Pergamon

Biochemical Pharmacology, Vol. 50, No. 10, pp. 1543–1549, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02020-0

PROPERTIES OF THE REVERSIBLE, K^+-COMPETITIVE INHIBITOR OF THE GASTRIC (H^+/K^+)-ATPase, SK&F 97574. I. *IN VITRO* ACTIVITY

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(*Received* 1 *February* 1995; *accepted* 1 *June* 1995)

Abstract—SK&F 97574 (3-butyryl-4-(2-methylamino)-8-(2-hydroxyethoxy)quinoline), is a potent inhibitor of the (H^+/K^+)-ATPase in membrane vesicles isolated from porcine gastric mucosa. It inhibits (H^+/K^+)-ATPase activity in lyophilised vesicles in a kinetically competitive manner with respect to the activating cation, K^+, with an inhibition constant (K_i) of 0.46 ± 0.003 μM. Inhibition of (H^+/K^+)-ATPase activity is freely reversible. Binding of SK&F 97574 was shown to be mutually exclusive and the previously reported reversible (H^+/K^+)-ATPase inhibitors, SCH 28080 and MDPQ. Therefore, despite its structural dissimilarity, SK&F 97574 appears to bind to the same lumenal region of the (H^+/K^+)-ATPase identified as the binding site for these compounds. SK&F 97574 is a weak base (pK_a = 6.86), and would therefore be expected to accumulate in the acidic compartment at the lumenal face of the parietal cell. In intact gastric vesicles (which have the lumenal face of the ATPase on the interior), SK&F 97574 inhibited ATP-dependent H^+-transport with a similar potency to ATPase activity. SK&F 97574 is therefore relatively membrane permeable, and would be predicted to gain access readily to its site of action *in vivo*. The effect of pH on inhibition of H^+/K^+-ATPase activity by SK&F 97574 is consistent with its being active only in its protonated form. The selectivity of SK&F 97574 for the gastric (H^+/K^+)-ATPase was tested by examining its ability to inhibit a closely related *p*-class pump, the (Na^+/K^+)-ATPase from dog kidney. SK&F 97574 was found to have a 60-fold greater sensitivity for the former enzyme. The (Na^+/K^+)-ATPase was not inhibited in a K^+-competitive manner by SK&F 97574, indicating an entirely different, probably nonspecific, mechanism.

Key words: gastric (H^+/K^+)-ATPase; inhibitor; mechanism; competitive; membrane vesicle

The gastric (H^+/K^+)-ATPase of the parietal cell is the ion pump responsible for acid secretion in the stomach, and as such has been identified as a pharmacological target for the development of drugs to treat acid-related diseases (e.g. [1]). Long-lasting inhibition of the (H^+/K^+)-ATPase by drugs in the substituted benzimadazole class, such as omeprazole and lanzoprazole, has been shown effective in the treatment of peptic ulcer disease (e.g. [2]). However, such agents irreversibly inactivate the ATPase, and the return of acid secretion following such inhibition probably requires *de novo* synthesis of new pumps [3]. A freely reversible inhibitor of the (H^+/K^+)-ATPase may allow greater control over the duration of suppression of acid secretion [4].

During active acid secretion, the gastric lumenal face of the gastric (H^+/K^+)-ATPase faces an extremely acidic environment (around 160 mM HCl). This provides the opportunity to target a weakly basic inhibitor to its site of action. Since the lumenal face of the (H^+/K^+)-ATPase possesses a binding site for the secondary transported ion, K^+, inhibitors that compete with K^+ for binding to the ATPase and contain the appropriate physico-chemical properties to accumulate at the lumenal face of the (H^+/K^+)-ATPase *in vivo* may have excellent potential to be used therapeutically as anti-secretory agents. A number of structural classes of reversible (H^+/K^+)-ATPase inhibitor have now been described (e.g. [1, 5, 6]), but none of these compounds have been successfully used clinically. In this and an accompanying paper, the *in vitro* and *in vivo* properties of a new reversible (H^+/K^+)-ATPase inhibitor, SK&F 97574,§ which has considerable potential therapeutic value, are described.

MATERIALS AND METHODS

Porcine gastric vesicles

Pig stomachs were obtained fresh from a local abattoir and transported to the laboratory on ice. Gastric vesicles were prepared from the fundic mucosa using an adaptation of the method of Saccomani [7] described by Keeling *et al.* [6]. Vesicles were frozen in liquid nitrogen and stored at −70°C before use. For H^+-transport measurements, intact vesicles were used, but for all other experiments vesicles were permeabilised by washing in hypotonic buffer and lyophilised overnight [6].

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§ Abbreviations: CDTA, 1,2-diaminocyclohexanetetraacetic acid; DMSO, dimethyl sulfoxide; MDPQ, 1-(2-Methylphenyl)-4-methylamino-6-methyl-2,3-dihydropyrrolo[2,3-c]quinoline; SK&F 97574, 3-butyryl-4-(2-methylamino)-8-(2-hydroxyethoxy)quinoline; 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; K_{mapp}, apparent Michaelis constant; and ΔpH, pH gradient.

Measurement of ATPase activity in lyophilised vesicles

Lyophilised vesicles (0–6 μg protein) were incubated for up to 30 min at 37°C in medium containing 0–10 mM KCl, 10 mM Pipes-Tris pH 7.0, 2 mM $MgSO_4$, and 2 mM Na_2ATP. All reactions were started by adding enzyme. Reactions were stopped and inorganic phosphate release determined using the method of Yoda and Hokin [8]. Briefly, one mL of ice-cold acid molybdate (3.6% w/v ammonium molybdate in 12% v/v perchloric acid) and 3 mL of N-butylacetate were added, and then the reaction tubes were mixed and the solvent and aqueous phases separated by centrifugation (2000 rpm for 5 min; Beckman J6B centrifuge). The inorganic phosphate released from ATP was measured from the absorbance at 310 nm of the phosphate molybdate complex in the solvent phase, in comparison to an inorganic phosphate standard curve (0–200 nmoles) prepared in each experiment. In each experiment, assay blanks were determined either in the absence of vesicles or KCl. ATPase activity was linear with respect to time for at least 30 minutes under all experimental conditions used.

The effect of inhibitors on the phosphate assay was corrected by using controls in which inhibitor was added to incubation mixture in which the enzyme and ATP had been replaced by 50 or 100 nmoles of phosphate. The effect of the inhibitor on the recovery of phosphate was used to correct for results obtained with enzyme incubations in the presence of inhibitor.

Dog kidney (Na^+/K^+)-ATPase (from Sigma) was assayed as described for the gastric (H^+/K^+)-ATPase, except that 100 mM NaCl was included in the reaction mixture.

Inhibition of ATPase activity in lyophilised vesicles

IC_{50} determination. ATPase activity, measured in the presence of 1 mM KCl, was determined in the presence of a range of SK&F 97574 concentrations (0–100 μM). The effect of SK&F 97574 on phosphate release was fitted to a four-parameter logistic equation [9] to obtain a value for IC_{50}.

K^+-kinetic experiments. To investigate the mechanism of action of SK&F 97574, ATPase activity was measured over a range of KCl concentrations (0–10 mM) in the presence of a range of concentrations of SK&F 97574 (0–3 μM). The results obtained for the K^+-stimulated component of ATPase activity (i.e. after subtraction of the activity obtained in the absence of K^+) were fitted to equations describing the patterns of competitive, non-competitive, and mixed inhibition [10] by least squares fitting using the computer programme GRAFIT® [11].

Reversibility of inhibition. The reversal of inhibition of (H^+/K^+)-ATPase activity on removal of SK&F 97574 was tested by incubating vesicles at a high concentration (90 μg protein/ml) in the presence or absence of SK&F 97574 at a concentration close to its IC_{50} value (2 μM). Samples were removed at various times and the amount of phosphate release measured. After 30 min, the incubation mixtures were diluted 20-fold into media containing either no SK&F 97574 or SK&F 97574 at the same concentration as in the original incubation. The concentration of all other components of the incubation except for inhibitor and enzyme were kept constant. The incubation was continued for a further 30 min, with further samples periodically removed to follow the effect of the dilution on the progress of the enzyme reaction.

(H^+/K^+)-ATPase inhibitor interactions

1. Yonetani-Theorell analysis. The interaction of SK&F 97574 and SCH 28080 was analysed using the method of Yonetani & Theorell [12]. ATPase assays were performed using lyophilised gastric vesicles and in the presence of 10 mM KCl and 0–1 μM SCH 28080 and 0–8 μM SK&F 97574. Other conditions were exactly as described above.

2. MDPQ fluorescence measurements. The interaction of the fluorescent pyrroloquinoloine inhibitor, MDPQ, with porcine gastric (H^+/K^+)-ATPase was measured at room temperature using a Perkin-Elmer LS5 fluorimeter and emission and excitation wavelength of 342 and 446 nm, respectively. Quenching of the ATP (i.e. enzyme phosphorylation) dependent component of enzyme-bound MDPQ fluorescence by K^+ or SK&F 97574 was performed essentially as described by Rabon *et al.* [13].

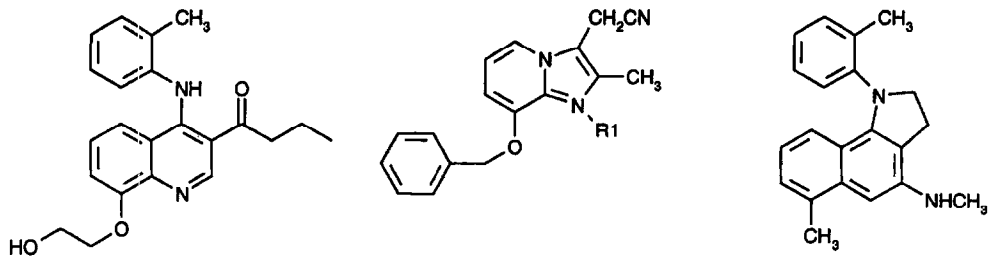
H^+-transport in intact gastric vesicles. ATP-dependent H^+-transport was monitored as the initial rate of fluorescence quenching of the monoamine fluorescent dye, acridine orange. Assays (total volume 1 mL) were performed in a medium containing 10 mM Pipes-Tris pH 7.0, 150 mM KCl, 2 mM $MgSO_4$, 2 mM Na_2ATP, 9 μM valinomycin, 4 μM acridine orange, and intact gastric vesicles (10–20 μg protein). Fluorescence was monitored using a Perkin-Elmer LS-5 fluorimeter using excitation and emission wavelengths of 490 and 540 nm, respectively. Fluorescence quenching was initiated by adding valinomycin to cuvettes containing all of the other reaction components. The effect of SK&F 97574 on passive H^+-conductance was assayed by measuring the initial rate of quench recovery after a pre-formed steady-state ΔpH was collapsed by adding an excess of the Mg^{2+}-chelator, CDTA. Test compound was added immediately before the CDTA.

Chemicals. SK&F 97574, MDPQ, SCH 28080, and N-Me SCH 28080 were synthesised in house. They were dissolved in DMSO and subsequently diluted in water. The maximum concentration of DMSO present in the assays was 0.5%, which had previously been shown to have no effect on enzyme activity. Other chemicals were obtained from Sigma or Aldrich, and were of the highest grade available.

RESULTS

In lyophilised procine gastric vesicles, the acyl-quinoline SK&F 97574 (Fig. 1) inhibited K^+-stimulated ATPase activity (Fig. 2). Under our standard test conditions, in the presence of 1 mM KCl (equivalent to around twice the K_{mapp} at that pH), the IC_{50} was found to be 1.6 ± 0.2 μM (mean ± SEM, $n = 5$ expts.). To assess the selectivity of SK&F 97574 for the gastric ((H^+/K^+)-ATPase, its effects were also tested against the (Na^+/K^+)-ATPase from dog kidney. SK&F 97574 inhibited the (Na^+/K^+)-ATPase, but only at much higher concentrations than required for the gastric (H^+/K^+)-ATPase (Fig. 2; IC_{50} = 95 ± 17 μM (mean ± SEM; $n = 4$ expts.). SK&F 97574 therefore has around 60-fold selectivity for the former enzyme.

The mechanism of inhibition of both (H^+/K^+)-ATPase and (Na^+/K^+)-ATPase activity by SK&F 97574 was investigated by measuring the effect of inhibitor on the response of enzyme activity to variations in the concentration of the activating cation, K^+ (Fig 3A,B). The results were fitted to three-dimensional forms of the equa-



SK&F 97574 **SCH 28080 R1 = H** **MDPQ**

Me-SCH 28080 R1 = CH_3

Fig. 1. Structures of reversible (H^+/K^+)-ATPase inhibitors.

tions describing competitive, non-competitive, and mixed patterns of inhibition [10] using the Grafit programme [11]. In the case of the (H^+/K^+)-ATPase, the data was best described by a competitive pattern of inhibition, since fitting to this model yielded a markedly lower reduced χ^2 value than fitting to either an uncompetitive or mixed pattern of inhibition. In six experiments, a mean K_i value of 0.46 ± 0.03 μM (±SEM) was obtained. Figure 3A shows the data from a single representative experiment.

In contrast, the K^+-kinetics of inhibition of (Na^+/K^+)-ATPase activity by SK&F 97574 did not fit any of the classical patterns of inhibition. Lineweaver-Burk plots of these data showed upward curvature of low K^+ concentrations and were parallel at high K^+ (Fig. 3B). This shows that, in addition to being far less sensitive to SK&F 97574, the (Na^+/K^+)-ATPase interacts with this compound through an entirely different, possibly non-specific, mechanism. The kinetics obtained against the (Na^+/K^+)-ATPase are similar to those described for an earlier reversible (H^+/K^+)-ATPase inhibitor [6].

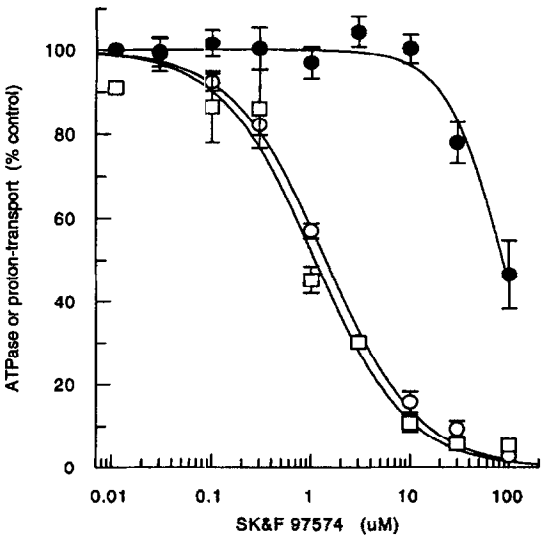


Fig. 2. Effect of SK&F 97574 on (H^+/K^+)-ATPase activity in lyophilised gastric vesicles (○), initial acidification in intact gastric vesicles (□), and (H^+/K^+)-ATPase activity from dog kidney (●). Experimental details were as described in Materials and Methods. Each curve represents the mean (±SEM) of 2–5 individual experiments.

In intact porcine gastric vesicles, the initial rate of ATP-dependent H^+-transport (measured by acridine orange fluorescence quenching) was inhibited with similar potency to ATPase activity (Fig. 2; IC_{50} = 1.0 ± 0.2 μM [mean ± range, n = 2 expts.]). By analogy with previous reversible (H^+/K^+)-ATPase inhibitors [5, 14], the site of inhibitory action of SK&F 97574 is likely to be on the interior of the vesicle membrane. Therefore, the similarity of the IC_{50} values obtained in intact and permeabilised vesicles suggests that SK&F 97574 is rapidly membrane permeable. This, together with the fact that SK&F 97574 is a weak base (pKa = 6.86), indicate that it may well be selectively accumulated at its site of action *in vivo*, the lumenal face of the parietal cell.

In vesicles in which a steady-state ΔpH had been previously established, SK&F 97574 inhibited at slightly lower concentrations than were found to inhibit initial H^+-transport (IC_{50} = 0.2 μM; mean ± SEM, n = 3 expts.). This probably reflects the accumulation of the protonated form of the compound within the acidic interior of the vesicles. However, at higher concentrations, SK&F 97574 caused an increase in the passive H^+-conductance of the membranes (shown by an increase in the rate of collapse of a pre-formed gradient after H^+-transport was stopped by chelating Mg^{2+} with CDTA). The concentration of SK&F 97574 required to double the rate of collapse of ΔpH was 11 ± 1 μM (mean ± range; n = 2 expts.). This uncoupling effect was almost certainly due to outward movement of the protonated form of SK&F 97574 from the vesicle interior.

In vivo, the acidic pH at the lumenal face of the parietal cell is such that SK&F 97574 would be completely protonated (since pH << pK_a). This contrasts with *in vitro* assays where, at pH 7.0, less than half of the compound would be in the protonated form. The effect of protonation of SK&F 97574 was therefore investigated by measuring its inhibition of (H^+/K^+)-ATPase activity at a range of pH (5.7–8.0). The permanently charged quaternary amine (H^+/K^+)-ATPase inhibitor, N-Me SCH28080 (8-benzyloxy-3-cyanomethyl-1,2-dimethylimidazo(1,2-a)pyridinium iodide; Fig. 1; [5]), was used as a standard to correct for changes in the protonation of the enzyme. In a preliminary set of experiments, the K_{mapp} for activation of the (H^+/K^+)-ATPase by K^+ was determined at each pH, yielding the following values: pH 5.7 = 2.86 mM, pH 6.01 = 1.82 mM, pH 6.49 = 0.91 mM, pH 6.83 = 0.61 mM, pH 7.17 = 0.39 mM, pH 7.62 = 0.19 mM, pH 8.01 = 0.068 mM. In subsequent experiments, the K^+ concentration was set equal to K_{mapp}. Under these conditions, $IC_{50} = K_i$ for competitive

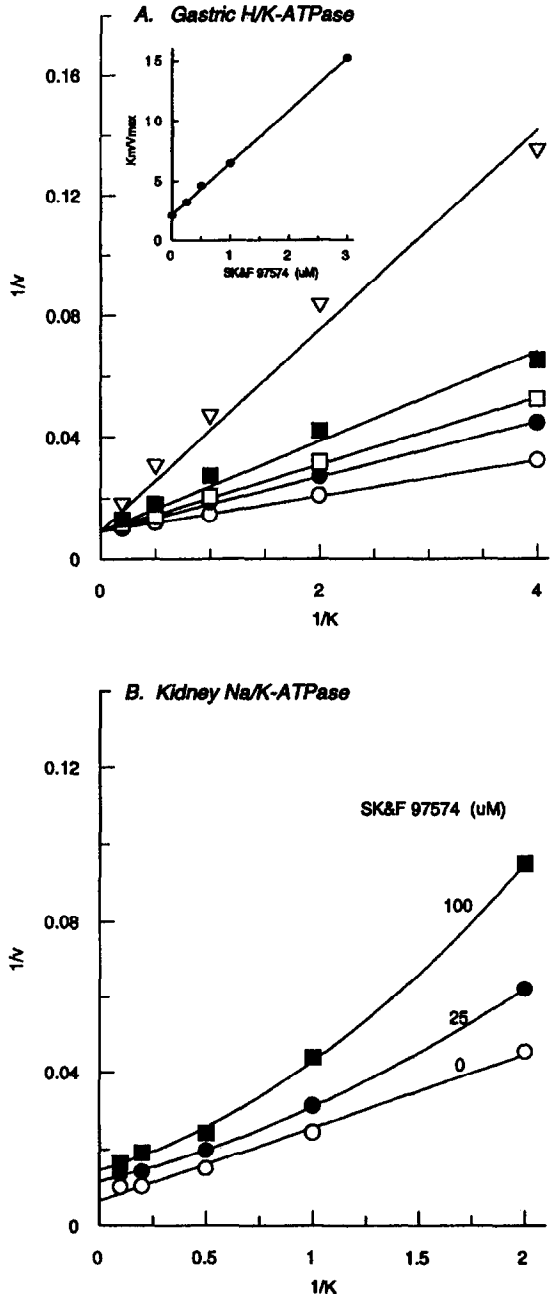


Fig. 3. Lineweaver-Burk plot of the kinetics of K^+-competitive inhibition of (A) (H^+/K^+)-ATPase activity in lyophilised gastric vesicles; and (B) (Na^+/K^+)-ATPase from dog kidney by SK&F 97574. In expeiment A, assays were performed in the presence of 0–10 mM KCl and 0 (○), 0.25 (●), 0.5 (□), 1 (■), and 3 (▽) μM SK&F 97574 Experimental details were otherwise as described in Materials and Methods. Data shown are from a single representative experiment. The fitted lines were obtained by least squares fits to a competitive pattern of inhibition, from which the following constants were obtained: K_m = 0.64 mM, V_{max} = 0.31 IU/mg, K_i = 0.47 μM. The inset shows a secondary plot of the linear relationship between K_m/V_{max} and inhibitor concentration, as expected for this pattern of inhibition. In experiment B, assays were performed in the presence of 0–10 mM KCl and 0 (○), 25 (●), or 100 (■) μM SK&F 97574. Experimental conditions were otherwise as described in Materials and Methods. Data shown are from a single representative experiment.

inhibitors such as SK&F 97574 and SCH 28080 [15], and so K_i could be determined reliably from a single inhibition curve. The relative potency of SK&F 97574 at each pH was then expressed as a ratio to that of N-Me SCH 28080 (Fig. 4). These experiments showed clearly that the relative potency of SK&F 97574 decreases with increasing pH. Moreover, a reasonable fit was obtained to the data by least squares fitting to a simple function based on the assumption that only the protonated form was active [11]. The fitted value of pK_i (i.e. the pH at which half-maximal activity occurs) of 6.79 corresponds well to the pK_a of SK&F 97574 (6.86). This suggests that SK&F 97574 binds to the (H^+/K^+)-ATPase only in the protonated form. Based on this assumption, the K_i for SK&F 97574·[H^+] under our standard assay conditions (pH 7.0) can be estimated to be 0.19 μM (since 42% of the compound will be protonated at that pH).

To test whether inhibition of the (H^+/K^+)-ATPase by SK&F 97574 could be freely reversed, progress curve experiments were performed in which the effect of removing inhibitor (by dilution) on enzyme velocity was tested (Fig. 5). In the initial part of the experiment, SK&F 97574 was present at a concentration equivalent to its IC_{50} value. When the enzyme reaction mixture was diluted 20-fold into medium lacking SK&F 97574 (and so the inhibitor was diluted to a concentration at which inhibition of the (H^+/K^+)-ATPase should not be detected), the enzyme velocity increased immediately to a value similar to that obtained in a control treatment incubated in the absence of inhibitor (Fig. 5), showing that inhibition was freely reversible.

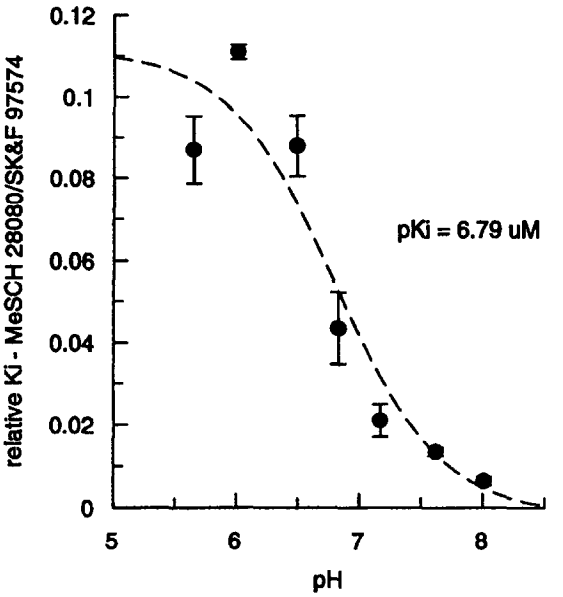


Fig. 4. The effect of pH on the potency of SK&F 97574. At each pH indicated, the K_i for SK&F 97574 and MeSCH 28080 was determined by measuring the inhibitor concentration required for 50% inhibition, when the K^+ concentration was equivalent to K_{mapp} at that pH. The potency of SK&F 97574 is expressed relative to the permanently charged N-Me SCH 28080 to correct for the effect of pH on the (H^+/K^+)-ATPase. Each data point represents the mean (±SEM or range) of 2–3 independent experiments. The fitted line was obtained by least-squares fitting of the data to a simple function on the assumption that only the protonated form of the inhibitor was active [11].

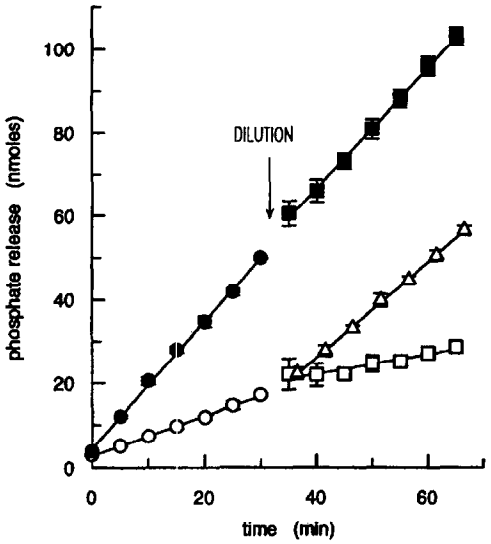


Fig. 5. Reversibility of inhibition of (H^+/K^+)-ATPase activity by SK&F 97574. Lyophilised gastric vesicles were initially incubated in the presence (○) or absence (●) of 2 μM SK&F 97574. At the point indicated, the incubation mixture was diluted 20-fold into media containing either no inhibitor (■,▽) or SK&F 97574 at the same concentration as the original incubation (□). Other conditions were as described in Materials and Methods. The data shown are from a single representative experiment.

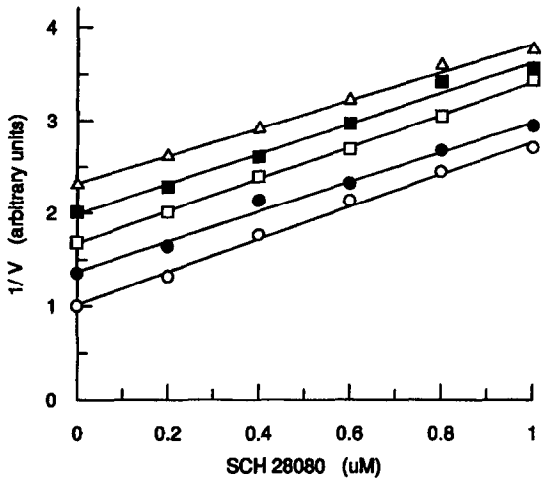


Fig. 6. Yonetani and Theorell analysis of the interaction between SK&F 97574 and SCH 28080. ATPase activity in lyophilised gastric vesicles was measured in the presence of (0–1.0 μM) SCH 28080 and 0 (○), 1 (●), 2 (□), 4 (■), or 8 (△) μM SK&F 97574 and 10 mM KCl. The data shown are the mean of two experiments. Other conditions were as described in Materials and Methods.

The interaction of previous structural classes of reversible inhibitor with the gastric (H^+/K^+)-ATPase have been investigated in detail (for a review see [1]). In particular, photo-affinity derivatives of SCH 28080 [16, 17] have allowed the binding site for this class of compound to be localised to a lumenal region of the pump between the first two trans-membrane spanning regions, designated M1/M2 [14]. We tested whether SK&F 97574 bound in a similar region of the (H^+/K^+)-ATPase using two approaches. Firstly, the method of Yonetani and Theorell [12] was used to examine the interactions between SK&F 97574 and SCH 28080 in their inhibition of (H^+/K^+)-ATPase activity (Fig. 6). In this analysis, parallel slopes in plots of inhibitor concentration reciprocal velocity are predicted for two ligands that bind to the enzyme in a mutally exclusive manner [12]. Figure 6 shows that this was the case for SK&F 97574 and SCH 28080. It therefore seems likely that SK&F 97574 binds to the same lumenal region of the (H^+/K^+)-ATPase as SCH 28080.

Additional data in support of this idea was obtained by examining the interaction of SK&F 97574 with the fluorescent pyrroloquinoline (H^+/K^+)-ATPase inhibitor, MDPQ, Fig. 1; [18, 19, 20]. This compound has been shown to specifically report its binding to the (H^+/K^+)-ATPase via an increase in fluorescence that is further enhanced when the enzyme is phosphorylated by adding Mg·ATP (in the E_2-P form; for a review, see ref. [13]). The MDPQ·E_2-P fluorescence is quenched by K^+ and SCH 28080, presumably due to displacement of bound MDPQ [19, 20]. We therefore investigated the effect of SK&F 97574 on MDPQ · E_2-P fluorescence using K^+ as a standard (Fig. 7). As found by Rabon *et al.* [19], K^+ reduced enzyme-bound MDPQ fluorescence in a saturable manner. In the experiment shown in Fig. 7A, a value for $K_{0.5[K+]}$ of 3.06 mM was obtained, compared to a value of 1.8 mM reported by Rabon *et al.* [19]. This is in good agreement, especially as our experiments were conducted at pH 7.0 compared to pH 7.4 for those of Rabon *et al.* [19] and, over that pH range, the K_{mapp} for K^+ fo the (H^+/K^+)-ATPase changes around two-fold (see above). Adding SK&F 97574 also caused a saturable quench of MDPQ·E_2-P fluorescence (Fig. 7B). However, in this case a linear component of MDPQ quenching was also observed, almost certainly due to spectral interference between the two compounds (Fig. 7B). When this component was subtracted, a value of $K_{0.5[574]}$ of 1.7 μM was obtained. It is interesting to note that the $K_{0.5}$ values obtained for K^+ and SK&F 97574 for quenching of MDPQ · E_2-P fluorescence are in similar ratio (around 5-fold) to their respective dissociation constants.

DISCUSSION

The results presented in this paper show that SK&F 97574 is a potent, reversible inhibitor of the porcine gastric (H^+/K^+)-ATPase. Like other reversible inhibitors of this enzyme, it inhibits in a kinetically competitive manner with respect to the activating cation of the (H^+/K^+)-ATPase, K^+. SK&F 97574 was also shown to have around 60-fold selectivity for the gastric (H^+/K^+)-ATPase relative to the closely related *p*-class pump, the (Na^+/K^+)-ATPase. In addition, the kinetics of inhibition of (Na^+/K^+)-ATPase activity did not fit any of the classical patterns of inhibition, indicating that the mechanism of action was probably nonspecific. It was previously shown that SK&F 96067, an analogue of SK&F 97574, inhibits the human (H^+/K^+)-ATPase with similar potency to the porcine enzyme [1], suggesting that SK&F 97574 may have the potential to be used clinically as an anti-secretory agent.

The kinetics of the interaction between SK&F 97574 and SCH 28080 strongly suggest that binding of these

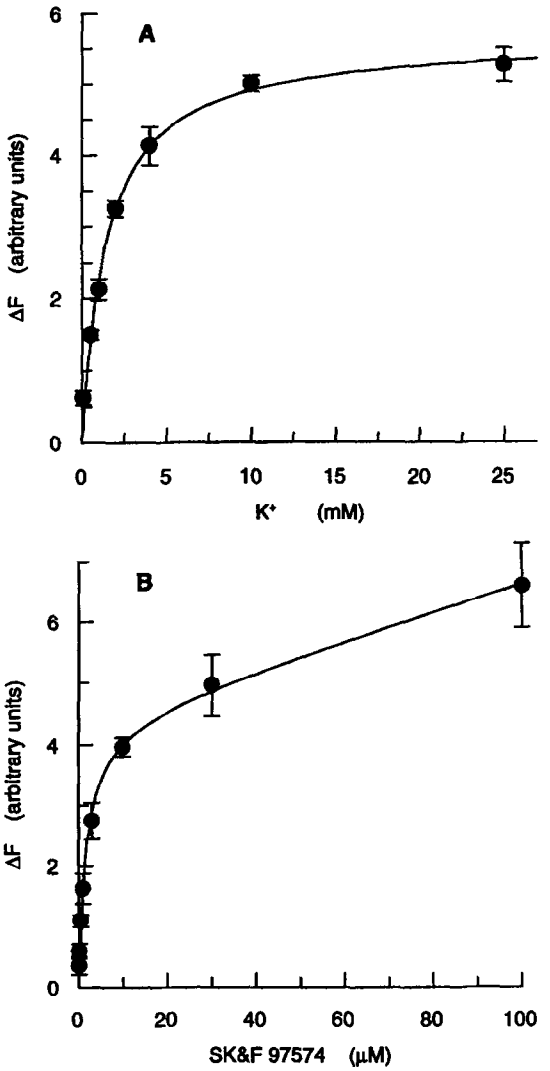


Fig. 7. Quenching of enzyme bound MDPQ fluorescence by (A) K^+ and (B) SK&F 97574. Lyophilised gastric vesicles (100 μg) were incubated in medium containing 50 mM Pipes-Tris pH 7.0 and 0.3 μM MDPQ. After the fluorescence had stabilised, ATP was added to a final concentration of 2mM. This led to a large enhancement of fluorescence. After this signal had stabilised, either KCl or SK&F 97574 was added at the final concentrations shown. The fluorescence signal was then allowed to stabilise and the MDPQ quench relative to enhancement obtained upon adding ATP calculated. In A, the data were fitted to a single Michaelis Menten function, yielding a $K_{0.5[K+]}$ value of 3.1 ± 0.2 mM. In B, the data were fitted to a single Michaelis Menten function plus a linear component (due to spectral interference from SK&F 97574), yielding a $K_{0.5[574]}$ value of 1.70 ± 0.15 μM. The data are from a single representative experiment. Other details are as described in Materials and Methods.

two ligands is mutually exclusive. It therefore seems probable that both compounds bind to the same, lumenally exposed region of the enzyme previously identified as the binding site of photo-affinity derivatives of SCH 28080 [14]. Additional support for this idea was obtained from the fact that SK&F 97574 was effective in displacing the fluorescent inhibitor, MDPQ, from binding to E_2-P, in a similar manner to K^+ and SCH 28080 [19]. Rabon *et al.* [19] have previously shown that the site of interaction of both MDPQ and K^+ with the (H^+/K^+)-ATPase is on the vesicle interior (since the quenching of MDPQ·E_2-P fluorescence in intact gastric vesicles by exogenously added K^+ is dependent upon increasing the K^+ permeability of the membranes by adding valinomycin). The quench of MDPQ·E_2-P fluorescence by SK&F 97574 showed saturation kinetics. In the absence of a measured K_d for MDPQ binding to E_2-P, it is not possible to compare the $K_{0.5}$ for MDPQ fluorescence quenching with the K_i measured for inhibition of catalytic activity. However, it is interesting to note that the ratio of the $K_{0.5}$ values obtained between inhibitor and K^+ for SCH 28080 ($K_{0.5[280]}/K_{0.5[K+]} = 3.7 \times 10^{-4}$; [19]) and SK&F 97574 ($K_{0.5[574]}/K_{0.5[K+]} = 5.4 \times 10^{-4}$; Fig. 7) were similar for both compounds, despite the fact that the K_i for SCH 28080 (0.024 μM; [5]) is around 20-fold lower than that for SK&F 97574. Binding of SCH 28080 to ATPase has been shown to be enhanced when the enzyme is in the E_2-P form [17]. The apparent discrepancy between K_i and $K_{0.5}$ for MDPQ quenching between the two compounds might therefore be due to differences in their relative affinity for the different conformational states of the ATPase (see ref. [13]). It is important to note that although the different classes of reversible inhibitor (i.e. SK&F 97574, SCH 28080, and MDPQ) appear to bind in a *kinetically* mutually exclusive manner, the actual location of the binding site for these ligands does not necessarily physically overlap. The kinetics merely show that the inhibitors compete for the same enzyme forms as K^+ and, presumably, once a single inhibitor (or K^+ ion) is bound, this induces a conformation change that prevents additional inhibitor binding.

In intact gastric vesicles, SK&F 97574 inhibited proton transport to a similar degree as ATPase activity in the absence of a pH gradient, and was more potent in the presence of a pre-formed ΔpH. Given the evidence that it binds to the lumenal side of the enzyme (i.e the vesicle interior *in vitro*), it must therefore be relatively membrane permeable, at least in its unprotonated form. Relatively high concentrations of SK&F 97574 (>10 μM) had an uncoupling effect on intact vesicles, showing that the protonated form of the compound has some membrane permeability. This probably explains why only a modest increase in potency was obtained when the effect of SK&F 97574 was tested in the presence of a preformed ΔpH.

The physico-chemical properties of SK&F 97574 (i.e. it is a lipophilic weak base) suggested that, *in vivo*, it may well accumulate into the acid compartment on the lumenal face of the parietal cell. Under these conditions, virtually all of the enzyme would be present as the protonated form. The effects of pH on inhibition were consistent with this being the inhibitor form that binds to the enzyme. Although the *in vivo* activity of any compound is largely dictated by its bioavailability and metabolism, the *in vitro* properties of SK&F 97574 suggest strongly that it has the potential to be an effective anti-secretory agent *in vivo*. An accompanying paper [21] shows that this is in fact the case.

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