British Journal of Pharmacology (2001) 134, 1655–1662 © 2001 Nature Publishing Group All rights reserved 0007–1188/01 $15.00

www.nature.com/bjp

Cibenzoline, an ATP-sensitive K^+ channel blocker, binds to the K^+-binding site from the cytoplasmic side of gastric H^+,K^+-ATPase

*,[1]Yoshiaki Tabuchi, [2]Hiroaki Yashiro, [2]Satomi Hoshina, [1]Shinji Asano & [2]Noriaki Takeguchi

[1]Molecular Genetics Research Center, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama City, Toyama 930-0194, Japan and [2]Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama City, Toyama 930-0194, Japan

1 Cibenzoline, (±)-2-(2,2-diphenylcyclopropyl-2-imidazoline succinate, has been clinically used as one of the Class I type antiarrhythmic agents and also reported to block ATP-sensitive K^+ channels in excised membranes from heart and pancreatic β cells. In the present study, we investigated if this drug inhibited gastric H^+,K^+-ATPase activity *in vitro*.

2 Cibenzoline inhibited H^+,K^+-ATPase activity of permeabilized leaky hog gastric vesicles in a concentration-dependent manner (IC_{50}: 201 μM), whereas no effect was shown on Na^+,K^+-ATPase activity of dog kidney (IC_{50}: >1000 μM). Similarly, cibenzoline inhibited H^+,K^+-ATPase activity of HEK-293 cells (human embryonic kidney cell line) co-transfected with rabbit gastric H^+,K^+-ATPase α- and β-subunit cDNAs (IC_{50}: 183 μM).

3 In leaky gastric vesicles, inhibition of H^+,K^+-ATPase activity by cibenzoline was attenuated by the addition of K^+ (0.5–5 mM) in a concentration-dependent manner. The Lineweaver-Burk plot of the H^+,K^+-ATPase activity shows that cibenzoline increases K_m value for K^+ without affecting V_{max}, indicating that this drug inhibits H^+,K^+-ATPase activity competitively with respect to K^+.

4 The inhibitory effect of H^+,K^+-ATPase activity by cibenzoline with normal tight gastric vesicles did not significantly differ from that with permeabilized leaky gastric vesicles, indicating that this drug reacted to the ATPase from the cytoplasmic side of the membrane.

5 These findings suggest that cibenzoline is an inhibitor of gastric H^+,K^+-ATPase with a novel inhibition mechanism, which inhibits gastric H^+,K^+-ATPase by binding its K^+-recognition site from the cytoplasmic side.

British Journal of Pharmacology (2001) **134,** 1655–1662

Keywords: Cibenzoline; hog gastric vesicle; H^+,K^+-ATPase activity; proton uptake; K^+-recognition site

Abbreviations: cibenzoline, (±)-2-(2,2,-diphenylcyclopropyl)-2-imidazoline succinate; K_{ATP} channel, ATP-sensitive K^+ channel; NAIBS, non-adrenergic idazoxan binding site; Pipes, piperazine-N,N′-bis(2-ethanesulfonic acid); SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo(1,2a)-pyridine; N-methylated SCH 28080, 3-(cyanomethyl)-2,3-dimethyl-8-(phenylmethoxy) imidazo(1,2a)-pyridine; SK&F96067, 3-butryrl-4-(2-methylphenylamino)-8-methoxyquinoline; SUR, sulphonylurea receptor

Introduction

The gastric H^+,K^+-ATPase is involved in acid secretion of the stomach (Sachs *et al*., 1976; Wallmark *et al*., 1985). This enzyme is a heterodimeric membrane protein which belongs to the family of P-type cation-transporting ATPases, such as Na^+,K^+-ATPase and Ca^{2+}-ATPase (Sachs *et al*., 1995), and catalyzes the electroneutral exchange of luminal potassium ions for cytosolic protons mediated by the hydrolysis of ATP (Sachs *et al*., 1976). Gastric H^+,K^+-ATPase is irreversibly inhibited by benzimizazole derivatives such as 5-methoxy-2-(((4-methoxy-3, 5-dimethyl-2-pyridinyl) methyl) sulphinyl)-1*H*-benzimidazole (omeprazole) and 2-((4-(3-methoxypropoxy)-3-methylpyridin-2-yl)methyl-sulphinyl)-1*H*-benzimidazole sodium salt (rabeprazole), which have been clinically used as anti-ulcer drugs (Fryklund *et al*., 1988; Morii *et al*., 1990; Richardson *et al*., 1998). The H^+,K^+-ATPase is reversibly inhibited by 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo(1,2a)-pyridine (SCH 28080) (Asano *et al*., 1999; Beil *et al*., 1986; Briving *et al*., 1988; Keeling *et al*., 1988; Scott *et al*., 1987; Wallmark *et al*., 1987) and 3-butyryl-4-(2-methylphenylamino)-8-methoxy-quinoline (SK&F96067) (Keeling *et al*., 1998; 1991). SCH 28080 and SK&F96067 are K^+-site inhibitors, which competitively bind to the K^+-high affinity site on the luminal side of the membrane (Beil *et al*., 1986; Briving *et al*., 1988; Keeling *et al*., 1988, 1991; Wallmark *et al*., 1987).

(±)-2-(2,2-diphenylcyclopropyl)-2-imidazoline succinate (cibenzoline, Figure 1), has been clinically used as one of the Class I type antiarrhythmic agents (Harron *et al*., 1992; Holck & Osterrieder, 1986; Millar & Williams, 1982; Touboul *et al*., 1986). This drug relieves arrhythmia by restricting fast inward Na^+ current (Millar & Williams, 1982) and blocking the slow inward Ca^{2+} channel in myocytes (Holck & Osterrieder, 1986). In addition, it has been reported that cibenzoline induces sporadic hypoglycemia as an extracardiac side effect (Gachot *et al*., 1988; Jeandel *et al*., 1988) and

*Author for correspondence; E-mail: ytabu@ms.toyama-mpu.ac.jp

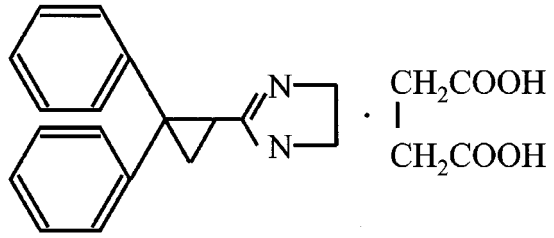

Figure 1 Chemical structure of cibenzoline (molecular mass = 380.44).

stimulates insulin secretion from pancreatic β cells (Bertrand *et al*., 1992; Gachot *et al*., 1988). It was recently shown that micromolar concentrations of cibenzoline blocked ATP-sensitive K^+ (K_{ATP}) channel in excised membranes from rat heart and pancreatic β cells (Horie *et al*., 1992; Ishida-Takahashi *et al*., 1996; Kakei *et al*., 1993). Molecular biological findings indicate that K_{ATP} channel of pancreatic β cells is composed of a sulfonylurea receptor (SUR) 1, a member of the ATP-binding cassette super family, and a K^+ channel (Kir6.2), which forms the ion-pore (Inagaki *et al*., 1995). And, cardiac K_{ATP} channel is composed of SUR2A, a subtype of SUR, and Kir6.2 (Chutkow *et al*., 1996). More recently, Mukai *et al*. (1998a, b) and Horie *et al*. (2000) reported that cibenzoline inhibits K_{ATP} channels by a novel inhibitory mechanism in which cibenzoline directly binds to the Kir6.2 subunit rather than the SUR1 subunit. This finding recurred our question whether the K^+ recognition site of K^+ channel structurally resembles with that of K^+-transporting pump.

Methods

Materials

Cibenzoline and N-methylated SCH 28080, 3-(cyanomethyl)-2,3-dimethyl-8-(phenylmethoxy) imidazo(1,2a)-pyridine were a generous gift from Fujisawa Pharmaceutical Co. (Osaka, Japan) and Toyama Chemical Co. (Tokyo, Japan), respectivly. HEK-293 cells (human embryonic kidney cell line) were a kind gift from Prof. Jonathan Lytton (University of Calgary, Calgary, Canada). Dog kidney Na^+,K^+-ATPase (Product number: A0142) was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan), and pcDNA3 vector from Invitrogen Co. (San Diego, CA, U.S.A.). All other reagents were of molecular biology or analytical grade.

Preparation of hog gastric vesicles

Hog gastric vesicles were prepared from mucosa in the fundic region of hog stomachs as described previously (Asano *et al*., 1989; Takeguchi *et al*., 1983). Brifely, hog gastric mucosa was homogenized in a buffer solution containing 250 mM sucrose, 1 mM EGTA and 5 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at $20{,}000 \times g$ for 30 min at 4°C, and the supernatant was re-centrifuged at $78{,}000 \times g$ for 30 min at 4°C. The pellet was suspended in the above solution, and centrifuged at $132{,}000 \times g$ for 1 h at 4°C through 250 mM sucrose layered on 250 mM sucrose containing 7% (w v^{-1}) Ficoll. The membrane fraction containing vesicles fractionated at the Ficoll interface was collected and called 'gastric vesicles'. When indicated, permeabilized leaky vesicles were prepared as following. Vesicles were diluted with 10 volumes of pure water, followed by immediate freezing in liquid nitrogen. Then, the vesicles were lyophilized and resuspended with the original volume of pure water.

Hog gastric vesicles used were originated from intracellular tubulovesicles (tubulocisternal network) and almost all of them have been well established to be 'inside-out' and tightly sealed by previous studies (Forte *et al*., 1980; Morii *et al*., 1984; Ogata & Yamasaki, 2000; Saccomani *et al*., 1977; Sachs *et al*., 1976; Wolosin & Forte, 1981). The cytosolic domains of H^+,K^+-ATPase including the ATP-binding site are present on the external side of the vesicles, and the addition of ATP into the vesicle solution induces H^+ uptake into the vesicle interior and K^+ release from vesicles (Asano *et al*., 1992; Forte *et al*., 1980; Lee *et al*., 1974; Sachs *et al*., 1976). Ion permeability in isolated gastric vesicles have been shown to be low by the measurement of solute influx (Rabon *et al*., 1980; Takeguchi *et al*., 1983; Wolosin & Forte, 1981). The addition of valinomycin (K^+ ionophore) or gramicidin (H^+/K^+ ionophore) to the gastric vesicle solution increases KCl influx into vesicles, resulting in 4–10 fold increase of H^+,K^+-ATPase activity (Asano *et al*., 1992; Forte *et al*., 1980; Saccomani *et al*., 1977; Sachs *et al*., 1976), because the intravesicular K^+ stimulates the ATPase activity. On the other hand, H^+,K^+-ATPase in permeabilized leaky gastric vesicles shows high levels of the activity without addition of the ionophores (Briving *et al*., 1988; Keeling *et al*., 1988). Furthermore, Briving *et al*. (1988) and Keeling *et al*. (1988) showed that N-methylated SCH 28080, which is an analogue of SCH 28080 with a permanent cation, was able to permeate the leaky vesicles but not normal tight vesicles. Thus, lyophilized vesicles are permeable to small molecules with permanent cations as well as ions.

Cell culture, transfection and preparation of HEK-293 membrane fraction

Cell culture of HEK-293 was carried out as described previously (Asano *et al*., 1996). H^+,K^+-ATPase α- and β-subunit cDNAs were prepared from rabbit gastric mucosa, and cloned in pcDNA3 vector as described elsewhere (Asano *et al*., 1996). Transfection was carried out in subconfluent HEK-293 cells with H^+,K^+-ATPase α- and β-subunit cDNAs (10 μg each) using the calcium phosphate method (Asano *et al*., 1996). Two days after the transfection, cells were harvested, and membrane fractions of the cells were prepared according to the method of Asano *et al*. (1996). In brief, cells were washed with phosphate-buffered saline and incubated in low ionic salt buffer (0.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4) for 10 min on ice. Phenylmethylsulphonyl fluoride (1 mM) and aprotinin (0.09 unit ml^{-1}) were added to the cell suspension. The cells were homogenized with 25 strokes in a Dounce homogenizer, and the homogenate was diluted with equal volume of a solution containing 500 mM sucrose and 10 mM Tris-HCl (pH 7.4). The cell suspension was centrifuged at $800 \times g$ for 10 min, and the supernatant was centrifuged at $100{,}000 \times g$ for 90 min, and the pellet (HEK membrane fraction) was suspended in a solution containing 250 mM sucrose and 5 mM Tris-HCl (pH 7.4). The ATPase

activity of the membrane fraction was not elevated by the addition of gramicidin, indicating that the membrane was leaky (Asano *et al*., 1996).

Assay of protein

Protein was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as standard.

Incubation of vesicles and membrane fractions with cibenzoline or N-methylated SCH 28080

Cibenzoline at final concentrations of 1–1,000 μM or N-methylated SCH 28080 at final concentrations of 0.01–300 μM was dissolved in each reaction buffer. Unless noted otherwise, vesicles or HEK membrane fractions were preincubated with each drug for 10 min at 37°C in the absence of ATP and Mg^{2+}. Then, ATP and Mg^{2+} were added to start the enzymatic reaction. An equal volume of reaction buffer without each drug was used as the control.

Measurement of H^+,K^+-ATPase activity

H^+,K^+-ATPase activity of normal tight or permeabilized leaky vesicles was measured in a 1 ml solution containing 5 μg of each vesicles, 3 mM $MgSO_4$, 3 mM ATP and 40 mM Tris-HCl (pH 6.4–8.0) in the presence or absence of KCl (0.5–15 mM). With the normal tight vesicles, to increase K^+ permeability of the vesicle membrane, 10 μg of valinomycin was added to the reaction solution. When leaky HEK membrane fractions were used, H^+,K^+-ATPase activity was measured in a 1 ml solution containing 50 μg of the membrane fractions (mM): $MgSO_4$ 3, ATP 1, NaN_3 5, ouabain 1 and Tris-HCl 40 (pH 7.4) in the presence or absence of 15 mM KCl. After incubation for 10 min (30 min for membrane fractions) at 37°C, the reaction was terminated by the addition of ice-cold stop solution (12% perchloric acid and 3.6% ammonium molybdate). Inorganic phosphate released was measured by the method of Yoda & Hokin (1970). The K^+-dependent ATPase activity was calculated as the difference between activities in the presence and absence of KCl, and defined as H^+,K^+-ATPase activity (Asano *et al*., 1989; 1996).

Measurement of Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity was measured in a 1 ml of solution containing 0.03 units of dog kidney Na^+,K^+-ATPase (mM), NaCl 120, KCl 15, $MgSO_4$ 3, ATP 3 and Tris-HCl 40 (pH 7.4) in the presence or absence of 100 μM ouabain. After incubation for 10 min at 37°C, the reaction was terminated by the addition of ice-cold stop solution, and inorganic phosphate released was measured as described above. The Na^+,K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of ouabain.

Proton uptake

Proton uptake into normal gastric vesicles was monitored by measuring the quenching of a fluorescence dye, acridine orange (Takeguchi & Yamazaki, 1986). Gastric vesicles were incubated in a 1 ml solution containing 150 mM KCl, 2 mM $MgCl_2$, 4 μM acridine orange, 10 μg of valinomycin and 1 mM Pipes [piperazine-N,N′-bis(2-ethanesulphonic acid)]-NaOH (pH 7.4) at 25°C. Proton uptake was started by the addition of 0.3 mM ATP. When time-course of inhibition of proton uptake by cibenzoline was evaluated, the drug was added 15 s after the ATP addition. Fluorescence of acridine orange was measured with excitation and emission wavelengths of 493 and 530 nm, respectively. Activity of proton uptake was taken as the initial rate of acridine orange quenching.

Results

Effects of cibenzoline on H^+,K^+-ATPase activity

Cibenzoline was examined for its ability to inhibit gastric H^+,K^+-ATPase activity in two different *in vitro* systems. With permeabilized leaky hog gastric vesicles, the value of H^+,K^+-ATPase activity in the absence of inhibitors was about 130 μmol Pi (mg protein)$^{-1}$ h^{-1}. Although low concentrations of cibenzoline (0.1–10 μM) did not inhibit the H^+,K^+-ATPase activity of the vesicles, higher concentrations of the drug markedly inhibited the H^+,K^+-ATPase activity in a concentration-dependent manner, with the IC_{50} value being 201 μM (Figure 2). To clarify the effective concentration range of cibenzoline under other *in vitro* conditions, we investigated effects of cibenzoline on H^+,K^+-ATPase activities of the membrane fraction obtained from HEK-293 cells expressing rabbit gastric H^+,K^+-ATPase α- and β-subunits. This HEK membrane was leaky. In this recombinant *in vitro* system, the value of H^+,K^+-ATPase activity in the absence of inhibitors was about 1.1 μmol Pi (mg protein)$^{-1}$ h^{-1}. On the other hand, the enzyme activity of mock-transfected cells was very low (0.06 μmol Pi (mg protein)$^{-1}$ h^{-1}) (Asano *et al*., 1996). Cibenzoline at concentrations from 30 to 1,000 μM

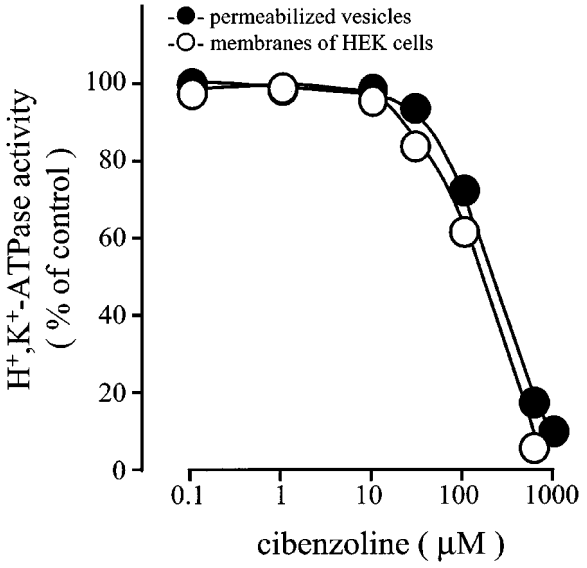

Figure 2 Effects of cibenzoline on the activities of H^+,K^+-ATPase from permeabilized leaky hog gastric vesicles and membrane fractions of HEK-293 cells co-transfected with rabbit gastric H^+,K^+-ATPase α- and β-subunit cDNAs. The HEK membrane was leaky. Each enzyme preparation was preincubated with cibenzoline for 10 min, and the K^+-dependent ATPase activity was measured. Data show means ± s.e.m. of 3–4 different experiments.

inhibited the H^+,K^+-ATPase activity of the HEK membrane fraction in a concentration-dependent manner, with its IC_{50} value being 183 μM (Figure 2).

Effects of cibenzoline on Na^+,K^+-ATPase activity

H^+,K^+-ATPase and Na^+,K^+-ATPase belong to the same family of P-type cation-transporting ATPases. To study the specificity of cibenzoline among the P-type cation-transporting ATPases, effects of this drug on Na^+,K^+-ATPase activity were studied. In dog kidney membrane preparation, the value of Na^+,K^+-ATPase activity in the absence of inhibitors was about 180 μmol Pi (mg protein)$^{-1}$ h^{-1}. Cibenzoline at concentrations from 0.1–1,000 μM had no effect on the Na^+,K^+-ATPase activity, indicating that this drug has specificity to gastric H^+,K^+-ATPase (Figure 3).

Effects of K^+ concentrations and pHs on inhibition of H^+,K^+-ATPase activity by cibenzoline

To study the inhibition mechanism of H^+,K^+-ATPase activity of permeabilized leaky gastric vesicles by cibenzoline, we measured H^+,K^+-ATPase activity as a function of the K^+ concentration in the presence of 10, 50 and 100 μM cibenzoline. Inhibition of H^+,K^+-ATPase activity by cibenzoline was attenuated with the addition of K^+ in a concentration-dependent manner. From Lineweaver-Burk plot between K^+-dependent ATPase activity and K^+ concentration, cibenzoline (0, 10, 50 and 100 μM) was shown to elevate the K_m value for K^+ (0.20, 0.54, 2.84 and 6.57 mM, respectively) without affecting V_{max} (171, 157, 161 and 166 μmol Pi (mg protein)$^{-1}$ h^{-1}, respectively), and the calculated K_i value for cibenzoline was 807 μM. These data indicate that cibenzoline is a competitive inhibitor with respect to K^+ (Figure 4).

Next, effects of medium pH on the inhibition of H^+,K^+-ATPase activity of leaky gastric vesicles by cibenzoline were studied. H^+,K^+-ATPase activities in the absence of cibenzoline at pH 6.4, 7.4 and 8.0 were about 90, 130 and 60 μmol Pi (mg protein)$^{-1}$ h^{-1}, respectively. As shown in Figure 5, the inhibition by cibenzoline did not depend on these pHs of the medium.

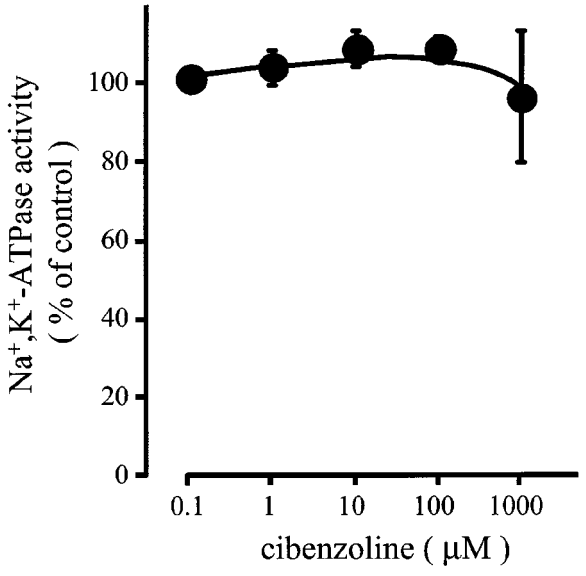

Figure 3 Effects of cibenzoline on the activity of Na^+,K^+-ATPase from dog kidney. The membrane fraction of dog kidney was preincubated with cibenzoline for 10 min, and the Na^+ and K^+-dependent ATPase activity was measured. Data show means±s.e.m of four different experiments.

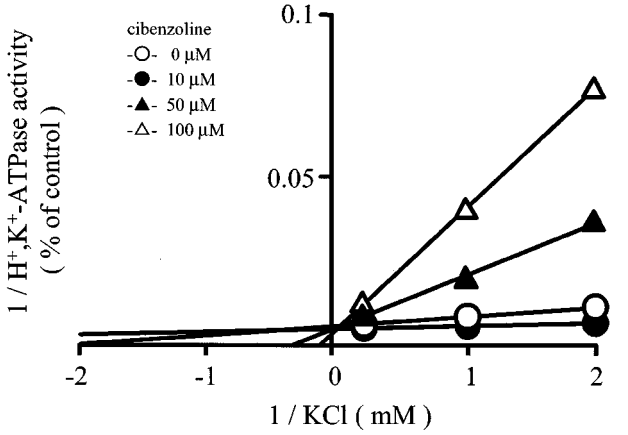

Figure 4 Lineweaver-Burk plots between H^+,K^+-ATPase activity of permeabilized leaky hog gastric vesicles and KCl concentration (0.5–5 mM) in the absence or presence of cibenzoline (10, 50 and 100 μM). Vesicles were preincubated with cibenzoline for 10 min in the presence of KCl, and the K^+dependent ATPase activity was measured. Data show means of 4–5 different experiments.

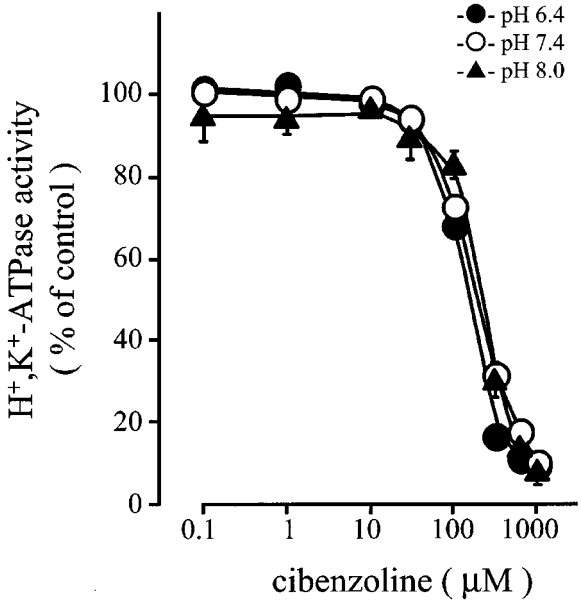

Figure 5 Effects of pH on inhibition of H^+,K^+-ATPase activity of permeabilized leaky hog gastric vesicles by cibenzoline. Vesicles were preincubated with cibenzoline for 10 min at various pHs (6.4–8.0), and the K^+-dependent ATPase activity was measured. Data show means±s.e.m. of 3–6 different experiments.

Identification of binding site of cibenzoline

To study whether the binding site of cibenzoline is located on the inner or outer surface of gastric vesicles, we used two kinds of vesicle preparations. One is normal tight vesicles.

The other is permeabilized leaky vesicles which have high permeability to ion (Briving *et al*., 1988; Keeling *et al*., 1988). Since the pKa value for imidazoline ring and distribution coefficient (n-octanol: water^{-1}) of cibenzoline are reported to be 10.6 and 0.126, respectively, the drug exists as a charged form at neutral pH and is difficult to permeate the membrane (Ishida-Takahashi *et al*., 1996). Here, these vesicles were preincubated in a test solution for 10 min and incubated for another 10 min for the measurement of the H^+,K^+-ATPase activity. If the binding site of the drug were located on the inner surface of vesicles, the inhibitory effect of the drug in tight vesicles would be smaller than that in leaky vesicles because the drug is difficult to permeate the membrane. On the other hand, if the binding site of cibenzoline were located on the outer surface of vesicles, inhibitory effects of the drug would be similar in both tight and leaky vesicles. The values of H^+,K^+-ATPase activities in the absence of inhibitors were about 40 and 130 μmol Pi (mg protein)$^{-1}$ h^{-1} in the tight and leaky vesicles, respectively. With tight vesicles, cibenzoline inhibited H^+,K^+-ATPase activity, with its IC_{50} value being 230 μM. A comparable inhibitory effect of cibenzoline was observed with leaky vesicles ($IC_{50}=201$ μM) (Figure 6A). These findings indicate that the binding site of cibenzoline is located on the outer surface of vesicles (on the cytoplasmic side).

N-methylated SCH 28080, an analogue of SCH 28080, has a permanent cation and binds to the inner surface of vesicles as in the case of SCH 28080 (Briving *et al*., 1988; Keeling *et al*., 1988). Here, we checked effects of N-methylated SCH 28080 on the H^+,K^+-ATPase activity using the present leaky and tight gastric vesicles. This drug markedly inhibited the H^+,K^+-ATPase activity of leaky vesicles, with its IC_{50} value being 0.73 μM. On the other hand, inhibition potency of this drug was dramatically attenuated in the tight vesicles ($IC_{50}=40$ μM) (Figure 6B). In contrast to cibenzoline which exists as a cationic form at neutral pHs (Figure 6A), these results confirm that binding site of N-methylated SCH 28080 is located on the inner surface of vesicles (on the luminal side).

Effects of cibenzoline on proton uptake

Proton uptake into tight gastric vesicles was monitored by measuring the quenching of acridine orange fluorescence. When ATP was added to the reaction medium, decrease in the fluorescence intensity was observed (data not shown). Pretreatment with cibenzoline (50–1000 μM) inhibited the proton uptake into vesicles in a concentration-dependent manner, with its IC_{50} value being 143 μM (Figure 7). Moreover, time-course of inhibition of proton uptake by the drug was tested. Proton uptake was inhibited immediately after addition of this drug (500 μM) (data not shown).

Discussion

In the present study, we discovered that cibenzoline inhibits gastric H^+,K^+-ATPase activity of hog gastric vesicles and the membrane fraction of HEK-293 cells transfected with the H^+,K^+-ATPase cDNAs. Cibenzoline is clinically used as one of the Class I type antiarrhythmic agents (Harron *et al*., 1992; Holck & Osterrieder, 1986; Millar & Williams, 1982; Touboul *et al*., 1986). In whole-cell mode of patch clamp with isolated cardiac myocytes of guinea pig, cibenzoline caused an inhibition of Ca^{2+} inward current ($IC_{50}=14$ μM) (Holck & Osterrieder, 1986). In addition, this drug blocked K_{ATP} channel in cell-attached mode of patch clamp of rat pancreatic β cells ($IC_{50}=1.5-5.2$ μM) (Horie *et al*., 1992; Ishida-Takahashi *et al*., 1996; Kakei *et al*., 1993). In the present study, IC_{50} value of cibenzoline for inhibition of gastric H^+,K^+-ATPase activity and H^+ uptake in *in vitro* systems were approximately 200 μM and 143 μM, respectively.

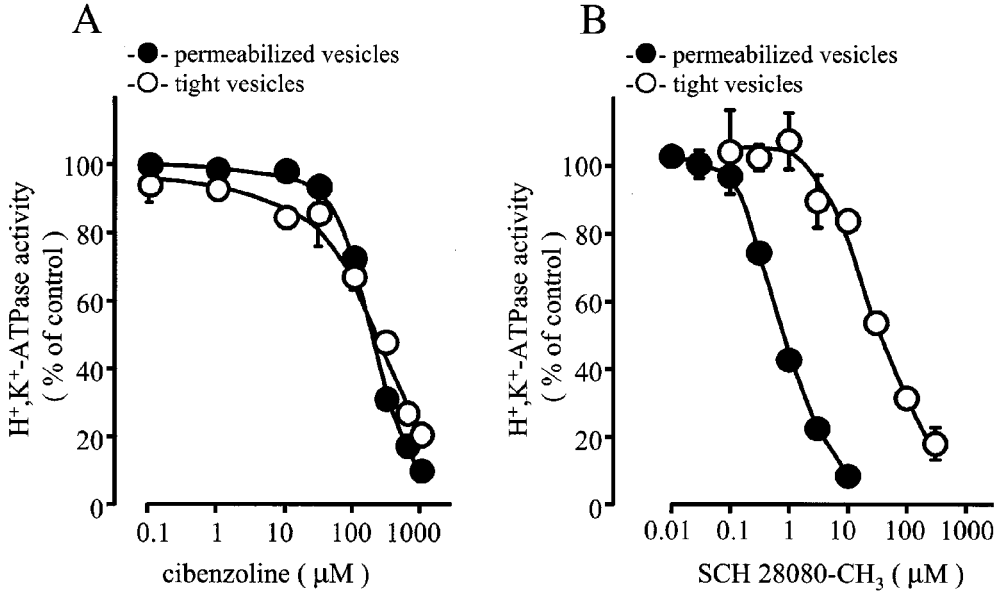

Figure 6 Inhibition of H^+,K^+-ATPase activity by (A) cibenzoline and (B) N-methylated SCH 28080 (SCH 28080-CH_3) in permeabilized leaky and normal tight hog gastric vesicles. Vesicles were preincubated with each drug for 10 min, and the K^+-dependent ATPase activity was measured. Data show means±s.e.m. of 3–4 different experiments.

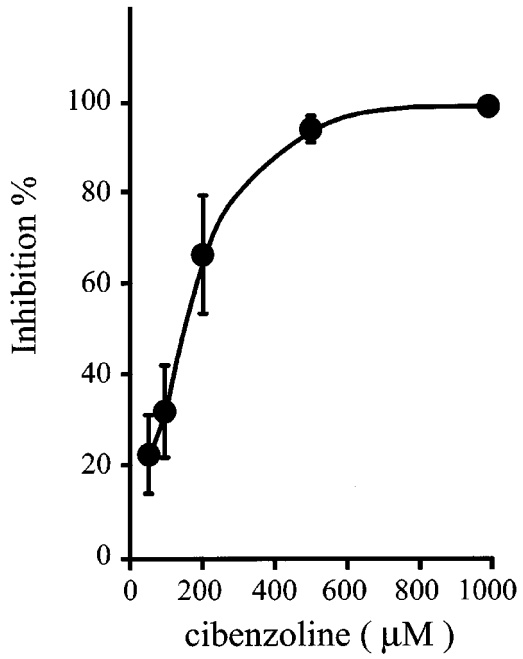

Figure 7 Effects of cibenzoline on proton uptake into normal tight hog gastric vesicles. The vesicles were preincubated with cibenzoline for 10 min, and proton uptake was measured. Data show means±s.e.m. of 3–5 different experiments.

The present difference of IC_{50} values between H^+,K^+-ATPase activity and H^+ uptake would reflect the different types of observation. Thus, the inhibitory effect of H^+,K^+-ATPase activity by cibenzoline was 14 and 38–133 times smaller than those of Ca^{2+} inward current and K_{ATP} channel, respectively. However, because cibenzoline did not inhibit Na^+,K^+-ATPase activity (IC_{50}: >1,000 μM), the inhibition of gastric H^+,K^+-ATPase activity by this drug was specific. In humans, the therapeutic mean maximum plasma concentration was 1.9 μM following the last dose (160 mg t.i.d. for 7 days) (Massarella *et al*., 1986). It is, therefore, considered that cibenzoline at clinically used doses does not inhibit gastric H^+,K^+-ATPase. In fact, it has not been reported that such doses of cibenzoline inhibit gastric acid secretion.

Inhibition of H^+,K^+-ATPase activity by cibenzoline was attenuated with the addition of K^+ with permeabilized leaky gastric vesicles, and the Lineweaver-Burk plot of the ATPase activity showed that this drug increased K_m value for K^+ without affecting V_{max}. Therefore, we suggest that cibenzoline is a competitive inhibitor respect to K^+ as in the cases of SCH 28080 (Beil *et al*., 1986; Keeling *et al*., 1988; Scott *et al*., 1987; Wallmark *et al*., 1987) and SK&F96067 (Keeling *et al*., 1991). The calculated K_i value for cibenzoline was 807 μM, which was much larger than those of SCH 28080 (K_i=0.056–0.12 μM) (Scott *et al*., 1987; Wallmark *et al*., 1987) and SK&F96067 (K_i=0.39 μM) (Keeling *et al*., 1991). These hydrophobic compounds of SCH 28080 and SK&F96067 permeate the membrane, accumulate in the acidic compartment as prontonated forms, and bind to the high-affinity K^+-binding site on the luminal side of the membrane (Beil *et al*., 1986; Briving *et al*., 1988; Keeling *et al*., 1988; 1991; Wallmark *et al*., 1987). N-methylated SCH 28080, a permanent cation, was shown to bind to the inner (luminal) surface of vesicles (Briving *et al*., 1988; Keeling *et al*., 1988). We found here that the inhibition of H^+,K^+-ATPase in tight vesicles by N-methylated SCH 28080 (IC_{50}=40 μM) was two orders of magnitude less effective than that in the present leaky vesicles (IC_{50}=0.73 μM), indicating that the inhibition of N-methylated SCH 28080 in tight vesicles was 55 times less effective than that in leaky vesicles. Because cibenzoline exists as a charged form at neutral pH, the drug is difficult to permeate the vesicle membrane (Ishida-Takahashi *et al*., 1996; Horie *et al*., 2000). In the present study, the inhibitory effect of H^+,K^+-ATPase activity by cibenzoline with normal tight gastric vesicles did not significantly differ from that with permeabilized leaky gastric vesicles. Moreover, this drug inhibited proton uptake in tight vesicles soon after addition of the drug. Therefore, it is suggested that the binding site of cibenzoline is on the cytoplasmic side of the membrane (external surface of the vesicles). Ishida-Takahashi *et al*. (1996) previously showed that the action of cibenzoline to K_{ATP} channel in the excised inside-out mode was acute in onset with a small IC_{50} (0.4 μM) compared with that in the cell-attached mode of patch clamp (IC_{50}=5.2 μM) in rat pancreatic β cells. These results indicate that the binding site of cibenzoline is located on the cytoplasmic side of the cell membrane (Horie *et al*., 2000).

It has been demonstrated that imidazoline derivatives such as several classical α-adrenoreceptor antagonists, act as stimulators of insulin secretion, and these effects do not result from antagonism of α-adrenoreceptors but from inhibition of K_{ATP} channels in the pancreatic β cell (Dunne, 1991; Le Brigand *et al*., 1999; Proks & Ashcroft, 1997; Plant & Henquin, 1990; Jonas *et al*., 1992). The K_{ATP} channel of pancreatic β cells is a complex of two proteins: a member of the ATP-binding cassette super family, SUR1, and a pore-forming subunit, Kir6.2 (Inagaki *et al*., 1995). Proks & Ashcroft (1997) indicated that K_{ATP} channels blocked by phentolamine, an imidazoline derivative, is mediated by Kir6.2 subunit. Mukai *et al*. (1998a, b) and Horie *et al*. (2000) reported that an imidazoline derivative, cibenzoline, inhibits K_{ATP} channels by direct binding of this drug to the cytoplasmic side of Kir6.2 subunit. In the present study, we showed that the binding site of cibenzoline is located on the cytoplasmic surface of gastric H^+,K^+-ATPase. It is interesting that cibenzoline binds to the cytoplasmic surface of both Kir6.2 subunit and H^+,K^+-ATPase α-subunit. Although the amino acid homology between Kir6.2 (Inagaki *et al*., 1995) and H^+,K^+-ATPase α-subunit (Shull & Lingrel, 1986) is extremely low, the three-dimensional structure of the K^+-binding site in Kir6.2 subunit is likely to have some extent of similarity with that of the K^+ binding site of H^+,K^+-ATPase α-subunit.

In conclusion, cibenzoline inhibits gastric H^+,K^+-ATPase by binding to the K^+-recognition site of the enzyme from the cytoplasmic side. In our knowledge, this is the first drug that inhibits gastric H^+,K^+-ATPase with this unique inhibitory action.

This work was supported in part by Grants-in-Aid for Scientific Research (to S. Asano and N. Takeguchi) from the Ministry of Education, Science, Sports and Culture of Japan.

References

ASANO, S., KAMIYA, S. & TAKEGUCHI, N. (1992). The energy transduction mechanism is different among P-type ion-transporting ATPases. *J. Biol. Chem.,* **267,** 6590 – 6595.

ASANO, S., MATSUDA, S., HOSHINA, S., SAKAMOTO, S. & TAKEGUCHI, N. (1999). A chimeric gastric H^+,K^+-ATPase inhibitable with both ouabain and SCH 28080. *J. Biol. Chem.,* **274,** 6848 – 6854.

ASANO, S., TABUCHI, Y. & TAKEGUCHI, N. (1989). Monoclonal antibody HK4001 completely inhibits K^+-dependent ATP hydrolysis and H^+ transport of hog gastric H^+,K^+-ATPase. *J. Biochem.,* **106,** 1074 – 1079.

ASANO, S., TEGA, Y., KONISHI, K., FUJIOKA, M. & TAKEGUCHI, N. (1996). Functional expression of gastric H^+,K^+-ATPase and site-directed mutagenesis of the putative cation binding site and the catalytic center. *J. Biol. Chem.,* **271,** 2740 – 2745.

BEIL, W., HACHBARTH, I. & SEWING, K.F. (1986). Mechanism of gastric antisecretory effect of SCH 28080. *Br. J. Pharmacol.,* **88,** 19 – 23.

BERTRAND, G., GROSS, R., PETIT, P., LOUBATIERES-MARIANI, M.M. & RIBES, G. (1992). Evidence for a direct stimulatory effect of cibenzoline on insulin secretion in rats. *Eur. J. Pharmacol.,* **214,** 159 – 163.

BRIVING, C., ANDERSSON, B.M., NORDBERG, P. & WALLMARK, B. (1988). Inhibition of gastric H^+/K^+-ATPase by substituted imidazo[1,2-a]pyridines. *Biochim. Biophys. Acta.,* **946,** 185 – 192.

CHUTKOW, W.A., SIMON, M.C., LE BEAU, M.M. & BURANT, C.F. (1996). Cloning, tissue expression, and chromosomal localization of SUR2, the putative drug-binding subunit of cardiac, skeletal muscle, and vascular K_{ATP} channels. *Diabetes,* **45,** 1439 – 1445.

DUNNE, M.J. (1991). Block of ATP-regulated potassium channels by phentolamine and other alpha-adrenoceptor antagonists. *Br. J. Pharmacol.,* **103,** 1847 – 1850.

FRYKLUND, J., GEDDA, K. & WALLMARK, B. (1988). Specific labeling of gastric H^+,K^+-ATPase by omeprazole. *Biochem. Pharmacol.,* **37,** 2543 – 2549.

FORTE, J.G., MACHEN, T.E. & OBRINK, K.J. (1980). Mechanisms of gastric H^+ and Cl^- transport. *Ann. Rev. Physiol.,* **42,** 111 – 126.

GACHOT, B.A., BEZIER, M., CHERRIER, J.F. & DAUBEZE, J. (1988). Cibenzoline and hypoglycaemia. *Lancet,* **2,** 280.

HARRON, D.W., BROGDEN, R.N., FAULDS, D. & FITTON, A. (1992). Cibenzoline. A review of its pharmacological properties and therapeutic potential in arrhythmias. *Drugs,* **43,** 734 – 759.

HOLCK, M. & OSTERRIEDER, W. (1986). Inhibition of the myocardial Ca^{2+} inward current by the class I antiarrhythmic agent, cibenzoline. *Br. J. Pharmacol.,* **87,** 705 – 711.

HORIE, M., HAYASHI, S., YUZUKI, Y. & SASAYAMA, S. (1992). Comparative studies of ATP sensitive potassium channels in heart and pancreatic beta cells using Vaughan-Williams class Ia antiarrhythmics. *Cardiobasc. Res.,* **26,** 1087 – 1094.

HORIE, M., WATANUKI, M., TSUJI, K., ISHIDA, H., ISHIDA-TAKAHASHI, A., YUZUKI, Y., SEINO, Y. & SASAYAMA, S. (2000). Blockade of cardiac ATP-sensitive K^+ channel by cibenzoline targets its pore-forming subunit. *J. Cardiovasc. Pharmacol.,* **35,** 434 – 442.

INAGAKI, N., GONOI, T., CLEMENT, 4TH J.P., NAMBA, N., INAZAWA, J., GONZALEZ, G., AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science,* **270,** 1166 – 1170.

ISHIDA-TAKAHASHI, A., HORIE, M., TSUURA, Y., ISHIDA, H., AI, T. & SASAYAMA, S. (1996). Block of pancreatic ATP-sensitive K^+ channels and insulinotrophic action by the antiarrhythmic agent, cibenzoline. *Br. J. Pharmacol.,* **117,** 1749 – 1755.

JEANDEL, C., PREISS, M.A., PIERSON, H., PENIN, F., CUNY, G., BANNWARTH, B. & NETTER, P. (1988). Hypoglycaemia induced by cibenzoline. *Lancet,* **1,** 1232 – 1233.

JONAS, J.C., PLANT, T.D. & HENQUIN, J.C. (1992). Imidazoline antagonists of alpha 2-adrenoceptors increase insulin release in vitro by inhibiting ATP-sensitive K^+ channels in pancreatic beta-cells. *Br. J. Pharmacol.,* **107,** 8 – 14.

KAKEI, M., NAKAZAKI, M., KAMISAKI, T., NAGAYAMA, I., FUKAMACHI, Y. & TANAKA, H. (1993). Inhibition of the ATP-sensitive potassium channel by class I antiarrhythmic agent, cibenzoline, in rat pancreatic beta-cells. *Br. J. Pharmacol.,* **109,** 1226 – 1231.

KEELING, D.J., LAING, S.M. & SENN-BILFINGER, J. (1988). SCH 28080 is a luminally acting, K^+-site inhibitor of the gastric $(H^+ + K^+)$-ATPase. *Biochem. Pharmacol.,* **37,** 2231 – 2236.

KEELING, D.J., MALCOLM, R.C., KAING, S.M., LEACH, C.A. & IFE, R.J. (1991). SK&F96067 is a reversible luminally acting inhibitor of the gastric H^+,K^+-ATPase. *Biochem. Pharmacol.,* **42,** 123 – 130.

LE BRIGAND, L., VIRSOLVY, A., MANECHEZ, D., GODFROID, J.J., GUARDIOLA-LEMAITRE, B., GRIBBLE, F.M., ASHCROFT, F.M. & BATAILLE, D. (1999). In vitro mechanism of action on insulin release of S-22068, a new putative antidiabetic compound. *Br. J. Pharmacol.,* **128,** 1021 – 1026.

LEE, J., SIMPSON, G. & SCHOLES, P. (1974). An ATPase from dog gastric mucosa: changes of outer pH in suspensions of membrane vesicles accompanying ATP hydrolysis. *Biochem. Biophys. Res. Commun.,* **60,** 825 – 832.

MASSARELLA, J.W., KHOO, K.C., SZUNA, A.J., SANDOR, D.A., MORGANROTH, J. & AOGAICHI, K. (1986). Pharmacokinetics of cibenzoline after single and repetitive dosing in healthy volunteers. *J. Clin. Pharmacol.,* **26,** 125 – 130.

MILLAR, J.S. & WILLIAMS, E.M.V. (1982). Effects on rabbit nodal, atrial ventricular and purkinje cell potentials of a new antiarrhythmic drug, cibenzoline, which protects against action potential shortening in hypoxia. *Br. J. Pharmacol.,* **75,** 469 – 478.

MORII, M., ISHIMURA, N. & TAKEGUCHI, N. (1984). Quasi-elastic light-scattering studies of conformational states of the H^+,K^+-ATPase. Intervesicular aggregation of gastric vesicles by disulfide cross-linking. *Biochemistry,* **23,** 6816 – 6821.

MORII, M., TAKATA, H., FUJISAKI, H. & TAKEGUCHI, N. (1990). The potency of substituted benzimidazoles such as E3810, omeprazole, Ro18-5364 to inhibit gastric H^+,K^+-ATPase is correlated with the rate of acid-activation of the inhibitor. *Biochem. Pharmacol.,* **39,** 661 – 667.

MUKAI, E., ISHIDA, H., HORIE, M., NOMA, A., SEINO, Y. & TAKANO, M. (1998a). The antiarrhythmic agent cibenzoline inhibits K_{ATP} channels by binding to Kir6.2. *Biochem. Biophys. Res. Commun.,* **251,** 477 – 481.

MUKAI, E., ISHIDA, H., KATO, S., TSUURA, Y., FUJIMOTO, S., ISHIDA-TAKAHASHI, A., HORIE, M., TSUDA, K. & SEINO, Y. (1998b). Metabolic inhibition impairs ATP-sensitive K^+channel block by sulfonylurea in pancreatic beta-cells. *Am. J. Physiol.,* **274,** E38 – E44.

OGATA, T. & YAMASAKI, Y. (2000). Scanning EM of resting gastric parietal cells reveals a network of cytoplasmic tubules and cisternae connected to the intracellular canaliculus. *Anat. Rec.,* **258,** 15 – 24.

PLANT, T.D. & HENQUIN, J.C. (1990). Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic beta-cells. *Br. J. Pharmacol.,* **101,** 115 – 120.

PROKS, P. & ASHCROFT, F.M. (1997). Phentolamine block of K_{ATP} channels is mediated by Kir6.2. *Proc. Natl. Acad. Sci. USA,* **94,** 11716 – 11720.

RABON, E., TAKEGUCHI, N. & SACHS, G. (1980). Water and salt permeability of gastric vesicles. *J. Membr. Biol.,* **53,** 109 – 117.

RICHARDSON, P., HAWKEY, C.J. & STACK, W.A. (1998). Proton pump inhibitors. Pharmacology and rationale for use in gastrointestinal disorders. *Drugs,* **56,** 307 – 335.

SACHS, G., CHANG, H.J., RABON, E., SCHACKMAN, R., LEWIN, M. & SACCOMANI, G. (1976). A nonelectrogenic H pump in plasma membranes of hog stomach. *J. Biol. Chem.,* **251,** 7690 – 7698.

SACHS, G., SHIN, J.M., BRIVING, C., WALLMARK, B. & HERSEY, S. (1995). The pharmacology of the gastric acid pump: the H^+,K^+-ATPase. *Annu. Rev. Pharmacol. Toxicol.,* **35,** 277 – 305.

SACCOMANI, G., STEWART, H.B., SHAW, D., LEWIN, M. & SACHS, G. (1977). Characterization of gastric mucosal membranes. IV. Fractionation and purification of K^+-ATPase-containing vesicles by zonal centrifugation and free-flow electrophoresis technique. *Biochim. Biophys. Acta,* **465,** 311 – 330.

SCOTT, C.K., SUNDELL, E. & CASTROVILLY, L. (1987). Studies on the mechanism of action of the gastric microsomal ($H^+ + K^+$)-ATPase inhibitors SCH 32651 and SCH 28080. *Biochem. Pharmacol.,* **36,** 97 – 104.

SHULL, G.E. & LINGREL, J.B. (1986). Molecular cloning of the rat stomach ($H^+ + K^+$)-ATPase. *J. Biol. Chem.,* **261,** 16788 – 16791.

TAKEGUCHI, N., JOSHIMA, R., INOUE, Y., KASHIWAGURA, T. & MORII, M. (1983). Effects of Cu^{2+}-o-phenanthroline on gastric ($H^+ + K^+$)-ATPase. Evidence for opening of a closed anion conductance by S-S cross-linking. *J. Biol. Chem.,* **258,** 3094 – 3098.

TAKEGUCHI, N. & YAMAZAKI, Y. (1986). Disulfide cross-linking ATPase opens Cl^- conductance, triggering proton uptake in gastric vesicles. *J. Biol. Chem.,* **261,** 2560 – 2566.

TOUBOUL, P., ATALLAH, G., KIRKORIAN, G., DE ZULOAGA, C., DUFOUR, A., AYMARD, M.F., LAVAUD, P. & MOLEUR. P. (1986). Electro physiologic effects of cibenzoline in humans related to dose and plasma concentration. *Am. Heart J.,* **112,** 333 – 339.

WALLMARK, B., LARSSON, H. & HUMBLE, L. (1985). The relationship between gastric acid secretion and gastric H^+,K^+-ATPase activity. *J. Biol. Chem.,* **260,** 13681 – 13684.

WALLMARK, B., BRIVING, C., FRYKLUND, J., MUNSON, K., JACKSON, R., MEDLEIN, J., RABON, E. & SACHS, G. (1987). Inhibition of gastric H^+,K^+-ATPase and acid secretion by SCH 28080, a substituted pyridyl(1,2a)imidazole. *J. Biol. Chem.,* **262,** 2077 – 2084.

WOLOSIN, J.M. & FORTE, J.G. (1981). Changes in the membrane environment of the ($K^+ + H^+$)-ATPase following stimulation of the gastric oxyntic cell. *J. Biol. Chem.,* **256,** 3149 – 3152.

YODA, A. & HOKIN, L.E. (1970). On the reversibility of binding of cardiotonic steroids to a partially purified (Na + K)-activated adenosinetriphosphatase from beef brain. *Biochem. Biophys. Res. Commun.,* **40,** 880 – 886.

(Received May 2, 2001
Revised September 7, 2001
Accepted September 28, 2001)