Biochemical Pharmacology, Vol. 60, pp. 1855–1863, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00509-8

Reversible and Potent Uncoupling of Hog Gastric $(H^+ + K^+)$-ATPase by Prodigiosins

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ABSTRACT. Prodigiosin, prodigiosin 25-C, and metacycloprodigiosin all strongly inhibited the acidification activity of $(H^+ + K^+)$-ATPase on membrane vesicles from hog gastric mucosa (IC_{50} = 32 to 103 pmol/mg protein). But, the prodigiosins, unlike omeprazole, showed little inhibitory effect on K^+-dependent ATPase (K^+-ATPase) activity, although at higher concentrations they inhibited K^+-ATPase activity with an IC_{50} of 1.5 to 3.0 μM. Furthermore, the inhibitory effect of the prodigiosins was rapid and completely reversible unlike that of omeprazole, and the mode of inhibition was non-competitive with respect to ATP. Hog gastric $(H^+ + K^+)$-ATPase itself showed an absolute requirement of halide (effectively, chloride) for acidification activity. Prodigiosins also showed a chloride requirement for inhibition of vesicular acidification, and quickly reversed the acidification of vesicular pH to neutrality even in the presence of *N,N′*-dicyclohexylcarbodiimide (DCCD), showing their ionophoric nature of acidification inhibitory activity. In fact, tributyltin chloride (TBT, an OH^-/Cl^- exchange ionophore) also inhibited vesicular acidification, but it inhibited K^+-ATPase activity too. Finally, the prodigiosins inhibited the acid secretion from parietal cells isolated from rabbit gastric mucosa. These results suggest that prodigiosins are potent reversible uncouplers of $(H^+ + K^+)$-ATPase that inhibit gastric acid secretion. BIOCHEM PHARMACOL **60**;12:1855–1863, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. adenosine triphosphatase; proton translocation; acid secretion; H^+/Cl^- symport

We showed [1] that prodigiosins, antibiotic red pigments produced by microorganisms including *Streptomyces* and *Serratia* [2], uncouple lysosomal H^+-ATPases (EC 3.6.1.35) (V-ATPase‖), resulting in an increase of lysosomal pH and the suppression of glycoprotein processing. Eventually, we found that prodigiosins also "uncouple" mitochondrial and bacterial H^+-ATPases (F-ATPases) [3] in addition to lysosomal H^+-ATPases [4], because of their H^+/Cl^- symport activity [5]. However, the F- and V-ATPases are essentially the same in structure [6–10], and are composed of a hydrophilic protrusion with catalytic activity (F_1- or V_1-portions) and membrane embedded proton-channel-forming sectors (F_0 or V_0 portions) consisting of multi-subunits of 5–6 for the catalytic portion and 4–5 for the channel portion, respectively. More importantly, they are generally insensitive to vanadate (although some V-ATPases, for example the avian osteoclastic plasma membrane type, are sensitive to vanadate [11, 12]) and appear not to form a phosphorylated intermediate through their reactions. Therefore, it is important and interesting to study the effect of prodigiosins on other types of proton pumps [e.g. P-ATPases like gastric $(H^+ + K^+)$-ATPase (EC 3.6.1.36) or respiratory proton pumps].

In this paper, we present evidence that prodigiosins also uncouple the $(H^+ + K^+)$-ATPase of plasma membrane vesicles from hog gastric mucosa (and inhibit acid secretion) in such a way that they reversibly inhibit acidification with little effect on ATP hydrolysis unlike the irreversible potent selective inhibitor of $(H^+ + K^+)$-ATPase, omeprazole, which inhibits both proton translocation and ATP hydrolysis.

MATERIALS AND METHODS

Materials

Stomachs of freshly slaughtered hogs were provided by the Meat Inspection Center of Kanazawa City. Rabbits (Japan white rabbits, male, 2 to 2.5 kg in body weight) were

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‖ *Abbreviations*: DCCD, *N, N′*-dicyclohexylcarbodiimide; DMEM, Dulbecco's modified Minimum Essential Medium; FBS, fetal bovine serum; F-ATPase, F-type H^+-ATPase; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; K^+-ATPase, K^+-dependent ATPase; PIPES, piperazine-*N,N′*-bis[2-ethanesulfonic acid]; P-ATPase, P-type H^+-ATPase; TBT, tributyltin chloride; TMAH, tetramethylammonium hydroxide; and V-ATPase, vacuolar type H^+-ATPase.

Received 25 January 2000; accepted 30 May 2000.

obtained from Shiraishi Laboratory Animal. Prodigiosin was prepared from the culture broth of *Serratia marcescens* (HY-3) as described [13]. Metacycloprodigiosin and prodigiosin 25-C were prepared from the culture broth of *Streptomyces hiroshimensis* as described [14]. Omeprazole was obtained from Fujisawa-Astra Japan, and acridine orange from Wako. Oxonol-V was supplied by Nippon Kanko Shikiso. DCCD and TBT also were obtained from Wako. Other reagents were purchased as commercial products, mostly from the Sigma Chemical Co.

Preparation of Hog Gastric Membrane Vesicles

Membrane vesicles containing $(H^+ + K^+)$-ATPase were purified from gastric mucosa of freshly slaughtered hogs according to the method described by Maeda *et al.* [15] with slight modifications: briefly, the fundic mucosal surfaces were flooded with 3 M NaCl and wiped dry to remove mucus cells. Then the gastric mucosa (350 g) was scraped from underlying connective tissue, homogenized in a 40% suspension (w/w) in 500 mL of 0.25 M sucrose, 5 mM PIPES–Tris (pH 6.8), using a juicer/mixer, and centrifuged at 8000 *g* for 20 min. After passing through a cheesecloth, the 8000 *g* supernatant of the homogenate was layered on 1 M [34% (w/v)] sucrose, 5 mM PIPES–Tris (pH 6.8), and centrifuged at 100,000 *g* for 50 min. The fraction at the interface between the supernatant and the sucrose layer was collected, diluted with 5 mM PIPES–Tris (pH 6.8), and centrifuged at 100,000 *g* for 50 min. The precipitate was resuspended in 5 mM PIPES–Tris (pH 6.8) (20 mL) and fractionated further by centrifugation (27,000 *g*, 16 hr) through discontinuous sucrose layers consisting of 0.25 M sucrose (5 mL), 7% (w/w) Ficoll 70–0.25 M sucrose (10 mL), 1 M sucrose (15 mL), and 1.75 M sucrose (2 mL) [all in 5 mM PIPES–Tris (pH 6.8)]. The fraction at the interface between 7% (w/v) Ficoll–0.25 M sucrose and 1 M [34% (w/v)] sucrose was collected, diluted with 5 mM PIPES–Tris (pH 6.8), and centrifuged at 100,000 *g* for 50 min. The resulting pellet was resuspended in 2 mL of the 5 mM PIPES–Tris (pH 6.8) and stored at -80° until used. The final preparation showed a specific activity of vanadate-sensitive K^+-ATPase of about 0.72 to 0.75 U/mg protein and little detectable ouabain-sensitive ATPase activity.

Proton Pump Assay

Proton pump (acidification) activity of the $(H^+ + K^+)$-ATPase in the membrane vesicles from hog stomach was detected by the ATP-dependent quenching of acridine orange fluorescence [15]. The assay buffer contained 150 mM KCl, 8 mM PIPES–TMAH (pH 6.8), 2 mM $MgCl_2$, 5 μM valinomycin, and 1.0 mM ATP-Na_2 in a final volume of 2.0 mL with the dye. The concentration of FCCP and Triton X-100 was 1 μM and 0.1% (w/v), respectively. The concentration of acridine orange was 2 μM. Fluorescence was measured at 37° with a Hitachi 310 or F-4500 spectrofluorometer with excitation and emission wavelengths at 480 nm (or 493 nm) and 530 nm, respectively.

Reversibility of Prodigiosin Action

Membrane vesicles of hog gastric mucosa (0.38 mg protein) were preincubated in a total volume of 100 μL containing valinomycin and acridine orange with or without the inhibitors (metacycloprodigiosin, 20 nM; prodigiosin 25-C, 100 nM) for 3 min at 37°. After preincubation, 1.9 mL of assay buffer with or without inhibitors (metacycloprodigiosin, 1.5 nM; prodigiosin 25-C, 5 nM) was added, the acidification of these membrane vesicles was started by adding ATP (1 mM), and the change of fluorescence was traced with excitation at 493 nm and emission at 530 nm.

ATPase Assay

ATPase assays were performed as described using the Malachite-Green method [16]. The assay buffer [15] contained 150 mM KCl, 8 mM PIPES–TMAH (pH 6.8), 2 mM $MgCl_2$, 5 μM valinomycin, 1.0 mM ATP-Na_2, and 5 μg/mL of protease inhibitors (leupeptin, antipain, chymostatin, and pepstatin), with or without the dyes in a final volume of 1.0 mL. Negative control buffer contained 150 mM choline chloride instead of KCl. Assays were started by adding membrane vesicles (about 2.38 μg protein/tube) and incubating at 37° for 10 min. The liberated phosphate was estimated by the Malachite-Green method [17]. K^+-ATPase activity was defined as the difference between the activities in KCl buffer and in the choline chloride (negative control) buffer.

Isolation of Parietal Cells from Rabbit Gastric Mucosa

Parietal cells of rabbit gastric glands were isolated essentially as described [18]. Parietal cells were obtained from the isolated gastric gland by combined collagenase digestion [19] and Percoll density gradient cell fractionation methods [20], with slight modifications. The primary culture of isolated parietal cells was performed according to the method of Chew *et al.* [21] with slight modifications. Briefly, the gastric mucosa isolated from 2 to 2.5 kg male rabbits after perfusion *in situ* with PBS under high pressure was then digested with collagenase to produce single cells. These cells were enriched in parietal cells by gradient separation, using Percoll.

Evaluation of Gastric Acid Secretion of Isolated Parietal Cells

The isolated parietal cells were cultured on glass coverslips coated with collagen type IV in a medium consisting of DMEM plus 10% FBS at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Acid secretion was followed essentially as described by Mangeat *et al.* [22] using the accumu-

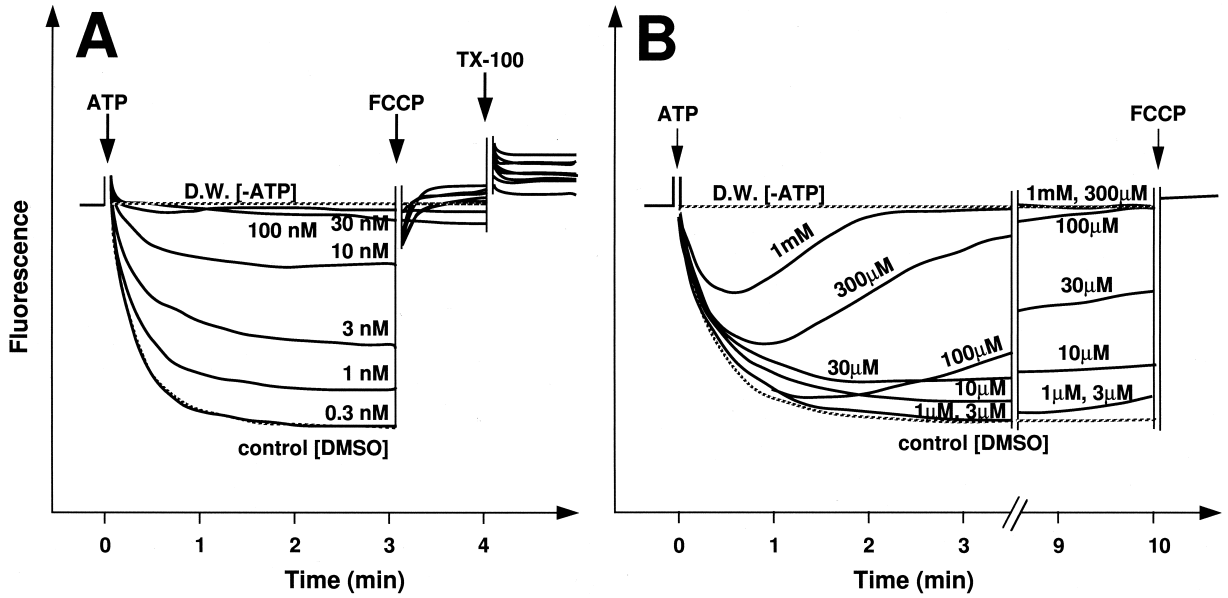

FIG. 1. Effect of metacycloprodigiosin, as compared with omeprazole, on the acidification activity of hog gastric mucosa membrane vesicles driven by $(H^+ + K^+)$-ATPase. The acidification activity of hog gastric membrane vesicles was assayed as described under Materials and Methods in incubation mixture with or without the indicated concentrations of metacycloprodigiosin (A) and omeprazole (B) (1% DMSO as solvent control) added 1 min before the addition of 1 mM ATP-Na_2. D.W., distilled water; FCCP, 1 μM: TX-100, 0.1% Triton X-100. Representative traces are from experiments performed at least three times.

lation of the fluorescent weak base, acridine orange, in apical acid vacuoles. Cells were preincubated in a medium containing 5 μM acridine orange, stimulated with histamine (10^{-4} M), then further incubated for 40 min, and viewed under a phase-contrast and fluorescence microscope (Axiovert+35M, Zeiss) using a B-type dichroic mirror (excitation filter, BP450–490; dichroic mirror, FT510; barrier filter, LP520).

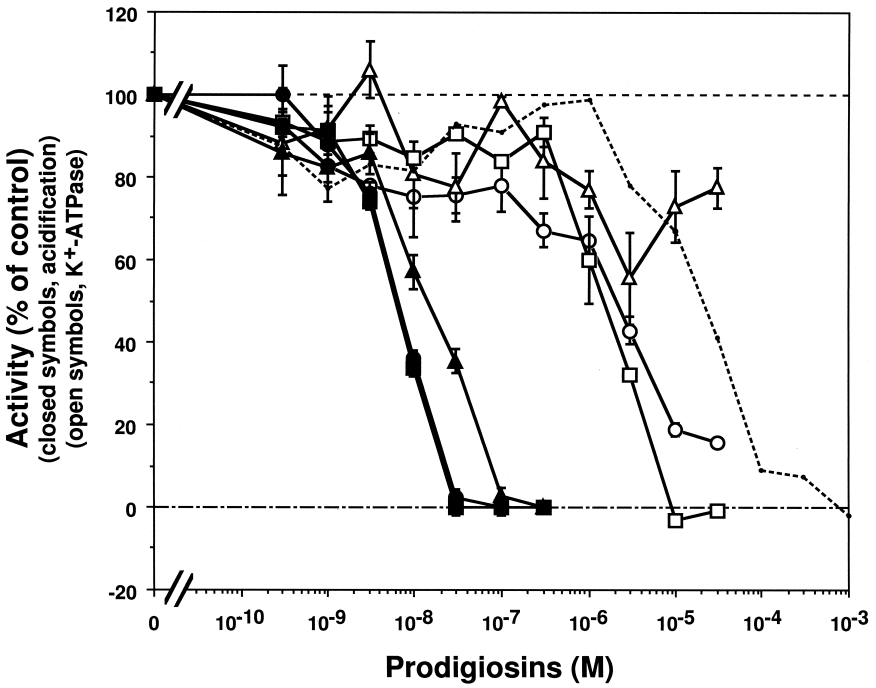

FIG. 2. Effects of prodigiosins on the acidification and K^+-ATPase activities of hog gastric mucosa membrane vesicles driven by $(H^+ + K^+)$-ATPase. The acidification activity of hog gastric membrane vesicles was assayed as described in the legend of Fig. 1 in incubation mixture with or without the indicated concentrations of prodigiosin, prodigiosin 25-C, or metacycloprodigiosin (1% DMSO as solvent control) added 1 min before the addition of 1 mM ATP-Na_2. K^+-ATPase activity was measured as described under Materials and Methods in incubation mixture with or without the indicated concentration of prodigiosins (1% DMSO as solvent control). The reaction was started by the addition of enzyme, and the mixture was incubated for 10 min at 37°. Symbols: (■,□) prodigiosin; (●,○) metacycloprodigiosin; and (▲,△) prodigiosin 25-C. Closed symbols represent acidification activity and open symbols K^+-ATPase activity, respectively. The dotted line (—●—) represents the concentration response of omeprazole on the K^+-ATPase activity expressed as the averages (with deviation of less than 5%) of duplicate experiments. The control value of the K^+-ATPase was about 0.72 to 0.75 U/mg protein. Representative data are from three independent experiments. Values are means ± SD.

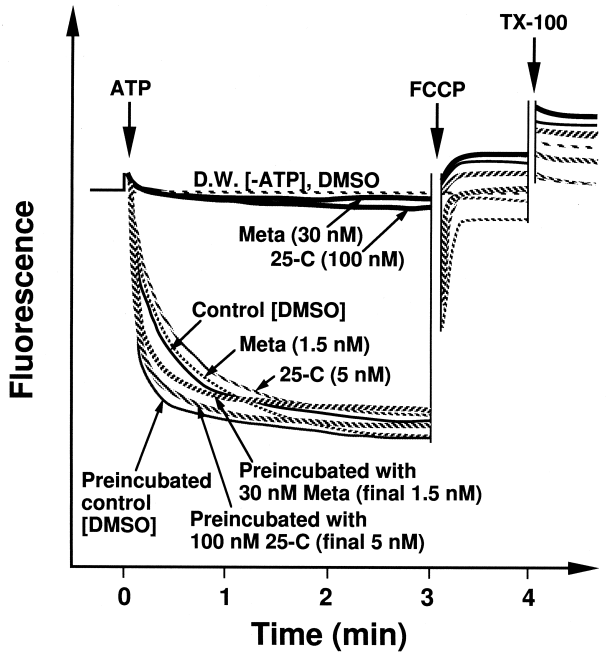

FIG. 3. Reversibility of the effects of prodigiosin analogues on the acidification activity of hog gastric $(H^+ + K^+)$-ATPase. Hog gastric membrane vesicles were added to 100 μL of preincubation mixture [8 mM PIPES–TMAH (pH 6.8), 0.15 M KCl, 2 mM $MgCl_2$, 5 μM valinomycin, 2 μM acridine orange with or without the indicated concentration of prodigiosin 25-C or metacycloprodigiosin (1% DMSO as solvent control)] and preincubated at 37° for 3 min; 1.9 mL of the dilution mixture [8 mM PIPES–TMAH (pH 6.8), 0.15 M KCl, 2 mM Mg_2, 5 μM valinomycin, 2 μM acridine orange] was added before the addition of 1 mM ATP-Na_2. ATP-dependent acidifications were measured as described in the legend to Fig. 1. 25-C, prodigiosin 25-C; Meta, metacycloprodigiosin; D.W., distilled water; FCCP, 1 μM; TX-100, 0.1% Triton X-100. Representative traces are from experiments performed at least three times.

Other Analytical Methods

The protein level was determined by the Amido Black/solid phase method of Schaffner and Weissmann [23] or a Bio-Rad protein assay kit according to the manufacturer's instructions (Bio-Rad), using BSA as the standard.

RESULTS

Inhibition of Acidification, But Not of K^+-Dependent ATP Hydrolysis Activity, of Hog Gastric Mucosa Membrane Vesicles by Prodigiosins

In this study, we used three types of prodigiosins: prodigiosin, metacycloprodigiosin, and prodigiosin 25-C. Figure 1 shows typical traces of the inhibition of the ATP-dependent acidification of hog gastric mucosa membrane vesicles by metacycloprodigiosin (panel A) and by omeprazole, a well known inhibitor of gastric $(H^+ + K^+)$-ATPase (panel B). Metacycloprodigiosin inhibited rapidly ($\ll$ 1 min) and concentration-dependently (1–30 nM) the acidification, which was reversed by a protonophore, FCCP. Omeprazole required a substantial period to express its inhibitory effect on the intravesicular acidification and to reverse intravesicular pH acidified by Mg-ATP. This lag time, which depended on the concentration of omeprazole (e.g. 3–5 min at 30 μM in our assays), reflects the time required for its conversion to active (SH-reagent) form and for subsequent reaction with the essential SH-group of the enzyme protein [24, 25]. Therefore, it is difficult to compare the effects of these two types of inhibitors directly and quantitatively. But it is clear that prodigiosins exert their acidification inhibitory effect without delay and at a lower concentration than omeprazole.

Figure 2 shows the concentration–responses of the inhi-

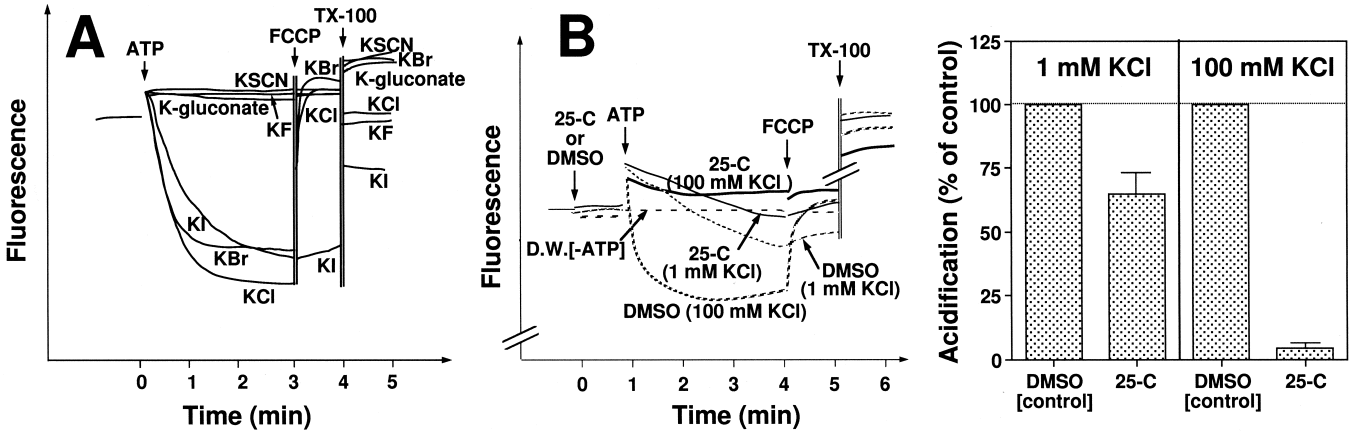

FIG. 4. Anion requirements of (A) acidification of hog gastric membrane vesicles and (B) its inhibition by prodigiosins. (A) Anion requirement of acidification. Hog gastric membrane vesicles were added to 2 mL of incubation mixture [8 mM PIPES–TMAH (pH 6.8), 0.15 M of potassium salt of the indicated anion, 2 mM $MgCl_2$, 5 μM valinomycin, 2 μM acridine orange] and incubated at 37° for 1 min before the addition of ATP-Na_2. ATP-dependent acidifications were measured as described in the legend to Fig. 1. ATP, 1 mM ATP-Na_2; FCCP, 1 μM; TX-100, 0.1% Triton X-100. (B) Anion requirement of metacycloprodigiosin for the inhibition of acidification. Hog gastric membrane vesicles were added to 2 mL of incubation mixture [8 mM PIPES–TMAH (pH 6.8), 2 mM $MgCl_2$, 5 μM valinomycin, 2 μM acridine orange with 100 nM prodigiosin 25-C (or 1% DMSO as solvent control)] containing either 1 mM KCl/99 mM K-gluconate or 100 mM KCl, and incubated at 37° for 1 min before the addition of ATP-Na_2. ATP-dependent acidifications were measured as described in the legend to Fig. 1. (Left and middle panels) fluorescence trace. (Right panel) bar presentation expressed as percent of control values (means ± SD of duplicate determinations). 25-C, 100 nM prodigiosin 25-C; DMSO, 1%; ATP, 1 mM ATP-Na_2; FCCP, 1 μM; TX-100, 0.1% Triton X-100. Representative data are from two independent experiments.

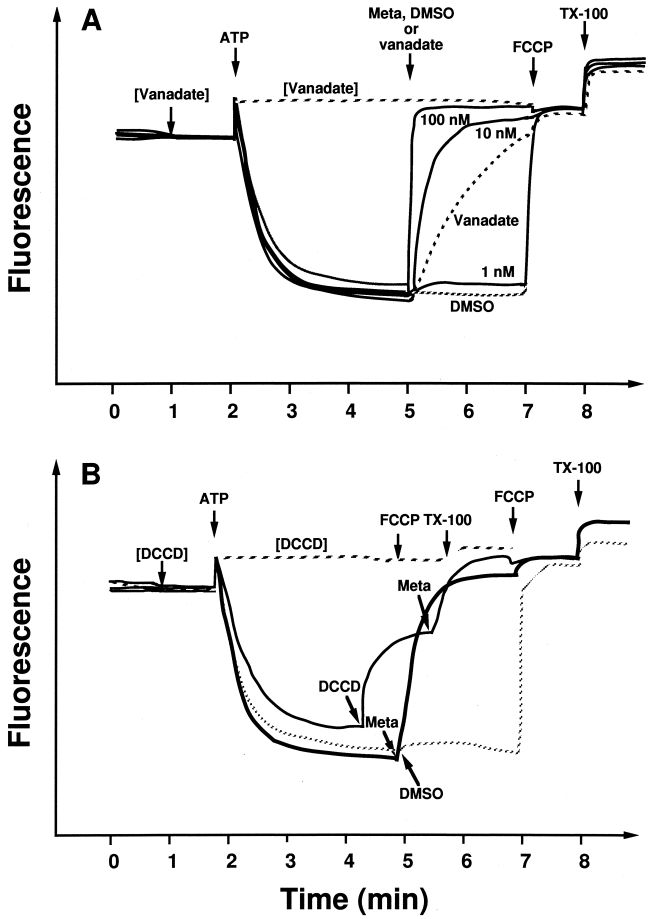

FIG. 5. Quick and concentration-dependent reversal of the acidification of vesicular pH by prodigiosins even in the presence of proton pump inhibitor. Hog gastric membrane vesicles were added to 2 mL of incubation mixture [8 mM PIPES–TMAH (pH 6.8), 0.15 M KCl, 2 mM $MgCl_2$, 5 μM valinomycin, 2 μM acridine orange] and incubated at 37° for 3 min in the presence of ATP. After 3 min, various concentrations of metacycloprodigiosin, DMSO, vanadate, or DCCD were added to the assay buffer. (A) Quick and concentration-dependent reversal of intravesicular pH by prodigiosins. Meta, 100, 10, or 1 nM metacycloprodigiosin; DMSO, 1%; vanadate, 100 μM sodium orthovanadate; ATP, 1 mM ATP-Na_2; FCCP, 1 μM; TX-100, 0.1% Triton X-100. Metacycloprodigiosin produced a quick and concentration-dependent reversal of acridine orange fluorescence. Vanadate (100 μM) added before ATP totally abolished the acidification activity. (B) Reversal of acidified intravesicular pH by prodigiosins even in the presence of proton pump inhibitor. Meta, 100 nM metacycloprodigiosin; DMSO, 1%; DCCD, 100 μM. Quick reversal of acridine orange fluorescence was observed by the addition of metacycloprodigiosin even in the presence of DCCD. DCCD (100 μM) added before ATP totally abolished the acidification activity. Representative traces are from two independent experiments.

bition of acidification by the three types of prodigiosin as compared with their effects on the K^+-ATPase activities of gastric mucosa membrane vesicles. All three prodigiosins inhibited vesicular acidification with similar IC_{50} values (6, 7, and 10 nM for prodigiosin, metacycloprodigiosin, and prodigiosin 25-C, respectively, at 190 μg protein/mL, which corresponded to the amount of prodigiosins that inhibit 50% of the activity (32, 37, and 53 pmol/mg protein, respectively). However, as was the case with V- and F-ATPases, they did not inhibit catalysis (K^+-ATPase activity) at concentrations (~ 1 μM) more than 10–100 times that of the IC_{50} of acidification inhibition (Fig. 2). At higher concentrations, prodigiosin and metacycloprodigiosin inhibited K^+-ATPase activity of hog gastric mucosa with IC_{50} values of 1–2 μM. Omeprazole inhibited K^+-dependent ATPase activity with an IC_{50} of about 30 μM (see the dotted line in Fig. 2), which was the same order of concentration required for the inhibition of acidification (Fig. 1B). Namely, omeprazole inhibited K^+-ATPase activity and acidification simultaneously, unlike prodigiosins.

Reversible Inhibition of Gastric Proton Pump by Prodigiosins

Figure 3 shows the results on the reversibility of prodigiosins on the acidification of hog gastric vesicles. The gastric vesicles were preincubated for 3 min with enough prodigiosin (20 and 100 nM for prodigiosin 25-C and metacycloprodigiosin, respectively) to strongly (~90%) inhibit acidification, as judged from Figs. 1 and 2, and then the mixtures were diluted 50-fold to lower the effective concentrations in the buffer (1.5 and 5 nM, respectively) enough to allow acidification of more than 90% of the control activity upon direct addition to the incubation buffer. As shown clearly in this figure, the acidification inhibitory activity of prodigiosins was hardly detectable after a 50-fold dilution of the incubation mixture. The apparent acceleration of acidification in the preincubated mixtures was most probably due to the higher concentration of K^+ in gastric vesicles attained by the entrance of K^+ from KCl buffer during preincubation in the presence of valinomycin. The kinetic analysis indicated a simple noncompetitive (with respect to ATP) type of inhibition [affecting V_{max} but not K_m for ATP (about 70 μM)], with apparent K_i values of 1.8, 1.8, and 2.0 nM (at 48 μg protein/mL) for prodigiosin, metacycloprodigiosin, and prodigiosin 25-C, respectively.

Chloride Requirement of Prodigiosins for the Inhibition of Acidification by Hog Gastric (H^++K^+)-ATPase

For the inhibition of V- and F-ATPases, prodigiosins require chloride ions [3–5]. Do prodigiosins also require chloride ions to inhibit the hog gastric proton pump? It has been reported that the hog gastric proton pump itself requires chloride ions and is not expressed in sulfate or gluconate buffer [26]. Figure 4A shows that hog gastric (H^++K^+)-ATPase requires halide ions in the order of chloride = bromide > iodide > gluconate (100:61:1.5 in the initial rate), but not fluoride or SCN^- for its activity; to our knowledge, this is the first comparative study of halide requirements of the gastric proton pump. This phenomenon has been explained as a collaboration between chloride channels and proton transport activities

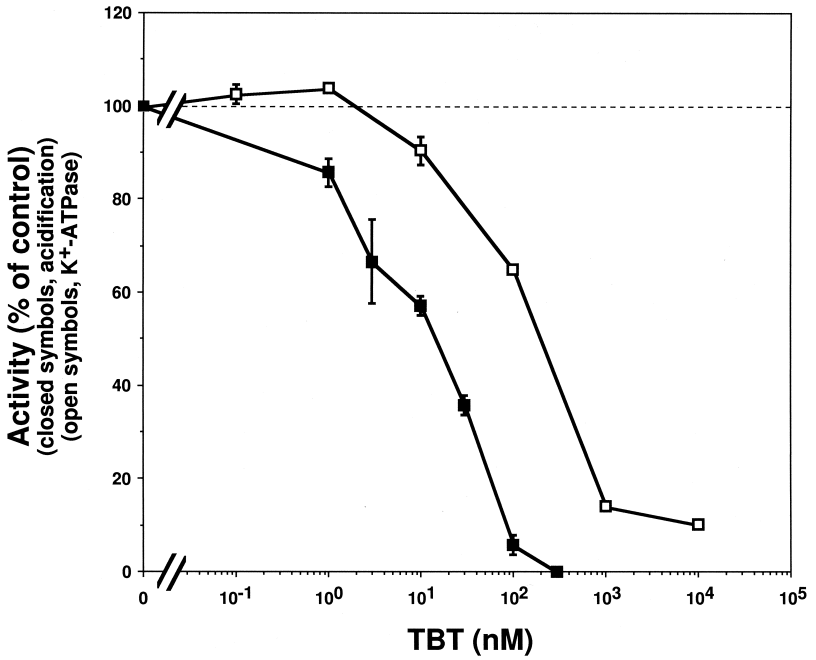

FIG. 6. Effects of TBT on the acidification and K^+-ATPase activities of hog gastric membrane vesicles driven by (H^++K^+)-ATPase. Acidification and K^+-ATPase activities were assayed, as described in the legend of Fig. 2, in incubation mixture with or without the indicated concentration of TBT (1% ethanol as solvent control). Symbols: (■) acidification; (□) K^+-ATPase. Representative data from the two independent experiments are presented as means ± SD of triplicate determinations. The control value of the K^+-ATPase activity was approximately 0.72 to 0.75 U/mg protein.

within (H^++K^+)-ATPase molecules [27, 28]: potassium channels and (H^++K^+)-ATPase provide a mechanism whereby in the absence of Cl^- the electroneutral (H^++K^+)-ATPase pump can generate an electrogenic flux of proton across the gastric mucosa and the Cl^- channel provides a path for the electrogenic secretion of Cl^- and, in conjunction with (H^++K^+)-ATPase and the potassium channel, a mechanism for HCl secretion [29, 30]. However, hog gastric (H^++K^+)-ATPase showed some acidification activity even at low concentrations of chloride. Hence, we tested the chloride requirement for the acidification inhibitory activity of prodigiosins by comparing their effects between buffers composed of either 1 mM chloride/99 mM gluconate or just 100 mM chloride (Fig. 4B). Prodigiosins required high concentrations of chloride ions to exert acidification inhibitory activity and exerted little activity in the 1 mM chloride/99 mM gluconate buffer.

Quick and Concentration-Dependent Reversal of the Acidification of Vesicular pH by Prodigiosins Even in the Presence of a Proton Pump Inhibitor

What is the mechanism of action of prodigiosins on the acidification of hog gastric vesicles? As shown in Fig. 5A, prodigiosins reversed the acidification of intravesicular pH quickly at the concentration at which acidification was strongly inhibited (more rapidly than vanadate or DCCD). Furthermore, this quick reversal was not affected by the blockade of the proton channel by preincubation with DCCD, which inhibits the enzyme irreversibly by covalently binding to it [31] (Fig. 5B). The rapid reversal of pH was observed, again, *only in the chloride buffer* (data not shown). These results are consistent with the idea that prodigiosins behave essentially as ionophores (H^+/Cl^- symporters; [5]).

Inhibition of Hog Gastric Proton Pump by TBT

TBT is widely used as an OH^-/Cl^- exchanger. Therefore, we can expect TBT to inhibit the acidification of hog gastric vesicles mediated by (H^++K^+)-ATPase. Figure 6 shows that indeed this was the case. TBT inhibited the acidification of hog gastric mucosa membrane vesicles with an IC_{50} of 5–15 nM. However, it also inhibited the K^+-ATPase activity of hog gastric mucosa membrane vesicles with an ic_{50} of 150 nM (at 48 μg protein/mL), as was the case for F- and V-ATPases [32–34]. This was probably due to the covalent modification activity of TBT, which resembled that of an SH-reagent [33, 35–38]; gastric K^+-ATPase is inhibited by SH-reagents such as omeprazole (after activation by mucosal acidity) [24, 25].

Inhibition of Acid Secretion by Prodigiosins in Parietal Cells Isolated from Rabbit Gastric Mucosa

Finally, prodigiosins were found to inhibit acid secretion of parietal cells isolated from rabbit gastric mucosa. As shown in Fig. 7, prodigiosins, like omeprazole, strongly inhibited the accumulation of orange fluorescence of weak base (acridine orange) within apical acid vacuoles of isolated parietal cells stimulated by a secretagogue histamine. Prodigiosins affected acridine orange accumulation within small lysosome-like vesicles, too, which was affected only marginally by omeprazole. TBT also inhibited strongly the acridine orange accumulation within both large apical vacuoles and small lysosome-like vesicles, probably reflecting depletion of cellular ATP.

DISCUSSION

In the present study, we examined the effects of prodigiosins on the activity of (H^++K^+)-ATPase on membrane

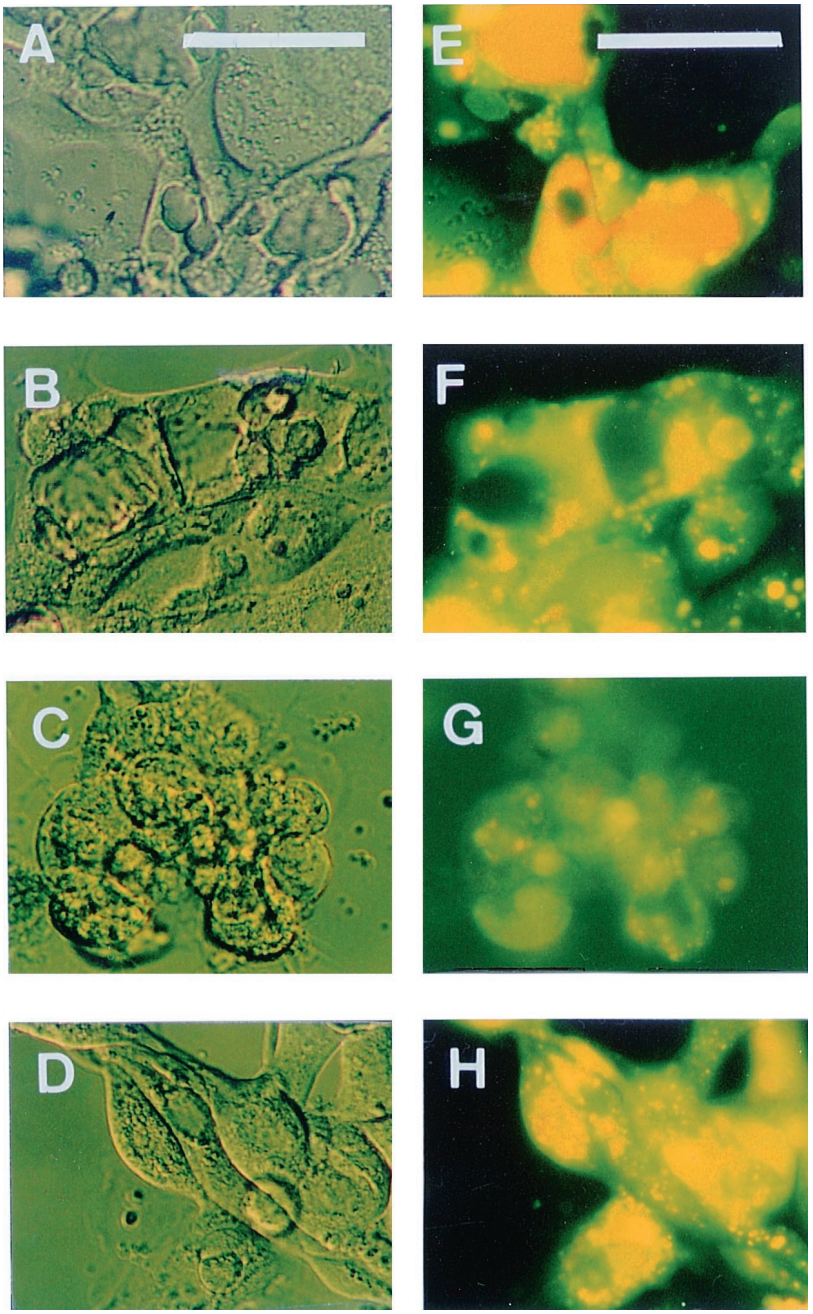

FIG. 7. Inhibition of acid secretion by prodigiosins in gastric parietal cells isolated from rabbit stomach. Parietal cells isolated from rabbit gastric glands were preincubated in medium containing 10^{-6} M quinacrine, stimulated with histamine (10^{-4} M), further incubated for 40 min in the presence of prodigiosin 25-C, TBT, or omeprazole, or in their absence (DMSO as solvent control) and viewed under a phase and fluorescence microscope using a B-type dichroic mirror. The pictures are representatives of the experiments performed twice. (A–D) phase contrast; (E–H) fluorescence. (A, E) control (1% DMSO); (B, F) 2.5 μM prodigiosin 25-C; (C, G) 2.5 μM TBT; (D, H) 100 μM omeprazole. Bars represent 50 μm. Accumulation of orange fluorescence in the large vacuoles disappeared after treatment of parietal cells with prodigiosin 25-C, with TBT, as well as with omeprazole. Representative data are from two independent experiments.

vesicles from hog gastric mucosa. The results clearly showed that: (i) prodigiosins also potently inhibit intravesicular acidification of hog gastric mucosa with IC_{50} values of 32–103 pmol/mg protein without inhibiting K^+-ATPase activity, (ii) prodigiosins inhibit ATPase activities of the gastric proton pump $[(H^+ + K^+)$-ATPase] only at high concentrations (IC_{50} = 1.5 and 3 μM for prodigiosin and metacycloprodigiosin, respectively), (iii) the effects of prodigiosins are rapid and reversible compared with that of omeprazole, a representative potent irreversible inhibitor of the gastric proton pump, and non-competitive (for ATP) with apparent K_i values of 1.8 to 2.0 nM, (iv) the gastric proton pump itself requires chloride ions for the acidification of gastric membrane vesicles, but prodigiosins require a high concentration of chloride ions to inhibit acidification, (v) prodigiosins quickly reverse the acidification of intravesicular pH even in the presence of a general covalent modifier of the proton pump, DCCD, suggesting that they themselves possess ionophoric activity to promote proton movement across vesicular membranes, and (vi) prodigiosins, in fact, prevent acid secretion of isolated parietal cells through inhibition of acid accumulation in apical acid vacuoles.

The results suggest that prodigiosins exhibit a more general inhibition of acidification. This is consistent with our findings that they exhibit ionophoric activity (H^+/Cl^- symport) even on liposomes [5]. In fact, prodigiosins also inhibit the acidification driven by the respiratory proton

pump, the proton translocation mechanism of which is totally different from that of H^+-translocating ATPases [39, *]. Recently, cycloprodigiosin has also been reported to show chloride-dependent uncoupling of proton pumping pyrophosphatase [40]. However, the results obtained thus far do not completely exclude the former possibility of interaction with the enzyme's *common* site required for proton translocation. In fact, at high enough concentrations, prodigiosins inhibit ATP hydrolysis too, suggesting their interaction with the enzyme molecules. We are now investigating the mode of inhibition of ATPase activity by prodigiosins *at higher concentrations*.

The new type of proton pump inhibitors (actually *uncouplers*) described in this paper will be useful not only for the clarification of the mechanism of acidification driven by gastric proton pumps but also for the clinical treatment of gastritis. To date, only omeprazole-type (e.g. omeprazole, lansoprazole) proton pump inhibitors have been used successfully for the treatment of peptic ulcer diseases (gastritis): these are acid-activated prodrugs that covalently bind to and inactivate the $(H^+ + K^+)$-ATPases [24, 25]. However, as a long duration of action appears to cause undesirable side-effects, quick and reversible drugs are awaited, the duration of action of which should be determined solely by pharmacokinetic properties [41]. Reversible-type proton pump inhibitors that compete for the potassium binding site of the enzyme have been reported [42]: SCH 28080 [43], SK&F 96067 [44], MDPQ [45], scopadulcic acid or scopadol [46], and benzimidazole class analogues, e.g. 4-(phenylamino)quinoline-3-carboxamides [47] and SK&F 97574 [3-butyryl-4-(2-methylamino)-8-(2-hydroxyethoxy) quinoline] [48]. Prodigiosins may constitute a separate group of strong reversible proton pump inhibitors: a group of H^+/Cl^- symporters that affect only the pH of acidic compartments (like gastric apical acid vacuoles) without any discernible effect on the cellular ATP level, unlike general uncouplers that affect cellular oxidative phosphorylation as well [1]. Therefore, we can expect wide range applicability of prodigiosins in this field. Furthermore, prodigiosins inhibit the growth of *Helicobacter pylori* cells [49]. It is not clear if this is due to inhibition of F-ATPase or of P-ATPase [50], but slightly higher concentrations of prodigiosins are required to inhibit the growth of *H. pylori* cells (MIC_{50} = 1 μM and MIC_{90} = 4 μM for prodigiosin 25-C) than to inhibit cell-free proton pump activity. Prodigiosins may also exhibit an additional protective effect against gastritis and peptic ulcer diseases, by suppressing vacuole formation, as does bafilomycin A_1 [51].

Our results also suggest that the conventional method of screening gastric proton pump inhibitors by ATPase inhibition assay does not pick up new classes of proton pump inhibitors. By screening the acidification inhibitor, one is able to find new groups of proton pump inhibitors. Furthermore, the ability of prodigiosins to perturb acid secretion may be useful for the treatment of bone resorption as is the case with omeprazole [52]. Such studies are now in progress in our laboratory.

We thank Mr. K. Yoshimura of the Meat Inspection Center of Kanazawa City for providing the fresh hog stomachs. We are also grateful to Professor N. Takeguchi and Dr. S. Asano (Toyama Medical and Pharmaceutical University) for donating test samples of membrane vesicles obtained from hog gastric mucosa and invaluable discussions, and Professor S. Okabe (Kyoto Pharmaceutical University) for instructions on the isolation of parietal cells of gastric mucosa glands from rabbits. The authors acknowledge the excellent technical assistance of Mses. K. Hirano, A. Kojima, and A. Hayashi (Faculty of Pharmaceutical Sciences, Kanazawa University). Thanks are also due to Drs. K. Yokoyama and K. Arai (Faculty of Pharmaceutical Sciences, Kanazawa University) for invaluable discussions. The authors would like to express special thanks to Professor Emeritus Y. Anraku (Graduate School of Science, The University of Tokyo) for critical reading of the manuscript. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

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