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SK&F 96067 IS A REVERSIBLE, LUMENALLY ACTING INHIBITOR OF THE GASTRIC ($H^+ + K^+$)-ATPase

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Abstract—SK&F 96067 [3-butyryl-4-(2-methylphenylamino)-8-methoxyquinoline] has been identified, from a novel class of 4-aminoquinolines, as a reversible inhibitor of the gastric ($H^+ + K^+$)-ATPase. This compound has been studied in gastric membrane vesicle preparations enriched in the ($H^+ + K^+$)-ATPase. At pH 7.0, SK&F 96067 inhibited K^+-stimulated ATPase activity competitively with respect to the activating cation K^+, with a K_i value of 0.39 ± 0.05 μM. Under comparable conditions, SK&F 96067 was 32 times more potent as an inhibitor of the gastric ($H^+ + K^+$)-ATPase relative to the closely related ($Na^+ + K^+$)-ATPase. Studies in intact gastric vesicles showed that SK&F 96067 also inhibited hydrogen ion transport. Using the initial rate of acridine orange quenching as the index of acidification, an IC_{50} of 0.84 ± 0.24 μM was observed. Steady state acidification, as measured by aminopyrine accumulation, was inhibited with greater potency ($IC_{50} = 0.06 \pm 0.01$ μM) consistent with the accumulation of this inhibitor into the intravesicular acidic space to a site of action on the inside (lumenal) face of the enzyme. Inhibition of ATPase activity in the presence of both SK&F 96067 and the K^+-competitive ($H^+ + K^+$)-ATPase inhibitor, SCH 28080, indicated that their binding was mutually exclusive, consistent with SK&F 96067 acting at the same lumenal binding site as does SCH 28080. The steady-state inhibition kinetics of SK&F 96067 against K^+-stimulated ATPase activity were followed as a function of pH. At pH 6.6 and 7.0 the inhibition was competitive with respect to the activating cation K^+. At pH 7.5 and 8.1 a mixed pattern of inhibition was detected. Thus, at alkaline pH values, the binding of SK&F 96067 and K^+ were no longer mutually exclusive. The potency of SK&F 96067 decreased as pH rose, consistent with the protonated form of the inhibitor being the preferred inhibitory species. A kinetic model is discussed, in which, at acidic pH, the protonated form of SK&F 96067 binds to the enzyme competitively with respect to K^+, whereas, at alkaline pH, the neutral form of SK&F 96067 can bind simultaneously with K^+.

The gastric ($H^+ + K^+$)-ATPase of the parietal cell is the ion pump responsible for the final step of acid secretion in the stomach [1] and, as such, this protein has been identified as a pharmacological target for drug development.

Inhibition of acid secretion by substituted benzimidazole sulphoxides such as omeprazole and pantoprazole has been shown to be long lasting in nature [2, 3]. These agents are covalent inhibitors of the ($H^+ + K^+$)-ATPase [4–6] and the subsequent return of acid secretion probably requires the *de novo* synthesis of a new ion pump [7]. Whilst such long lasting inhibition of acid secretion has been shown to be highly effective in the treatment of duodenal ulcer disease [8], there is still discussion as to the optimum degree and duration of acid suppression in the treatment of this and other acid-related diseases [9]. A freely reversible inhibitor of the ($H^+ + K^+$)-ATPase may allow greater flexibility to optimize the duration of acid suppression in acid-related diseases of the gastrointestinal tract.

The gastric ($H^+ + K^+$)-ATPase is a membrane spanning protein which, during active acid secretion, faces a unique, highly acidic, environment outside the cell. This provides the opportunity to target an inhibitor to a lumenal (extracellular) site on this protein. The presence of a protonatable group on the inhibitor with a suitable pK_a could result in the protonated form of the inhibitor accumulating in this acidic compartment. Furthermore, the positively charged, protonated, form of the inhibitor might also be capable of binding to the cationic binding site responsible for the uptake of K^+ by the ($H^+ + K^+$)-ATPase.

The gastric ($H^+ + K^+$)-ATPase can be isolated from pig gastric mucosa in the form of intact vesicles. The majority of these vesicles are orientated such that the ATP-hydrolysing (cytoplasmic) face of the pump faces outwards and pump activity leads to an acidification of the vesicle interior [10]. Activity of the ($H^+ + K^+$)-ATPase can be followed as a K^+-stimulated ATPase activity, provided that K^+ is allowed free access to its stimulatory site inside the vesicle. This is achieved either by the addition of a K^+-ionophore or by prior lyophilization of the vesicles, although in the latter case H^+ ion accumulation can no longer be observed. In intact gastric vesicles, acidification can be monitored directly by following the accumulation of a weak base, such as aminopyrine or the fluorescent dye acridine orange.

In this report we describe the effects of SK&F 96067† (Fig. 1) on vesicular preparations of the

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† Abbreviations: K_{is}, dissociation constant for inhibitor and free enzyme; K_{ii}, dissociation constant for inhibitor and K^+-bound enzyme; DMSO, dimethyl sulphoxide; CDTA, 1,2-diaminocyclohexanetetraacetic acid; SK&F 96067, 3-butyryl-4-(2-methylphenylamino)-8-methoxyquinoline; SCH 28080, 8-benzyloxy-3-cyanomethyl-2-methylimidazo(1,2-*a*)pyridine; N-Me SCH 28080, 8-benzyloxy-3-cyanomethyl-1,2-dimethylimidazo(1,2-*a*)pyridinium iodide; SEM, standard error of the mean.

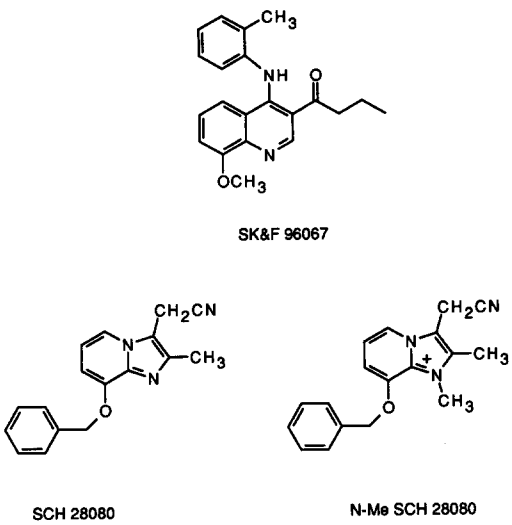

Fig. 1. The structures of SK&F 96067, SCH 28080 and N-Me SCH 28080.

$(H^+ + K^+)$-ATPase which support the idea that SK&F 96067 is a reversible inhibitor of the $(H^+ + K^+)$-ATPase acting selectively at a lumenal (extracellular) site on this ion pump.

MATERIALS AND METHODS

Preparation of gastric vesicles

Gastric vesicles were prepared from pig fundic mucosa by the method of Saccomani *et al.* [11] as described previously [5]. Briefly, tissue was homogenized in isotonic medium and the microsomal fraction obtained by differential centrifugation. This material was separated on a discontinuous density gradient and intact gastric vesicles taken from the interface between the 0.25 M sucrose and 0.25 M sucrose plus 9% Ficoll layers. Where intact vesicles were required for acidification studies, this fraction was frozen in liquid nitrogen and was stored at −70°. To increase the accessibility of the vesicle interior to K^+, other vesicles were centrifuged in hypotonic buffer and freeze dried overnight. The resulting lyophilized vesicles were resuspended in 10 mM Pipes/Tris buffer pH 7.0 and stored at −70°. Dilution of enzyme prior to use was in 10 mM Pipes/Tris buffer, which had been adjusted to the pH to be used in the subsequent assays. The protein content of the $(H^+ + K^+)$-ATPase preparations was determined by the method of Lowry *et al.* [12] using bovine serum albumin (Fraction V) as standard.

Determination of ATPase activity

Lyophilized vesicles (3–6 μg protein) were incubated for 30 min at 37° in the presence of 10 mM Pipes/Tris buffer pH 6.61–8.08, 0–5 mM KCl, 2 mM $MgSO_4$, 2 mM Na_2ATP. In all cases enzyme was used to start the incubation. At the end of the incubation, the inorganic phosphate released from ATP was determined [13], using a phosphate standard curve (0–200 mmol) prepared each day. $(H^+ + K^+)$-ATPase inhibitors were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 20 mM and were initially diluted in water containing 40 mM HCl. Further dilution was in water. The concentration of DMSO in the assays was less than or equal to 0.5% (v/v) which had no effect on K^+-stimulated ATPase activity.

$(N^+ + K^+)$-ATPase activity

Dog kidney enzyme (0–10 μg protein, Sigma Chemical Co., Poole, U.K.) was incubated as described for the gastric ATPase except that 100 mM NaCl was included in the incubation. The K^+-kinetics of SK&F 96067 on the $(Na^+ + K^+)$-ATPase were also investigated by determining K^+-activation (0–10 mM KCl) in the presence of different inhibitor concentrations (0–100 μM).

Kinetics experiments

At each of the pH values studied, the patterns of inhibition of SK&F 96067 and N-Me SCH 28080 were determined relative to the activation of $(H^+ + K^+)$-ATPase activity by K^+. Assays were performed in duplicate for three to five different inhibitor concentrations at each of four to eight different K^+ concentrations. At each inhibitor concentration, the basal ATPase activity, determined in the absence of KCl, was subtracted from the data which was then converted into International Enzyme Units (1 μmole product formed/min)/mg protein (I.U./mg). In order to achieve a range of pH values in the ATPase assays, stock Pipes/Tris buffers were prepared at room temperature with pH values of 6.6, 7.0, 7.58 and 8.3. Stock ATP solutions were adjusted to the appropriate pH value and the lyophilized gastric vesicles were diluted in 10 mM Pipes/Tris buffer at the appropriate pH. The actual pH values of the assays were determined experimentally at 37° in the presence of all the components of the assay. These pH values were found to be 6.61, 7.0, 7.58 and 8.08.

Control experiments showed that in the range pH 6.61–8.08, the amount of phosphate formed, in the presence and absence of the inhibitors used, was linearly related to both the time of incubation (up to 30 min) and the amount of gastric vesicles assayed (up to 10 μg protein/assay).

Determination of inhibition pattern and inhibition constants

For each experiment at each pH value, the K^+-stimulated ATPase activities were plotted graphically according to the double reciprocal method of Lineweaver and Burk [14]. Best fit lines were drawn by eye through these data and the pattern of inhibition assessed qualitatively. Values were obtained for the slopes and intercepts of each of these lines which were then replotted as a function of inhibitor concentration. The linearity of these secondary plots was used as a qualitative assessment that the inhibition conformed to a simple linear model. To obtain values for the kinetic constants describing the inhibition pattern, data for the K^+-stimulated ATPase activity were fitted directly to

equations for competitive, uncompetitive and mixed (non-competitive) patterns of inhibition described by Cleland [15], using the computer programme GraFit [16]. The pattern of inhibition chosen was that giving the lowest sum of squares provided that all the inhibition constants were well defined. If the inclusion of the additional inhibition constant in the mixed fit resulted in only a marginal decrease in the reduced chi-square term relative to the competitive or uncompetitive fits, then an F-test was performed. The mixed pattern was chosen if the probability that the additional constant could be omitted was less than 0.05. Inhibition constants were as defined by Cleland [17], where K_{is} was obtained from the slope of the Lineweaver–Burk plot and represented the dissociation constant of inhibitor from K^+-free enzyme and K_{ii} was obtained from the intercept of the Lineweaver–Burk plot and represented the dissociation constant of inhibitor from K^+-bound enzyme. The lines shown on all figures are those of the best computer fit to the data.

Analysis of kinetics as a function of pH

At each pH, mean values for the inhibition constant K_{is} were obtained for SK&F 96067 and N-Me SCH 28080. Where there was no value of K_{is} (one experiment at pH 8.08 which gave an uncompetitive pattern of inhibition) the experiment was omitted from the analysis. The ratio of these constants was taken as the relative potency of SK&F 96067.

$$\text{Relative potency} = \frac{K_{is} \text{ for N-Me SCH 28080}}{K_{is} \text{ for SK\&F 96067}}.$$

The logarithm of the relative potency was then plotted as a function of pH and compared with the proportion of protonated SK&F 96067 over the same pH range.

Acidification in intact gastric vesicles

Acridine orange fluorescence quenching. The initial rate of hydrogen ion transport in intact gastric vesicles was monitored using the distribution and quenching of the fluorescent probe acridine orange, which, being a weak base, accumulates into acidic compartments where its fluorescence signal is quenched. Assays were performed in plastic fluorimeter cuvettes at room temperature (20–25°) in a total volume of approximately 1 mL. Assays comprised 10 mM Pipes/Tris buffer, pH 7.0, 150 mM KCl, 2 mM $MgSO_4$, 2 mM ATP, 9 μM valinomycin, intact gastric vesicles (10–20 μg protein/assay), 4 μM acridine orange. Valinomycin was dissolved in methanol, which had no effect on the control rates of acidification at the concentrations used. Fluorescence was monitored using a Perkin-Elmer LS-5 fluorimeter with attached chart recorder. Wavelength and slit widths were as follows: excitation 490 nm, 5 nm; emissions 540 nm, 2.5 nm. Assays were started by the addition of valinomycin to the cuvette containing all other components. The initial rate of decrease of fluorescence was recorded. To quantify any effects of SK&F 96067 on the hydrogen ion leak rate, the following method was followed. Intact gastric vesicles were allowed to generate a pH gradient as described above. Once the maximum extent of acidification had been achieved, CDTA (4 mM) was added to the cuvette to prevent any further ATP hydrolysis and hydrogen ion pumping. Under these circumstances, the preformed pH gradient collapsed at a rate determined by the hydrogen ion leak rate of the membrane. This was quantified as the rate of increase in acridine orange fluorescence signal following the addition of CDTA. The effects of SK&F 96067 added at the same time as the CDTA, were determined.

Aminopyrine accumulation in intact gastric vesicles. Steady state acidification in intact vesicles was followed by the distribution of the radiolabelled probe aminopyrine. Incubations consisted of intact gastric vesicles (25 μg protein/mL), 10 mM Pipes/Tris buffer, pH 7.0, 2 mM $MgSO_4$, 2 mM Na_2ATP, 150 mM KCl, 9 μM valinomycin, 3 μM [dimethylamine-^{14}C]aminopyrine (100–120 mCi/mmol, Amersham International, Amersham, U.K.) and 0.1 mg/mL bovine serum albumin. Valinomycin was dissolved in methanol which, at the concentration used in the assay (0.5%), did not affect control aminopyrine accumulation. 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 washing solution, after which the radioactivity that had accumulated into the vesicle 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), an intravesicular volume of 2 μL/mg protein was assumed [11]. Control aminopyrine accumulation ratios were routinely in the range 800 to 1200. Data were fitted to the 4-parameter logistic equation [18] with the minimum of the inhibition curve constrained to equal 1 (i.e. the ratio where no accumulation had occurred).

RESULTS

In lyophilized gastric vesicles at pH 7.0, SK&F 96067 was found to inhibit K^+-stimulated ATPase activity, in the presence of 1 mM KCl, with an IC_{50} value of 1.9 ± 0.2 μM (SEM, N = 9 experiments). In order to assess the reversibility of inhibition, high concentrations of lyophilized gastric vesicles (30 μg protein/mL) were incubated for 30 min with 1 μM SK&F 96067 under the conditions of the ATPase assay before being diluted twenty-fold without altering the concentrations of the components of the assay other than enzyme and SK&F 96067. At the end of the first 30 min period, the amount of ATP hydrolysed in the presence of SK&F 96067 was reduced by 44 ± 3% (SEM, N = 3 experiments). The rate of phosphate production was then followed as a function of time for 25 min after dilution. During this period, the rate of ATP hydrolysis in SK&F 96067 pre-treated enzyme was found to have recovered to be 104 ± 8% of that seen in the absence of SK&F 96067. Thus inhibition by SK&F 96067 was fully reversed by dilution. Steady state kinetic analysis at pH 7.0, showed that the inhibition of

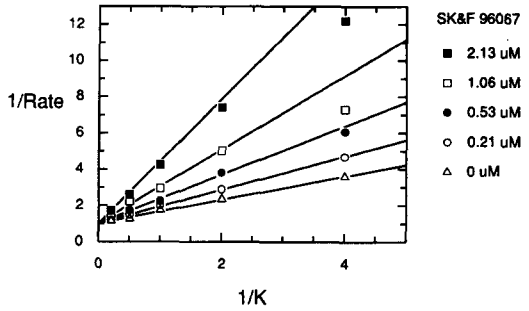

Fig. 2. Inhibition of $(H^+ + K^+)$-ATPase by SK&F 96067 at pH 7.0. K^+-stimulated ATPase activity was determined at pH 7.0 over a range of K^+ and SK&F 96067 concentrations as shown. Data were then plotted after the method of Lineweaver and Burk [14]. The ordinate axis is the reciprocal of the enzyme rate (in μmol/min/mg protein) and the abscissa is the reciprocal of the K^+ concentration (in mM). The lines drawn represent the best fit to a competitive pattern of inhibition with the following parameter estimates: $V_{max} = 0.97$ I.U./mg, $K_m = 0.62$ mM, $K_{is} = 0.49\ \mu$M. Values shown are the means of duplicate enzyme assays.

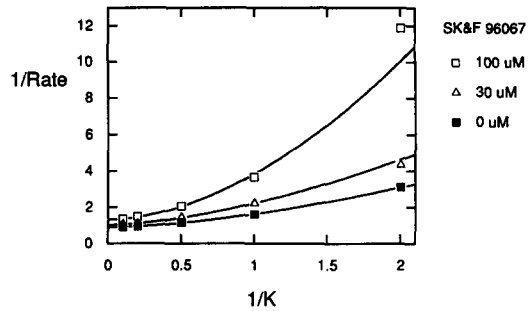

Fig. 3. Inhibition of $(Na^+ + K^+)$-ATPase by SK&F 96067 at pH 7.0. Na^+ and K^+-stimulated ATPase activity was determined at pH 7.0 over a range of K^+ and SK&F 96067 concentrations as shown. Data were then plotted after the method of Lineweaver and Burk [14]. The ordinate axis is the reciprocal of the enzyme rate (in μmol/min/mg protein) and the abscissa is the reciprocal of the K^+ concentration (in mM). The lines shown are the best fit to activation curves with a Hill coefficient greater than unity. Note that the intercept on the ordinate axis increases with inhibitor concentration. Values shown are the means of duplicate enzyme assays.

ATPase activity by SK&F 96067 was competitive with respect to the activating cation K^+ (Fig. 2) with a K_i value of $0.38 \pm 0.05\ \mu$M (SEM, N = 7 experiments). This was consistent with SK&F 96067 interacting at the K^+-uptake site on the lumenal face of the $(H^+ + K^+)$-ATPase. The K_m for K^+ in these experiments was determined to be 0.51 ± 0.04 mM.

In order to assess the selectivity of SK&F 96067 for the gastric $(H^+ + K^+)$-ATPase, its inhibitory effect on the closely related $(Na^+ + K^+)$-ATPase was also determined. At pH 7.0, in the presence of 100 mM NaCl and 1 mM KCl, SK&F 96067 inhibited ATPase activity with an IC_{50} value of $60 \pm 10\ \mu$M (SEM, N = 7 experiments), indicating a thirty-two-fold selectivity for the gastric $(H^+ + K^+)$-ATPase. Steady state kinetic experiments on the $(Na^+ + K^+)$-ATPase activity could not be analysed using the standard equations for inhibition, by virtue of the sigmoidal activation of ATP hydrolysis by K^+. However, K^+-activation curves in the presence of SK&F 96067 showed a clear intercept effect (Fig. 3) indicating that the inhibition of $(Na^+ + K^+)$-ATPase activity was not competitive with respect to K^+.

The effects of SK&F 96067 on hydrogen ion transport were assessed in intact gastric vesicle preparations. Effects on the initial rate of acidification were monitored by the accumulation and quenching of the fluorescent dye acridine orange. SK&F 96067 inhibited the initial rate of acridine orange quenching with an IC_{50} of $0.84 \pm 0.28\ \mu$M (range, $N = 2$ experiments, Fig. 4). To ensure that this was due to an effect on the $(H^+ + K^+)$-ATPase and not to an impaired ability of the vesicles to support a pH gradient, the effects of SK&F 96067 on the hydrogen ion leak rate were also determined. There was no effect of SK&F 96067 on the leak rate at concentrations around the IC_{50} for the inhibition of acidification, although at higher concentrations a concentration related increase in leak rate was

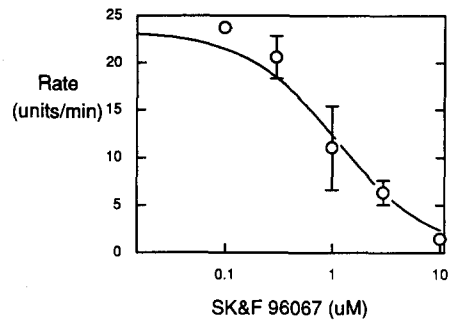

Fig. 4. Effect of SK&F 96067 on the initial rate of acidification in intact gastric vesicles. The initial rate of acidification was determined in the presence of a range of concentrations of SK&F 96067. SK&F 96067 was added to the cuvette 2–5 min before the addition of valinomycin. The rates of acidification were calculated from the initial slope of the fluorescence trace and were expressed in arbitrary fluorescence units per min. The data shown are means ± range from N = 2 determinations. The fitted line gave an IC_{50} of 1.1 μM for this experiment in which control acidification rate was 22 ± 6 fluorescence units/min.

observed (data not shown). The concentration of SK&F 96067 required to double the leak rate was $9 \pm 1\ \mu$M (range, N = 2 experiments). Steady state acidification was monitored by the accumulation of the weak base, aminopyrine. SK&F 96067 inhibited the aminopyrine accumulation ratio with an IC_{50} of $0.06 \pm 0.01\ \mu$M (range, N = 2 experiments, Fig. 5). This greater potency suggested the SK&F 96067 itself was accumulated into the vesicle under these circumstances and was consistent with a site of action on the lumenal (intravesicular) face of the enzyme.

Additional data supporting a lumenal site of action for SK&F 96067 were obtained using the K^+-competitive inhibitor SCH 28080. This compound

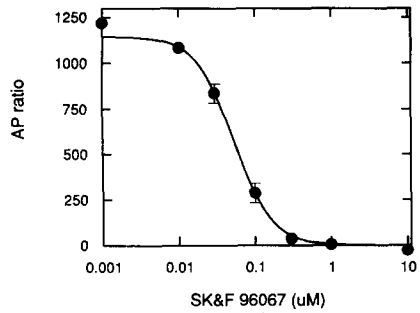

Fig. 5. Effect of SK&F 96067 on steady state acidification in intact gastric vesicles. The aminopyrine accumulation (AP) ratio was determined as described in Materials and Methods over a range of concentrations of SK&F 96067. The IC_{50} value in this experiment was 0.053 μM. Error bars show the range from duplicate determinations. Control AP ratio in this experiment was 1110 ± 8.

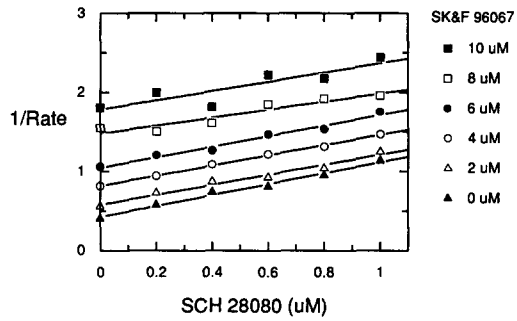

Fig. 6. Yonetani–Theorell plot for SK&F 96067 and SCH 28080. K^+-stimulated ATPase activity was determined at pH 7.0 in the combined presence of SK&F 96067 and SCH 28080 as shown. Parallel lines indicate mutually exclusive binding of the two inhibitors. Values shown are from single determinations of enzyme activity.

has previously been shown to act at the lumenal face of the $(H^+ + K^+)$-ATPase. The K^+-stimulated ATPase activity of lyophilized gastric vesicles was determined in the presence of combinations of concentrations of SK&F 96067 and SCH 28080. These data were then analysed by the method of Yonetani and Theorell [19]. The set of parallel lines so produced (Fig. 6) indicated that the two inhibitors could not bind to the enzyme at the same time and was consistent with SK&F 96067 acting at the same lumenal site as SCH 28080.

Since SK&F 96067 is a weak base (pK_a 6.53), it will tend to accumulate into acidic compartments. Thus, under conditions of acid transport, its protonated form should accumulate at the lumenal (acidic) face of the $(H^+ + K^+)$-ATPase. In view of this, it is of interest to assess whether protonation affects the ability of SK&F 96067 to inhibit the $(H^+ + K^+)$-ATPase. Inhibition was assessed over the pH range 6.61 to 8.08, where the proportion of SK&F 96067 in the protonated form is expected to vary between 45 and 3%. In order to correct for any

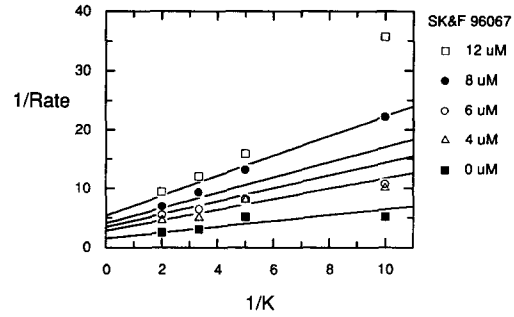

Fig. 7. Inhibition of K^+-stimulated ATPase activity by SK&F 96067 at pH 8.08. K^+-stimulated ATPase activity was determined at pH 8.08 over a range of K^+ and SK&F 96067 concentrations as shown. Data were then plotted after the method of Lineweaver and Burk [14]. The ordinate axis is the reciprocal of the enzyme rate (in μmol/min/mg protein) and the abscissa is the reciprocal of the K^+ concentration (in mM). The lines drawn represent the best fit to a mixed pattern of inhibition with the following parameter estimates: V_{max} = 0.64 I.U./mg, K_m = 0.32 mM, K_{is} = 4.9 μM, K_{ii} = 4.8 μM.

changes to the enzyme over this pH range the permanently cationic inhibitor, N-Me SCH 28080, (Fig. 1) was studied in parallel and the potency of SK&F 96067 expressed relative to this compound. At all pH values, the slopes and intercepts of the Lineweaver–Burk plots were a linear function of inhibitor concentration (data not shown). This implied that only one molecule of inhibitor could interact with the enzyme at any one time and that the inhibition equations fitted to these data were appropriate.

At all of the pH values studied, N-Me SCH 28080 inhibited K^+-stimulated ATPase activity competitively with respect to K^+. In only one experiment of four at pH 8.08 were the data better fitted to a mixed pattern of inhibition than to the competitive pattern. However, in this case, the value of K_{ii} was seventy-fold higher than that of K_{is} and this constant was only poorly defined (its error estimate was 50% of the value of K_{ii}). It was therefore concluded that overall the inhibition by N-Me SCH 28080 at this pH was best described by a competitive pattern.

At pH 6.61, SK&F 96067 inhibited K^+-stimulated ATPase in a manner that was essentially competitive with respect to K^+. In one experiment of the four, a mixed rather than competitive pattern of inhibition was found. Therefore it was concluded that the overall pattern of inhibition at this pH was competitive. At pH 7.0 the inhibition of K^+-stimulated ATPase activity was competitive with respect to K^+ (Fig. 2). In two experiments of seven at this pH the data were better fitted to a mixed pattern of inhibition than to the competitive pattern. However, in both cases, the value of K_{ii} was twenty-seven-fold higher than that of K_{is} and this constant was only poorly defined (the error estimate of K_{ii} was 39–50% of its value), it was therefore concluded that overall the inhibition by SK&F 96067 at this pH was best described by a competitive pattern. At pH 7.58 a clear intercept effect was observed on the Lineweaver–Burk plots in addition to the effect on

Table 1. Effect of pH on kinetic constants

pH	SK&F 96067 K_{is} (μM)	SK&F 96067 K_{ii} (μM)	N-Me SCH 28080 K_{is} (μM)	Relative potency
6.61	0.97 ± 0.16	—	0.060 ± 0.016	0.062 ± 0.019
7.0	0.49 ± 0.02	—	0.026 ± 0.004	0.053 ± 0.011
7.58	2.97 ± 0.73	8.1 ± 1.9	0.084 ± 0.029	0.028 ± 0.012
8.08	3.1 ± 1.8	6.0 ± 0.8	0.041 ± 0.006	0.013 ± 0.008

Relative potency is defined as K_{is} for N-Me SCH 28080 divided by K_{is} for SK&F 96067. Data shown are the means ± SEM or range (N = 2–4 experiments).

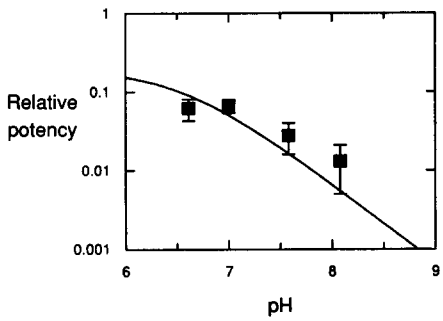

Fig. 8. Relative potency of SK&F 96067 as a function of pH. The potency of SK&F 96067 relative to that of N-Me SCH 28080 was plotted as a function of pH. The line drawn through the points represents the expected behaviour of a compound with the same pK_a as SK&F 96067 (6.53) which is only active as the protonated form. The pK_a determines the horizontal placement of this line. The position of this curve has been arbitrarily adjusted in the vertical direction to pass close to the data points at pH 6.61 and 7.0. The error bars represent the SEM of the relative potency as shown in Table 1.

the slope, indicating a mixed pattern of inhibition. This was confirmed by the kinetic analysis in each of the three experiments. At pH 8.08 the shift in kinetic mechanism of SK&F 96067 away from a competitive pattern continued (Fig. 7). In two of three experiments a mixed pattern was identified. In the third experiment, an uncompetitive pattern of inhibition was indicated.

The combined data for the effect of pH on the inhibition constants of SK&F 96067 and N-Me SCH 28080 are shown in Table 1. As the pH increased from 6.61 to 8.08, the relative affinity of SK&F 96067, as defined by the inhibition constant K_{is}, decreased progressively (Fig. 8). This was consistent with the protonated form of SK&F 96067 being the major inhibitory species.

DISCUSSION

There have been a number of reports of reversible inhibitors of the gastric $(H^+ + K^+)$-ATPase. One of the most extensively studied classes of compounds is that of the imidazopyridines, best exemplified by SCH 28080 [20, 21]. These compounds appear to inhibit the ATPase by interacting at a site on the lumenal (extracellular) face of the ion pump [22]. Since they are also K^+-competitive inhibitors of ATPase activity [20–22] it has been speculated that the inhibitor binding site is at, or very close to, the site associated with the uptake of K^+ by the $(H^+ + K^+)$-ATPase.

Another structural class of $(H^+ + K^+)$-ATPase inhibitor is that of the 4-amino-quinolines. These compounds have been shown to be effective inhibitors of the $(H^+ + K^+)$-ATPase *in vitro* and of acid secretion *in vivo* [23]. One of these compounds, SK&F 96067, has now been studied in more detail.

Like SCH 28080, SK&F 96067 was a reversible, K^+-competitive inhibitor at pH 7.0. Thus, at this pH, K^+ and inhibitor could not bind to the $(H^+ + K^+)$-ATPase simultaneously. Furthermore, the Yonetani/Theorell analysis indicated that both inhibitors could not bind at the same time. Although mutually exclusive binding cannot be taken as proof of a common site, the interactions of SCH 28080, SK&F 96067 and the active cation, K^+ were consistent with the hypothesis that all three interacted at a common site.

The $(Na^+ + K^+)$-ATPase is also an ion pump with a lumenal K^+-uptake site. This protein is also closely related to the $(H^+ + K^+)$-ATPase in evolutionary terms, sharing a 63% identity in primary amino acid sequence [24]. Nevertheless, SK&F 96067 showed a thirty-two-fold selectivity for the $(H^+ + K^+)$-ATPase over the $(Na^+ + K^+)$-ATPase, suggesting that the inhibitor binding sites on these two proteins differed significantly. This also suggested that one might expect to be able to produce effective inhibition of $(H^+ + K^+)$-ATPase activity *in vivo* without having any effects on the $(Na^+ + K^+)$-ATPase activity.

Two methods were used to assess the effects of SK&F 96067 on hydrogen ion transport. The lack of an effect on the hydrogen ion leak rate at concentrations below 10 μM, showed that the reduction in acidification observed at lower concentrations resulted from a direct inhibition of the $(H^+ + K^+)$-ATPase in these systems. The initial rate of acidification was followed using acridine orange quenching. Since the initial rate was being measured, acidification of the vesicle interior had only just commenced. Thus one might not expect there to be a significant pH gradient upon which the weak base SK&F 96067 could accumulate. Consistent with this, the IC_{50} of 0.84 μM was similar to that observed for the inhibition of K^+-stimulated ATPase activity at pH 7.0. Steady state acidification was measured by

the accumulation of the radiolabelled weak base, aminopyrine. Under these circumstances a large pH gradient was able to form upon which SK&F 96067 could accumulate. Here, the potency of SK&F 96067 was more than 10 times greater against aminopyrine accumulation supporting the view that the $(H^+ + K^+)$-ATPase was sensitive to the intravesicular concentration of SK&F 96067. This was consistent with a lumenal site of action for this compound. Correspondingly, structure/activity studies on a range of 4-aminoquinolines showed that those with properties that prevented free membrane permeability were weak inhibitors in this assay [23].

The weak base accumulation of SK&F 96067 would result in high concentrations of the protonated form of this compound at its site of action. In view of this, it is of interest to assess whether protonation affects the ability of SK&F 96067 to inhibit the $(H^+ + K^+)$-ATPase.

The inhibition constant K_{is}, representing the affinity of SK&F 96067 for the enzyme in the absence of bound K^+, was followed as a function of pH and compared with the proportion of SK&F 96067 in the protonated form. However, any changes seen in the K_{is} for SK&F 96067 could have been due either to protonation of the inhibitor or to a pH-dependent change to the $(H^+ + K^+)$-ATPase itself which affected inhibitor binding. To correct for changes to the enzyme, a second inhibitor, N-Me SCH 28080, was also studied. The affinity of this compound, being a permanent cation, could only be sensitive to changes to the enzyme and not to its own charged state. It was assumed that N-Me SCH 28080 bound to the same site on the $(H^+ + K^+)$-ATPase and therefore reported on enzyme changes at the appropriate site. This was supported by two observations. Firstly, N-Me SCH 28080 was also a K^+-competitive inhibitor of the $(H^+ + K^+)$-ATPase at pH 7.0 and secondly the binding of both SK&F 96067 and N-Me SCH 28080 were mutually competitive with a third K^+-competitive inhibitor, SCH 28080 (Fig. 6, [22]). Thus, by considering the ratio of potency between SK&F 96067 and N-Me SCH 28080 as a function of pH, those changes due to the protonation of SK&F 96067 could be followed. As seen in Fig. 8, the relative potency of SK&F 96067 decreased as the pH increased. This was consistent with the protonated form of SK&F 96067 being more potent. The line on this figure shows the way in which relative potency might be expected to vary for an inhibitor with a pK_a of 6.53 if only the protonated form was active and the neutral form had no affinity for the enzyme. Whilst the experimental data are in broad agreement with this hypothesis, the relative potency does not decrease by quite the theoretically expected amount at high pH values. This could be accounted for if the neutral form of SK&F 96067 had a finite potency for the $(H^+ + K^+)$-ATPase. Thus as the proportion of protonated form decreased, that inhibition due to the neutral form would be revealed. Such binding for the neutral form of SK&F 96067 would still be competitive with respect to K^+. An alternative explanation might be that the pK_a of SK&F 96067 on the enzyme was not the same as that determined in free solution. Indeed, SK&F 96067, in common with many of the reversible $(H^+ + K^+)$-ATPase inhibitors identified to date, is strongly hydrophobic and might be expected to bind to a hydrophobic site on the enzyme. In such an environment, the pK_a could differ significantly from that in free aqueous solution. Whether or not the neutral form of SK&F 96067 could bind to the $(H^+ + K^+)$-ATPase, the decrease in relative potency as the pH increased, indicated that the protonated form of SK&F 96067 was the preferred inhibitory species.

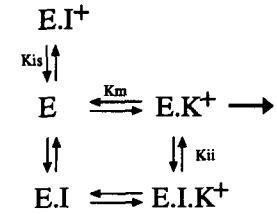

Fig. 9. Kinetic scheme for the interaction of SK&F 96067 with the $(H^+ + K^+)$-ATPase. Free enzyme is able to bind either protonated SK&F 96067 (I^+) with a dissociation constant, K_{is} or K^+ with an apparent Michaelis constant, K_m. These interactions describe the competitive inhibition seen at neutral and acidic pH values. Under alkaline conditions, the enzyme/K^+ complex is also able to bind the neutral form of SK&F 96067 (I) with a dissociation constant of K_{ii}. The binding of I to the free form of the enzyme is also shown. This interaction would contribute to the overall K_{is} term and could account for the deviation of data in Fig. 8 above the theoretical line.

The change in the kinetic mechanism of SK&F 96067 as the pH increased was not expected. Such a change was not seen for N-Me SCH 28080 nor was it seen when a similar analysis was performed on the K^+-competitive inhibitor, SCH 28080 [22]. The detection of mixed inhibition kinetics at pH 7.58 and above indicated that the binding of SK&F 96067 and K^+ were no longer mutually exclusive. That this effect was not observed for N-Me SCH 28080 and SCH 28080 suggests that the change was due to the particular inhibitor being studied rather than to changes in the binding site on the enzyme. This behaviour might be explained if, unlike the other two inhibitors, the neutral form of SK&F 96067 were also able to bind to the $(H^+ + K^+)$-ATPase. Thus, as the competitive inhibition due to the decreasing amount of protonated SK&F 96067 became less important, so the mixed inhibition due to the neutral form binding with K^+ might be revealed.

The linearity of the secondary plots of slope and intercept as a function of inhibitor concentration suggested that only one molecule of inhibitor could bind to the ion pump at any one time. A scheme consistent with this observation is shown in Fig. 9. The protonated form of SK&F 96067 interacts in a K^+-competitive manner, whilst the neutral form is able to bind in both the presence and absence of K^+. At pH 7.0 and below, the competitive effect dominates the overall behaviour.

In vivo, the lumenal binding site for SK&F 96067 on the $(H^+ + K^+)$-ATPase will normally face a highly acidic environment. Because SK&F 96067 is a weak base (pK_a 6.53), this favours the accumulation

of the protonated form of SK&F 96067 at its site of action. Thus the observation that the protonated form of SK&F 96067 is the preferred inhibitory species is consistent with the major form of the inhibitor present at that site. The mechanism of action of SK&F 96067 under conditions *in vivo* is more difficult to predict. On the assumption that the change in kinetic mechanism away from a competitive pattern at pH values above 7.0 was due to the effect of the neutral form of SK&F 96067, then it is reasonable to assume non-competitive effects would not occur *in vivo*, owing to the negligible concentrations of neutral SK&F 96067 at acidic pH values. Thus one might expect SK&F 96067 to inhibit acid secretion *in vivo* in a competitive manner with respect to K^+.

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