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Inhibition of gastric H^+/K^+-ATPase by substituted imidazo[1,2-*a*]pyridines

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A hydrophobic imidazopyridine, SCH 28080 (3-cyanomethyl-2-methyl-8-phenylmethoxy)imidazo[1,2-*a*]pyridine) has previously been shown to inhibit gastric acid secretion in vivo and in vitro. Studies of isolated gastric H^+/K^+-ATPase have demonstrated that SCH 28080 reversibly inhibited the enzyme and competitively interacted with the K^+-stimulated ATPase and *p*-nitrophenylphosphatase activities of the H^+/K^+-ATPase. To elucidate the mechanism of inhibition further, for example to establish whether the inhibitor interaction occurs on the luminal or the cytosolic side of the enzyme or if compound pK_a influences inhibition, SCH 28080 and three analogues have been studied. We have examined the effects on K^+-stimulated ATPase activity in isolated ion-permeable membrane vesicles at different pH values and KCl concentrations. In ion-tight membrane fractions the effect on acid formation was estimated. The results are in agreement with the hypothesis that the protonated, and thus positively charged, form of SCH 28080 is the active species, and that the inhibitory effect is exerted by binding of the compound to the luminal side of the H^+/K^+-ATPase.

Introduction

Gastric H^+/K^+-ATPase, which has been shown to be the proton pump of the gastric mucosa, is located in the apical membrane of the parietal cell. The enzyme contains a catalytic subunit of approx. 94 kDa [1]. The complete nucleotide sequence of the cDNA for rat stomach H^+/K^+-ATPase has been determined, and the amino acid sequence of the protein deduced. The H^+/K^+-ATPase has eight postulated membrane-spanning sequences as calculated from hydropathy analysis, and in analogy with Na^+/K^+-ATPase and Ca^{2+}-ATPase [2].

Catalysis involves an aspartyl-phosphate intermediate, and the hydrolysis of Mg^{2+}-ATP is inhibited by vanadate ions. Protons are translocated from the cytosolic to the luminal side of the parietal cell, and potassium ions in the reverse direction, in a 1 : 1 relationship and at the expense of hydrolysis of ATP [3,4].

During the catalysis of active transport, the enzyme cycles between two conformational states, generally called E_1 and E_2, where E_1 is the conformation with a high affinity for the proton to be translocated, while E_2 has a high-affinity site for luminal potassium ions to be translocated and released on the cytosolic side of the apical membrane.

A hydrophobic imidazopyridine, SCH 28080 (3-cyanomethyl-2-metyl-8-(phenylmethoxy)im-

Abbreviations: ATP, adenosine triphosphate; Ficoll, nonionic synthetic polymer of sucrose; Pipes, 1,4-piperazinediethanesulphonic acid; Trizma Base, tris(hydroxymethyl)aminomethane.

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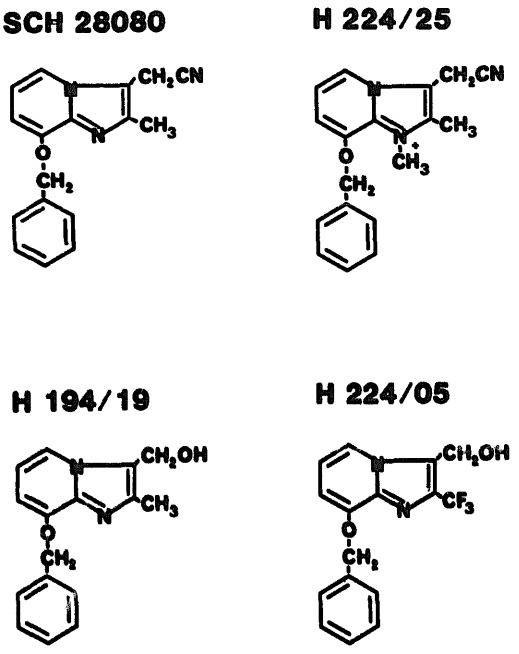

Fig. 1. The four compounds used in this study: SCH 28080 (3-cyanomethyl-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine), H 224/25 (3-cyanomethyl-2,3-dimethyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine), H 194/19 (3-hydroxymethyl-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine) and H 224/05 (3-hydroxymethyl-2-trifluoromethyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine).

idazo[1,2-*a*]pyridine), has previously been shown to inhibit gastric acid secretion in vivo and in vitro [5,6].

Previous studies on isolated H^+/K^+-ATPase showed that the compound reversibly inhibited the enzyme and competitively interacted with the K^+-stimulated ATPase and *p*-nitrophenylphosphatase activities of the H^+/K^+-ATPase [7].

To elucidate the mechanism of inhibition further, the effects of potassium and proton ion concentrations, as well as of compound pK_a, have been investigated. We have studied the inhibition in isolated ion-tight and permeable H^+/K^+-ATPase enriched gastric vesicles [8] and in isolated gastric glands [9] and we have used SCH 28080 and three analogues, H 224/25 (3-cyanomethyl-2,1-dimethyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine), H 194/19 (3-hydroxymethyl-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine), and H 224/05 (3-hydroxymethyl-2-trifluoromethyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine) as inhibitors (see Fig. 1). The compound H 224/25 is a close N-methylated analogue of SCH 28080 and a permanent cation. H 194/19 and H 224/05 are 3-hydroxymethyl analogues between themselves sterically very similar but with quite separate pK_a values due to the 2-trifluoromethyl substitution of H 224/05.

Materials

Gastric membrane vesicles. Gastric membrane vesicles containing H^+/K^+-ATPase were prepared from hog stomach by differential and zonal density gradient centrifugation according to previously published methods [8]. The density gradient was prepared accordingly: 100 ml 8.5% sucrose, 200 ml 7.5% Ficoll in 8.5% sucrose and 300 ml 30% sucrose, all three solutions in 2 mM Pipes adjusted to pH 7.4 with Trizma Base.

The membrane fraction which was fractionated above the Ficoll interface, designated fraction GI, was employed in these studies.

Permeable membrane vesicles. The GI fraction was diluted with 2 mM Pipes-Tris (pH 7.4) to obtain a 1% sucrose concentration. The vesicle preparation was re-centrifuged at $100\,000 \times g$; the resulting pellets were suspended in 2 mM Pipes-Tris, (pH 7.4) and subsequently lyophilized.

Ion-tight membrane vesicles. The GI fraction was diluted in ice-cold 60% (w/w) sucrose to a final concentration of 30% sucrose, instantly frozen and stored at −80 °C.

Gastric glands. Isolated gastric glands were prepared from rabbits, as previously described elsewhere [9]. The minced gastric corpus mucosa from one rabbit was digested with collagenase (12250 units) at 37 °C for about 60 min. After digestion the glands were rinsed three times and resuspended to a concentration of 80 mg wet weight per ml.

Reagents. All chemicals were of the highest grade available. [*dimethylamine*-^{14}C]Aminopyrine (88 mCi/mmol) was obtained from NEN Research Products; SCH 28080 was a gift from Schering Co. (New Jersey, U.S.A.); H 194/19, H 224/05, H 224/25 were synthesized by Hässle Laboratories (Mölndal, Sweden).

Inhibitors. The inhibitors were dissolved and diluted in methanol. Aliquots were pipetted into the incubation media. The final methanol concentration was 1%, which per se had no effect on the enzyme or the gland preparations.

Methods

Determination of ATPase activity. Briefly, this involved preincubation of membrane vesicles (10 μg) and inhibitor for 30 min in 2 mM Pipes-Tris (pH 7.4) and 10 mM KCl at 37°C. An ATP solution was added to give a final concentration of 2 mM ATP and 2 mM $MgCl_2$. The ATPase activity was estimated as release of inorganic phosphate from ATP, as described by LeBel et al. [10]. K^+-stimulated activity was obtained by subtracting the basal Mg^{2+} activity from the enzyme reaction in the presence of K^+ and Mg^{2+}.

Determination of proton transport. Proton transport in ion-tight gastric vesicles was measured as the uptake of Acridine orange, using an Aminco DW-2 dual beam spectrophotometer set at 491 nm and 547 nm [11]. The incubation mixture contained 2 mM Hepes-NaOH (pH 7.4), 2 mM $MgCl_2$, 150 mM KCl, 10 μM Acridine orange and 40 μg protein with or without inhibitor in a final volume of 1 ml. ATP was added to give a final concentration of 2 mM, and 15 μg of valinomycin was added when a stable absorbance was achieved.

Determination of aminopyrine accumulation in gastric glands. The glands were suspended in a medium, pH 7.4, containing (in mM): NaCl, 132.5; KCl, 5.4; $MgCl_2$, 1.2; $CaCl_2$, 1.0; NaH_2PO_4, 1.0; Na_2HPO_4, 5.0; indomethacin, 0.01; glucose 2 mg/ml and albumin 2 mg/ml. Acid formation in the glands was monitored by the uptake of the weak base ^{14}C-aminopyrine [9]. Secretagogue activation of the acid formation was carried out by the addition of 10^{-4} M histamine.

Determination of kinetic variables of the H^+/K^+-ATPase. Four μg/ml membrane protein was incubated in 10 mM Pipes-Tris buffer (pH 7.5 or pH 6.5), 2 mM Na_2ATP, 2 mM $MgCl_2$. In the experiments conducted at pH 7.5, the KCl concentration varied between 0.1 and 9.0 mM and the SCH 28080 concentration between 0.01 and 0.25 μM. In the experiments performed at pH 6.5, the KCl concentrations varied between 0.6 and 15.0 mM and the SCH 28080 concentrations between 0.002 and 0.05 μM.

Protein determination. Protein was determined according to Bradford, using the Bio-Rad Protein Assay kit [12].

Result analysis. All assays were performed in duplicate on three preparations unless otherwise indicated.

The IC_{50} values are defined as the drug concentration that gives 50% inhibition of K^+-stimulated enzyme activity or the drug concentration that gives 50% inhibition of the maximum stimulated aminopyrine accumulation. The 50% inhibitory values were calculated by regression analysis according to Marquardt and Levenberg [13,15] as used in Research System 1, BBN Research System, Cambridge, MA.

Study design. The ATPase activity of the H^+/K^+-ATPase is stimulated by K^+-binding to the luminally oriented high-affinity K^+-site of the E_2 conformation. Previous studies showed that SCH 28080 is kinetically competitive with respect to the K^+-activation of ATPase activity. In this study we wished to determine whether the E_2 conformation of the H^+/K^+-ATPase also has a high-affinity binding-site for luminal SCH 28080. If so, the observed inhibition of K^+-stimulated H^+/K^+-ATPase activity is exerted by the binding of the drug to the luminal side of the enzyme after penetration of the parietal cell apical membrane. To be able to assess the validity of this hypothesis, we have compared the inhibitory effect of SCH 28080 with the effects of another potent inhibitor, H 224/25 (Fig. 1). The latter compound is a permanent cation and, as such, is not inclined to penetrate the membrane of ion-tight vesicles. The results of these experiments would indicate the 'sidedness' of the inhibition.

Previous studies of inhibition by SCH 28080 indicated that the drug was active when protonated. To investigate the effect of protonation further, we observed the inhibition of H^+/K^+-ATPase by SCH 28080 and its corresponding permanent cation at pH 7.3 and at pH 6.4. Since the pK_a value of [SCH 28080]H^+ is 5.5 (Brändström, A. et al., unpublished results), we would expect the potency of the drug to increase as the pH decreases, while the inhibitory effect of the permanent cation should be unaffected by pH. We also compared the steady-state kinetic constants for enzyme inhibition by SCH 28080 at pH 7.5 and pH 6.5. To support the hypothesis that the protonated compound is the active inhibitor, we observed

the effect of compound pK_a on inhibition of the K^+-stimulated ATPase reaction. For this investigation we used two analogues of SCH 28080, namely H 194/19 and H 224/05. The 2-trifluoromethyl derivate, H 224/05, is sterically very similar to the potent inhibitor H 194/19, but dramatically different electronically. Theoretical estimation [14] of the pK_a of [H 224/05]H^+ gives a value of 2, as compared to 6.1 for [H 194/19]H^+. If the protonated compound is the active inhibitor, H224/05 would not, in contrast to H 194/19, inhibit H^+/K^+-ATPase activity at pH 7.4.

Results

Establishment of the side of inhibitor interaction with the H^+/K^+-ATPase

In order to determine the 'sidedness' of inhibitory action, we studied the effects of SCH 28080 and the corresponding cation analogue on three different proton-pump-containing preparations. In permeable vesicles, we estimated the effect on K^+-stimulated ATPase activity, in ion-tight vesicles the effect on proton transport activity and in gastric gland preparations the effect on parietal cell acid formation.

Enzyme activity in permeable vesicles

When the K^+-activated ATPase activity, measured as release of inorganic phosphate from ATP at pH 7.4, is inhibited by SCH 28080 or H 224/25 at 10 and 150 mM KCl, the IC_{50} values for the two compounds at each KCl concentration are very similar. At 10 mM KCl the IC_{50} values are 0.87 ± 0.19 μM and 0.33 ± 0.04 μM for SCH 28080 and H 224/25 and at 150 mM KCl 20.0 ± 6.9 μM for SCH 28080 and 20.5 ± 13.2 μM for H 224/25, (see Fig. 2). The control K^+-stimulated ATPase activities at 10 mM KCl and 150 mM KCl were 49.5 ± 3.8 and 30.6 ± 5.2 μmol $P_i \cdot h^{-1} \cdot$ (mg protein)$^{-1}$, the Mg^{2+}-stimulated ATPase activity was 6.3 ± 3.0 μmol $P_i \cdot h^{-1} \cdot$ (mg protein)$^{-1}$ ($n = 6$).

Proton transport in ion-tight vesicles

Inhibition of the initial rate of H^+ accumulation, as measured by uptake of acridine orange in valinomycin-treated vesicles, was estimated at 150 mM KCl. In contrast to inhibition of K^+-activated ATPase activity in permeable vesicles at 150 mM KCl, the IC_{50} values for SCH 28080 and H 224/25 were separated by almost two orders of magnitude, 0.7 μM and 60 μM (see Fig. 3).

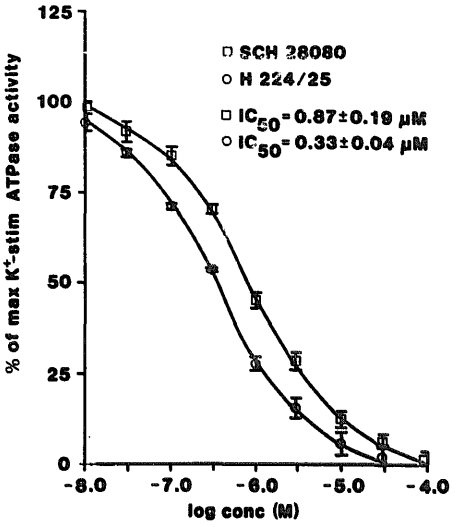

Fig. 2. Inhibition of K^+-stimulated ATPase activity by SCH 28080 and H224/25 at 10 mM KCl. Results are presented as % of control activity ± S.E. The IC_{50} value was calculated from each individual experiment, and is given as mean ± S.D. Control activities ($n = 6$) for K^+ stimulation were 49.5 ± 3.8 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$. Mg^{2+}-stimulated activity was 6.3 ± 3.0 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$.

Acid formation in gastric glands

The distribution of aminopyrine (AP) was used as an index of acid formation in the glands. SCH

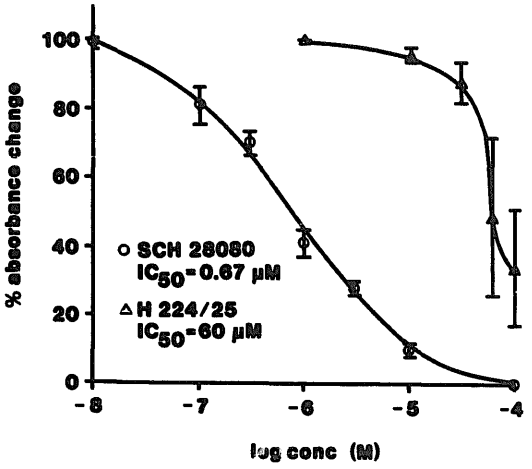

Fig. 3. Effect on initial rate of H^+-accumulation in tight membrane vesicles by SCH 28080 and H224/25. Accumulation was estimated by absorption change of Acridine orange at 491 and 547 nm. The initial slope of the control was set to 100% transport activity. The IC_{50} values were determined from the mean inhibitory curve. Experimental conditions as described in Methods.

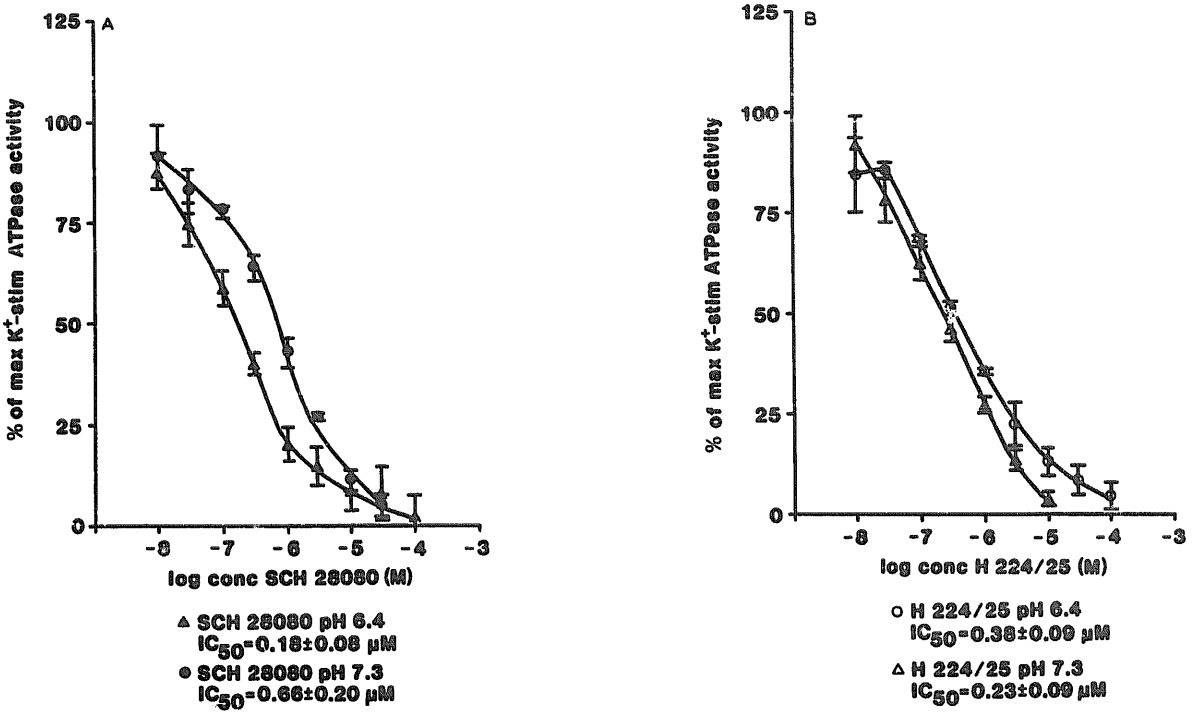

Fig. 4. Inhibition of K^+-stimulated ATPase activity by SCH 28080 (A) and H 224/25 (B) at pH 7.3 and pH 6.4. An IC_{50} value was calculated from each individual experiment and is presented as mean ± S.D. ($n = 3$). Control activities at pH 7.3 for K^+-stimulated and Mg^{2+}-stimulated ATPase activity were 65.5 ± 21.2 and 3.5 ± 4.1 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ ($n = 3$). The corresponding activities at pH 6.4 were 49.1 ± 12.9 and 1.16 ± 3.93 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ ($n = 4$).

28080 inhibits acid formation in stimulated gastric glands, as reported earlier, and the IC_{50} value was 0.2 μM irrespective of the agonist used [5]. In our current study, the permanent cation analogue (H 224/25) had no effect on histamine-stimulated acid formation up to a concentration of 10^{-4} M.

Effects of pH

To investigate the effect of compound protonation on H^+/K^+-ATPase activity, we studied the inhibition of enzyme activity at two different proton concentrations, pH 7.3 and pH 6.4, in permeable vesicles. The inhibitory effect of [SCH 28080]H^+ ($pK_a = 5.5$) was compared to that of its permanent cation analogue, H 224/25. The IC_{50} value for SCH 28080 (for the inhibition of K^+-stimulated ATPase) is lower at pH 6.4 (0.18 ± 0.08 μM) than at pH 7.3 (0.66 ± 0.20 μM). In contrast, the IC_{50} values for the permanent cation, H 224/25, are 0.38 ± 0.09 μM and 0.23 ± 0.09 μM at pH 6.4 and 7.3, respectively, and thus not significantly changed at the two different proton concentrations (see Fig. 4).

Previous studies of isolated H^+/K^+-ATPase established the inhibitory action of SCH 28080 to be K^+-competitive [5]. In this present report, we estimated the steady-state kinetic constants for inhibition of the K^+-stimulated ATPase activity at pH 7.5 and pH 6.5, (see Table I). The inhibition constant at pH 7.5 is 0.049 μM and that at pH 6.5 is 0.011 μM. However, the K_m value for K^+

TABLE I

KINETIC CONSTANTS: EFFECT OF SCH 28080 ON K^+-ACTIVATED ATPase ACTIVITY AT pH 7.5 AND pH 6.5

The kinetic constants presented are means ± S.D. for three different enzyme preparations. Two determinations were made for each preparation. V_{max} and K_m values were determined from the slopes and intercepts calculated by linear regression on the corresponding Eadie-Hofstee plots. The K_i values were calculated from secondary plots of K_m app/V_{max} versus SCH 28080 concentrations.

	ATPase activity	
	pH 7.5	pH 6.5
V_{max} (μmol·mg^{-1}·h^{-1})	56.2 ±14.3	36.9 ±3.1
K_m (K^+) (mM)	0.337 ± 0.028	2.06 ± 0.65
K_i (nM)	49.0 ± 6.0	11.0 ±0.9

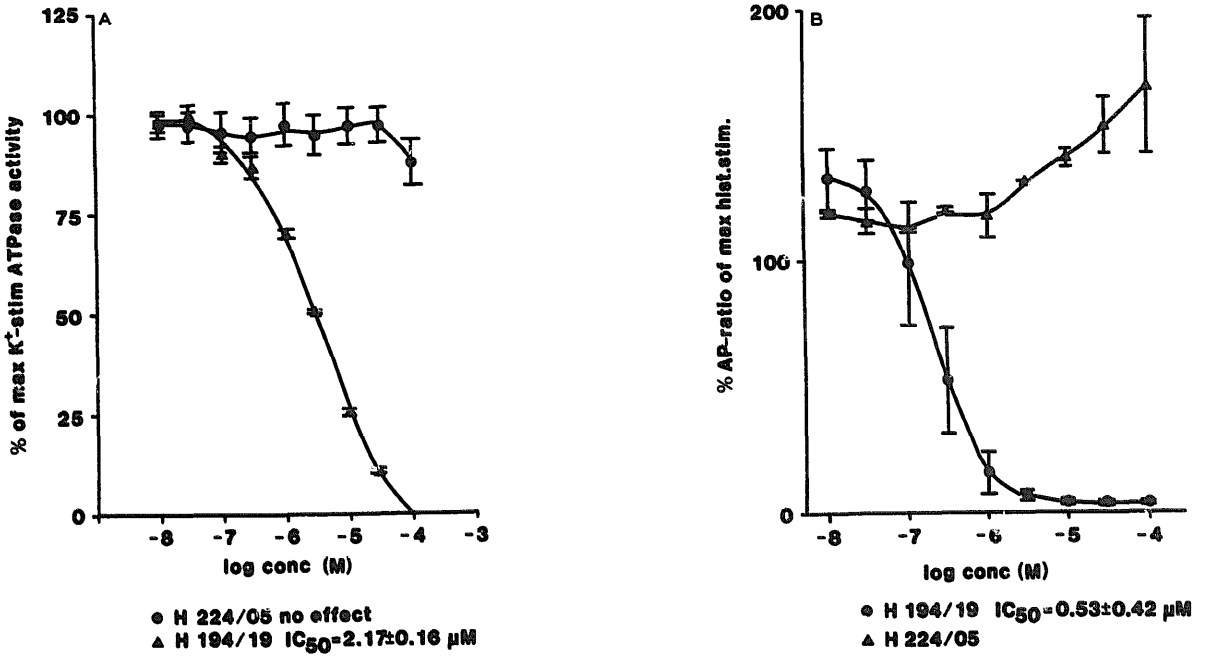

Fig. 5. (A) Effects on K^+-stimulated ATPase activity of H 194/19 and H 224/25. Results are presented as % of control K^+-stimulated ATPase activity ± S.D. The IC_{50} value is mean ± S.D. from three individual curves. Control activities were 111.7 ± 35.5 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ and 7.0 ± 0.6 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ for K^+- and Mg^{2+}-stimulated ATPase activity, respectively. (B) Effects on acid formation in gastric glands. The glands were stimulated by 10^{-4} M histamine. Results are expressed as % aminopyrine accumulation ratio, and the maximal histamine stimulation is set to 100%. The IC_{50} value for H194/19 is mean ± S.D. from three experiments. Control AP-accumulation ratios were 19.6 ± 9.7 (basal) and 122.5 ± 44.4 (histamine 10^{-4} M).

increases from 0.34 mM at pH 7.5 to 2.1 mM at pH 6.5.

Effects of compound pK_a

Another way of studying the effect of compound protonation on enzyme activity is to compare the IC_{50} values for enzyme inhibition by two sterically equivalent but electronically different analogues. H 194/19, a 3-hydroxymethyl analogue of SCH 28080 inhibits K^+-stimulated ATPase activity with an $IC_{50} = 2.2 \pm 0.2$ μM, in permeable vesicles. The pK_a of [H 194/19]H^+ is 6.1, as estimated by potentiometric pK_a determination [15].

When the 2-substituent is also changed from a methyl to a trifluoromethyl group, as in compound H 224/05, the pK_a is approx. 2 and no inhibitory effect can be observed in the concentration range of 10^{-8} to 10^{-4} M (see Fig. 5A). Acid formation in glands is inhibited by H 194/19, ($IC_{50} = 0.53 \pm 0.42$ μM), while H 224/05 produces no inhibition even at 10^{-4} M, (see Fig. 5B).

Discussion

That SCH 28080 inhibits gastric acid formation has been reported in several publications [5,6]. The action of SCH 28080 is similar to that of omeprazole, as inhibition of acid formation is carried out by inhibition of the proton pump [7]. However, in contrast to omeprazole, the inhibition of H^+/K^+-ATPase by SCH 28080 is K^+-competitive, and readily reversible in isolated H^+/K^+-ATPase preparations.

Gastric H^+/K^+-ATPase has two affinities for K^+, due to the existence of two enzyme conformations, E_1 and E_2. The E_1 form has a low-affinity site for K^+, and it is from this enzyme form that K^+ is released to the cytosol of the parietal cell. A high-affinity site ($K_m = 0.3$ mM at pH 7.4 and 2 mM ATP) for K^+ is exposed to the luminal side, and this is the K^+-uptake site of the E_2 conformation.

The outside of isolated ion-tight vesicles corresponds to the cytosolic side of the parietal cell. Thus, when ion-tight vesicles are used for trans-

port studies, it is possible to discriminate between the two potassium sites by using an inhibitor that most likely can not penetrate the vesicle membrane. In the inhibition experiments on proton transport in ion-tight vesicles, the permanent cation analogue, H 224/25, was two orders of magnitude less effective than SCH 28080.

These results, combined with those from the permeable membrane preparation, demonstrate that SCH 28080 interacts with the luminal high affinity K^+-site, since inhibition of the cytosolic K^+-site would be expected to give similar IC_{50} values for both H 224/25 and SCH 28080.

Control experiments with permeable membrane preparations show that the high KCl concentration (150 mM) used in the transport studies does not by itself cause a difference in potency between the two drugs. The inhibition by H 224/25, albeit observed only at high concentrations, can be explained by interaction with the low-affinity K^+ site exposed to the medium or, alternatively, with the high-affinity K^+-site, by slow leakage of the compound into the interior of the vesicle. Since no inhibition of acid formation in gastric glands by H 224/25 can be observed after 60-min incubation at 37°C, the first explanation seems more plausible.

In an earlier report from our laboratory (Ref. 7), it was shown that buffering of the acid space of the gastric glands gave a 10-fold decrease in the potency of SCH 28080, and one possible explanation is that the active species of the drug is the protonated form. The availability of a permanent cation analogue enabled us to study the effect of pH on the inhibition of H^+/K^+-ATPase in more detail.

Inhibition of H^+/K^+-ATPase by H 224/25 at pH 7.3 was more pronounced than at pH 6.4, however, this was not significant. The IC_{50} values for SCH 28080 under the same experimental conditions decreased from 0.66 μM to 0.18 μM. Since the pK_a of $[SCH\ 28080]H^+$ is 5.5, the IC_{50} value at pH 6.4, assuming the protonated form is the active species, can be calculated to be 8-times less than the IC_{50} at pH 7.3. We observe a significant 4-fold increase in potency of SCH 28080 at a higher proton concentration, although this does not amount to what could theoretically be expected. The effect on K_i for SCH 28080 inhibition of potassium-stimulated ATPase activity is in accordance with these data. A 10-fold increase in the concentration of the protonated compound only gives a 5-fold decrease in K_i. This inconsistency might indicate that protonation of H^+/K^+-ATPase amino acid side-chains counteracts the increase in potency of SCH 28080.

Two sterically equivalent analogues, H 194/19 and H 224/05, were used to study the influence of compound pK_a on inhibitory effect. The IC_{50} for inhibition of K^+-stimulated ATPase activity by SCH 28080 and H 194/19 at pH 7.4 is 0.87 and 2.2 μM, respectively. Consequently, the change in the 3-substituent from a cyanomethyl to hydroxymethyl had a minor influence on the inhibitory effect. Dramatic effects are observed when compound pK_a is decreased to about 2, which is the result of a change in the 2-substituent from a methyl to a trifluoromethyl group (H 224/05). This study has shown that H 224/05 does not inhibit the K^+-stimulated ATPase reaction at pH 7.4 at all, which is in accordance with a very low concentration of active inhibitory, i.e. protonated compound.

In conclusion, SCH 28080 is a potent inhibitor of gastric H^+/K^+-ATPase. The protonated form is the active inhibitor and the K^+-competitive inhibitor effect is exerted by high-affinity binding of the drug to a luminally oriented site of the E_2 conformation.

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