a weak base ($pK_a = 5.6$), it is important to understand the effect of protonation on the inhibition of ATPase activity. This is especially true since much of the kinetic analysis of SCH 28080 performed to date has been at neutral pH where it is essentially unprotonated, whereas, under physiological conditions, any SCH 28080 at the lumenal face of the enzyme will be almost completely protonated. To this end the effect of pH on the inhibition of K^+-stimulated ATPase activity was studied.

As the pH was reduced from 7.4 to 6.1, the K_m for K^+ and the K_i for the competitive inhibition of the *N*-methylated analogue of SCH 28080 both increased in parallel. It is tempting to speculate that this was due to the protonation of a single group somewhere close to the lumenal K^+ binding site. However, the kinetic cycle of the $(H^+ + K^+)$-ATPase is complex and particular care must be taken in interpreting experimental K_m values as true affinities. Nevertheless the fact that these two ligands, despite not changing their degree of protonation, had kinetic constants that varied with pH, complicated the analysis of the data for SCH 28080 itself. By expressing the affinity of SCH 28080 relative to that of its *N*-methylated analogue, those changes that resulted from pH effects on the enzyme were hopefully discounted. Any variation in the relative affinity with pH should result from changes in the degree of protonation of SCH 28080. The agreement between the changes in relative potency and the

calculated proportion of SCH 28080 in the protonated form suggested that it was the protonated species that was responsible for the inhibition of ATPase activity. This finding is in agreement with the increased potency of SCH 28080 in conditions of active proton pumping and pH gradient formation (unpublished data this laboratory), where one would expect the protonated form of the inhibitor to accumulate on the lumenal (acidic) side of the acid secreting membrane.

The term "K-site" inhibitor would imply that these compounds antagonise the effects of K^+ on the $(H^+ + K^+)$-ATPase. K^+ is believed to stimulate ATPase activity by increasing the rate of dephosphorylation of the phosphoenzyme (E–P) intermediate [10]. However, previous work failed to show any effect of 10 μM SCH 28080 on the initial rate of K^+-stimulated dephosphorylation at 0° and it was concluded that SCH 28080 interacted with a dephosphoform of the enzyme [8]. In contrast, we have shown that, by increasing the inhibitor concentration from 10 μM to 100 μM and by decreasing the K^+ concentration from 10 mM to 0.5 mM, SCH 28080 was able to block the K^+-stimulated dephosphorylation. This suggested that, at 0°, the association rate constant for SCH 28080 binding was sufficiently low that high inhibitor concentrations were required to ensure significant binding in the short time of the K^+ dephosphorylation experiment. Consistent with this interpretation, at room temperature, the association rate constant was sufficiently fast that 50 nM SCH 28080 was able to block the K^+-stimulated dephosphorylation provided that 30–60 sec was allowed for association to occur.

However, if the sole action of SCH 28080 was to bind to the E_2–P form of the enzyme, thereby blocking dephosphorylation, one would not expect this compound to reduce the steady state level of phosphoenzyme as was observed. At saturating concentrations of SCH 28080, a finite phosphoenzyme level and basal ATPase activity remained. Assuming that these were not due to the presence of a contaminating, SCH 28080-insensitive activity in the gastric vesicle preparation, it appeared that the SCH 28080-bound enzyme (E.S.) retained limited catalytic activity albeit at a reduced rate and with an altered balance between the phospho- and dephosphoforms of the enzyme. The reduction in steady state phosphoenzyme may therefore reflect a reduction in the rate of the $E_2 \cdot S$ to $E_1 \cdot S$, which must precede rephosphorylation by ATP. A more detailed study on the effects of SCH 28080 on the catalytic phosphorylation of the $(H^+ + K^+)$-ATPase may clarify this action.

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