Prodigiosins uncouple lysosomal vacuolar-type ATPase through promotion of H^+/Cl^- symport

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We reported previously [Kataoka, Muroi, Ohkuma, Waritani, Magae, Takatsuki, Kondo, Yamasaki and Nagai (1995) FEBS Lett. **359**, 53–59] that prodigiosin 25-C (one of the red pigments of the prodigiosin group produced by micro-organisms like *Streptomyces* and *Serratia*) uncoupled vacuolar H^+-ATPase, inhibited vacuolar acidification and affected glycoprotein processing. In the present study we show that prodigiosin, metacycloprodigiosin and prodigiosin 25-C, all raise intralysosomal pH through inhibition of lysosomal acidification driven by vacuolar-type (V-)ATPase without inhibiting ATP hydrolysis in a dose-dependent manner with IC_{50} values of 30–120 pmol/mg of protein. The inhibition against lysosomal acidification was quick and reversible, showing kinetics of simple non-competitive (for ATP) inhibition. However, the prodigiosins neither raised the internal pH of isolated lysosomes nor showed ionophoric activity against H^+ or K^+ at concentrations where they strongly inhibited lysosomal acidification. They required Cl^- for their acidification inhibitory activity even when driven in the presence of K^+ and valinomycin, suggesting that their target is not anion (chloride) channel(s). In fact, the prodigiosins inhibited acidification of proteoliposomes devoid of anion channels that were reconstituted from lysosomal vacuolar-type (V-)ATPase and *Escherichia coli* phospholipids. However, they did not inhibit the formation of an inside-positive membrane potential driven by lysosomal V-ATPase. Instead, they caused quick reversal of acidified pH driven by lysosomal V-ATPase and, in acidic buffer, produced quick acidification of lysosomal pH, both only in the presence of Cl^-. In addition, they induced swelling of liposomes and erythrocytes in iso-osmotic ammonium salt of chloride but not of gluconate, suggesting the promotion of Cl^- entry by prodigiosins. These results suggest that prodigiosins facilitate the symport of H^+ with Cl^- (or exchange of OH^- with Cl^-) through lysosomal membranes, resulting in uncoupling of vacuolar H^+-ATPase.

INTRODUCTION

Vacuolar-type H^+-ATPases (V-ATPases) reside on the membranes of acidic organelles such as synaptic vesicles, chromaffin granules, platelet-dense granules, secretary granules, lysosomes and the *trans*-Golgi network and maintain an acidic environment by pumping H^+, using the energy of ATP hydrolysis [1–4]. The acidic pH within such organelles has been suggested to be responsible for a wide variety of important cellular functions, such as endocytosis, exocytosis and intracellular trafficking, as well as for cell differentiation, cell growth and cell death [5–8]. V-ATPases are present also on the plasma membranes of some types of cells, thereby pumping H^+ out of the cells to acidify the extracellular milieu as well as to maintain cytosolic pH (osteoclasts, macrophages, activated neutrophils, renal epithelial cells and some tumour cells) [2]. Recently, bafilomycins [9], concanamycins [10] and destruxins [11,12], originally found in microbial metabolites, have proved to be potent selective inhibitors of V-ATPase and have become the most useful probes in clarifying the role of vacuolar (or extracellular) acidification by V-ATPases [13].

We reported previously, that prodigiosin 25-C, one of the red pigments of the prodigiosin group produced by micro-organisms such as *Streptomyces* and *Serratia*, raises lysosomal pH and suppresses glycoprotein processing as a result of its uncoupling effect on vacuolar (lysosomal) H^+-ATPase without showing apparent protonophoric activity [14]. Although prodigiosin 25-C seems to affect mitochondrial F-type (F-)ATPase and produce swelling of mitochondria besides the Golgi apparatus, it did not affect the cellular ATP level, showing relatively specific perturbation of vacuolar pH when treated *in vivo*, which makes it a promising drug for the analysis of vacuolar function. In fact, prodigiosin 25-C selectively inhibits T-cell proliferation induced by concanavalin A [15,16], exerts immunomodulating activities in immunized mice [17–19] and suppresses the bone resorption by osteoclasts [20], most likely explained by the uncoupling effect on vacuolar H^+-ATPases.

Here we present full details of the effect of prodigiosin-group antibiotics on the V-type H^+-ATPases, mostly using rat liver lysosomes as vacuolar organelles and show evidence that prodigiosin antibiotics are a new group of H^+/Cl^- symporters (or OH^-/Cl^- exchangers) that uncouple (dissociate) H^+ translocation from otherwise coupled catalysis in such a way that they inhibit acidification but neither catalysis nor membrane potential formation.

A preliminary account of this work was presented at the 22nd

Abbreviations used: $\Delta\psi$, transmembrane potential gradient; ΔpH, transmembrane pH gradient; diS-C_3-(5), dipropylthiadicarbocyanine iodide; DTT, dithiothreitol; F-ATPase, F-type H^+-ATPase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FD, fluorescein-labelled dextran; FD-dextranosomes, FD-loaded lysosomes; NEM, *N*-ethylmaleimide; TBT, tributyltin chloride; TMAH, tetramethylammonium hydroxide; TMG, tritosomal (Triton-filled lysosomes) membrane ghosts; TX-100, Triton X-100; V-ATPase, vacuolar-type H^+- ATPase.

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Meeting of the Japan Bioenergetics Group held in Nagoya on 18 December 1996 [21].

EXPERIMENTAL

Materials

Rats (Wistar, male) were obtained from Sankyo Labo Service (Tokyo, Japan). Outdated erythrocytes from normal subjects were supplied from the Blood Center of Japan Red Cross (Kanazawa) via Kanazawa University Hospital. Prodigiosin was prepared from the culture broth of *Serratia marsescens* [22] and metacycloprodigiosin and prodigiosin 25-C were from *Streptomyces hiroshimensis* as described in [23]. Triton WR-1339 was obtained from Ruger Chemical Company (Irvington, NJ, U.S.A.). n-Octyl β-D-thioglucoside was purchased from Dojin (Kumamoto, Japan). Fluorescein-labelled dextran (FD) was either synthesized as described in [24] or supplied in the form of FITC-dextran (M_r 70000) from Sigma (St. Louis, MO, U.S.A.). Acridine Orange was supplied by Wako (Tokyo, Japan) and dipropylthiadicarbocyanine iodide [diS-C_3-(5)] was supplied from Nippon Kanko Shikiso (Okayama, Japan). Phosphatidylcholine from soybean phospholipids was obtained from Sigma (Sigma type II-S, a so-called 'asolectin') and used without purification. *Escherichia coli* phospholipid was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.), and purified as described by Newman [25]. Protease inhibitors of microbial origin were obtained from the Peptide Research Institute (Osaka, Japan). Bafilomycin A_1 was either kindly provided by Professor K. Altendorf (University of Osnabrück, Osnabrück, Germany) or obtained from Wako (Tokyo, Japan). Other reagents were purchased as commercial products mostly from Sigma (St. Louis, MO, U.S.A.) and used without further purification.

Preparation of lysosomes (dextranosomes) and chromaffin granules

Preparation of FD-loaded lysosomes (FD-dextranosomes) from rat liver was performed as described [26], with the exception that the Percoll washout procedure was omitted and the lysosomal layer in the Percoll gradient was used for the experiments. Bovine chromaffin granules were obtained as described in [27].

Reconstitution of lysosomal H^+ pump into proteoliposomes

Reconstitution of the lysosomal H^+ pump into proteoliposomes from solubilized lysosomal H^+-ATPase was performed as described in [28]: briefly, membrane vesicles from Triton-filled lysosomes (tritosomal membrane ghosts, TMG) [29,30] were solubilized with n-octyl β-D-thioglucoside in the presence of asolectin and incorporated into *E. coli* phospholipid liposomes by dilution, freeze–thawing and sonication in 0.15 M KCl/10 mM dithiothreitol (DTT)/20 mM Mops/tetramethylammonium hydroxide (TMAH), pH 7.0, containing 5 μg/ml each of the protease inhibitors [28].

H^+ pump assay

The formation of a pH gradient (inside acid) was measured by means of the fluorescence quenching of FD incorporated into lysosomes (for intact lysosomes) [31–33] or the permeant basic dye Acridine Orange (for reconstituted proteoliposomes). The formation of a transmembrane potential gradient ($\Delta\psi$) (inside-positive) was measured by the fluorescence quenching of diS-C_3-(5) (for intact lysosomes) [34]. The assay buffer, unless otherwise indicated, contained 0.1 M KCl, 0.2 M sucrose, 20 mM Hepes/TMAH, pH 7.5, 0.5 mM $MgCl_2$ and 1 mM Na_2ATP in a final volume of 2.0 ml with or without the dyes. The concentrations of the dyes were 1 μM for Acridine Orange and 0.5 μM for diS-C_3-(5). Fluorescence was measured at 37 °C with a Hitachi 850 or F-4500 spectrofluorimeter with excitation at 495 nm and emission at 520 nm for FD, 480 and 520 nm for Acridine Orange, and 620 and 670 nm for diS-C_3-(5) respectively with a 5 nm slit width for both monochrometers. The initial velocity of acidification was defined by the rate of ATP-dependent quenching of FD fluorescence [$\Delta F(\%)/10$ s].

ATPase assay

ATPase assays (bafilomycin A_1-sensitive ATPase) were performed as described in [30]. The assay buffer contained 0.1 M KCl, 0.2 M sucrose, 20 mM Hepes/TMAH, pH 7.5, 0.5 mM $MgCl_2$ and 1 mM Na_2ATP in a final volume of 1.0 ml and the reaction mixture was incubated at 30 °C for 40 min. The liberated phosphate was estimated by the Malachite Green method [35]. All data shown are the averages (with deviation of less than 5%) for duplicated experiments.

Preparation of liposomes and erythrocytes for swelling assay

Liposomes were prepared from phosphatidylcholine essentially as described in [36,37]. Briefly, phosphatidylcholine (Sigma type II-S phospholipids) was suspended (50 mg/ml) in 20 mM Hepes/TMAH, pH 7.5, containing 0.25 M sucrose (usually 2 ml), vortex-mixed vigorously and sonified at room temperature for 5 min. Human erythrocytes were washed several times, and resuspended at 10% (v/v) in 0.15 M NaCl (or sodium gluconate) containing 1 mM acetazolamide (an inhibitor of carbonic anhydrase).

Swelling assay

The effect of prodigiosins on the permeability of liposomal and erythrocyte membranes to anions was evaluated by the swelling of liposomes and erythrocytes in iso-osmotic ammonium salts, which was monitored by the decrease in A_{550} [38]. Briefly, liposomes prepared in 2 mM Hepes/TMAH (pH 7.5)/0.25 M sucrose, and erythrocytes, washed in 0.15 M NaCl (or sodium gluconate)/1 mM acetazolamide, were loaded at 1 mg/ml and 0.033% (v/v) (122 mg of protein/ml) respectively in 0.2 M ammonium salt, pH 7.0 (containing 1 mM acetazolamide for erythrocytes) of either chloride (NH_4Cl) or gluconate (ammonium gluconate) and then, after 30–60 s, prodigiosins (DMSO as solvent control) or tributyltin chloride (TBT) (ethanol as solvent control) were added while monitoring A_{550}.

Other analytical methods

Protein was determined by the Amido Black/solid-phase method of Schaffner and Weissman [39] or Coomassie Brilliant Blue/liquid-phase method of Bradford [40] using a Bio-Rad (Richmond, VA, U.S.A.) protein assay kit according to the manufacturer's instruction manual, with BSA as the standard.

RESULTS

Prodigiosins raise lysosomal pH through inhibition of acidification, but not ATP hydrolysis, mediated by V-ATPase

In this study we used three types of prodigiosins: prodigiosin, metacycloprodigiosin and prodigiosin 25-C (Figure 1). They have a general structure of methoxybipyrrolopyrromethene with a hydrocarbon side chain attached to the second carbon atom of the 'right-hand' pyrrole ring. As all the prodigiosins tested

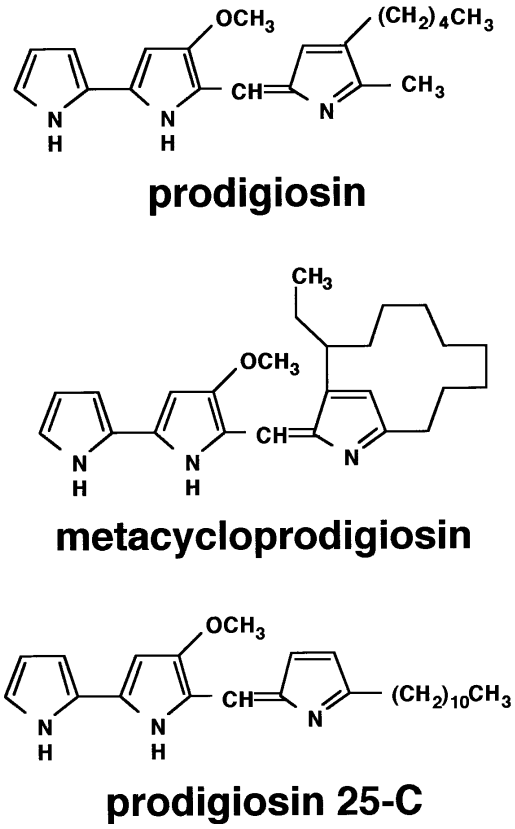

Figure 1 Chemical structures of prodigiosin, metacycloprodigiosin and prodigiosin 25-C

showed essentially the same effects, we present in most cases only the results obtained with one of them in this paper. Like prodigiosin 25-C [14], all the prodigiosins tested increased lysosomal pH in cultured cells. From their weak basic nature (pK_a 7.35 for prodigiosin 25-C [23]), prodigiosins may well raise lysosomal pH. However, unlike 40 mM NH_4Cl, which increases the internal pH of isolated lysosomes to near neutrality [33], the prodigiosins hardly raised, but sometimes rather slightly decreased, the intralysosomal pH at the micromolar concentrations used (Figure 2A for prodigiosin 25-C). Instead, they inhibited ATP-dependent acidification of isolated lysosomes, which resulted in an increase of lysosomal pH to neutrality in the long run (in KCl), like bafilomycin A_1, although the extent of pH increase seems to be slightly less than that of bafilomycins. Figure 2(B) shows the dose response of inhibition of lysosomal acidification by metacycloprodigiosin.

Figure 3 shows the dose responses of the prodigiosins as compared with their effect on the bafilomycin A_1-sensitive ATPase activities of lysosomal H^+-ATPase (V-ATPase). The IC_{50} values for the inhibition of lysosomal acidification by prodigiosin, metacycloprodigiosin and prodigiosin 25-C were between 5 and 20 nM (5, 10 and 20 nM respectively) under these conditions (170 mg of protein/ml) (30–120 pmol/mg of protein for 50 % inhibition) (Figure 3A). On the other hand, none of the prodigiosins tested inhibited bafilomycin A_1-sensitive ATPase activity at least up to concentrations ($\approx 1\,\mu M$) of more than 50–100 times that of the IC_{50} of acidification inhibition, which contrasts with the effect of bafilomycins, which inhibit both acidification and ATPase activities with a comparable IC_{50} of in the nanomolar range (Figure 3B; for bafilomycin A_1).

Reversibility of inhibition of lysosomal acidification by prodigiosins

The rapid inhibitory effects of prodigiosins are completely reversible. As shown in Figure 4, the inhibitory effect of 100 nM metacycloprodigiosin on the lysosomal acidification was almost totally reversed by 20-fold dilution of the prodigiosin to 5 nM after 2 min of preincubation with lysosomes.

Kinetics of inhibition of lysosomal acidification by prodigiosins

The inhibition of lysosomal acidification by prodigiosins is of a simple non-competitive type. Figure 5(A) shows the Lineweaver–Burk plot of the inhibition of lysosomal acidification by metacycloprodigiosin, and Figure 5(B) shows the Dixon plot, which gives a single apparent K_i value. The K_i values of acidification inhibition were 49.0, 58.2 and 60.8 nM (at about 350 μg of protein/ml) for prodigiosin, metacycloprodigiosin and prodigiosin 25-C respectively.

Prodigiosins do not show detectable ionophoric activity against H^+ or K^+

Prodigiosins hardly affected intralysosomal pH at the concentrations where a strong inhibitory effect on lysosomal acidification was detectable (Figure 2). Furthermore, prodigiosins did not show detectable ionophoric activity against H^+ or K^+ on lysosomal membranes as shown in Figure 6 (for prodigiosin 25-C). In KCl buffer, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin, in combination but not alone, raised the intralysosomal pH rapidly, whereas none of the prodigiosins, either alone or in combination with valinomycin or FCCP, affected the intralysosomal pH, suggesting very weak protonophoric activity of prodigiosins. The fluorescence quenching by prodigiosins certainly involves their direct quenching effect on the FD fluorescence at high concentrations, but the physical interaction of prodigiosins with FD fluorescence does not fully explain their quenching effect, because far less of a quenching effect was observed in gluconate buffer (see below).

The prodigiosin effect does not depend on anion (chloride) channels but requires Cl^-

There are several ways of inhibiting lysosomal acidification besides direct inhibition of the catalytic activity of H^+-ATPase molecules, among which is the blocking of anion (for example, chloride) channel(s), thereby inhibiting the movement of anions that allow continued intravesicular acidification. To clarify this point, we first examined the effect of prodigiosins on lysosomal acidification promoted by V-ATPase in the presence of valinomycin (namely acidification produced by extrusion of internal K^+ in exchange for H^+ uptake driven by V-ATPase). As shown in Figure 7(A), prodigiosins inhibited lysosomal acidification in chloride buffer even in the presence of valinomycin, suggesting that the acidification inhibitory effect of prodigiosins was not due to anion-channel inhibition. However, prodigiosins still required Cl^-, and their acidification inhibitory effect was hardly observed in gluconate buffer (Figure 7B): prodigiosins inhibited lysosomal acidification with almost linear dependency on the Cl^- concentration with no obvious saturation, similarly to the situation with TBT (results not shown). It was also noteworthy that the quenching effect of prodigiosins on the FD fluorescence was only slight in gluconate buffer.

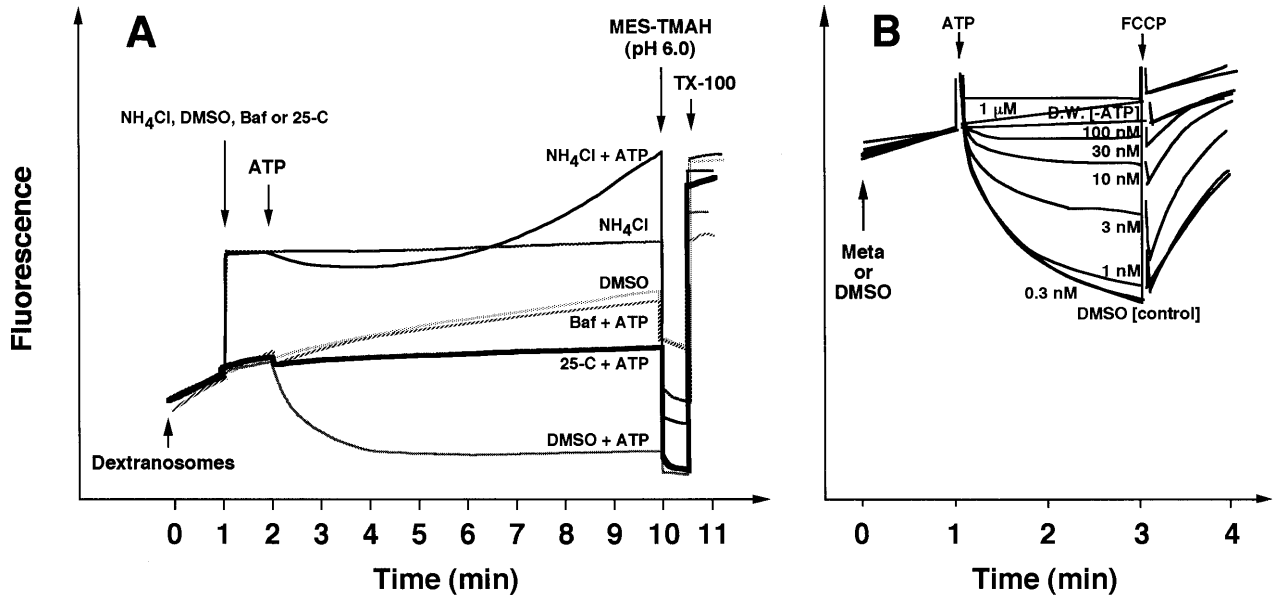

Figure 2 Effects of prodigiosins on (A) the internal pH and (B) the acidification of isolated lysosomes

Lysosomes (dextranosomes) loaded with FD were incubated in the incubation mixture [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/0.2 M sucrose/0.5 mM $MgCl_2$] at 37 °C, and the FD fluorescence was recorded continuously. (**A**) To the incubation mixture was added NH_4Cl, DMSO, prodigiosin 25-C or bafilomycin A_1 at 1 min, with or without ATP at 2 min. The concentrations were: DMSO, 1%; NH_4Cl, 40 mM; bafilomycin A_1 (Baf), 100 nM; Na_2ATP (ATP), 1 mM; prodigiosin 25-C (25-C), 250 nM; TX-100, 0.1%; Mes/TMAH, pH 6.0 (MES-TMAH), 50 mM. The increase in FD fluorescence by the combined addition of NH_4Cl and ATP is due to release of FD from lysosomes osmotically disrupted through massive accumulation of NH_4Cl [33]. (**B**) To the incubation mixture was added the indicated concentrations of metacycloprodigiosin 1 min before the addition of Na_2ATP (1 mM). D.W., distilled water. Prodigiosins strongly, rapidly (< 1 min) and dose-dependently (1–250 nM) inhibited ATP-dependent acidification of isolated lysosomes, which was reversed by a protonophore, FCCP.

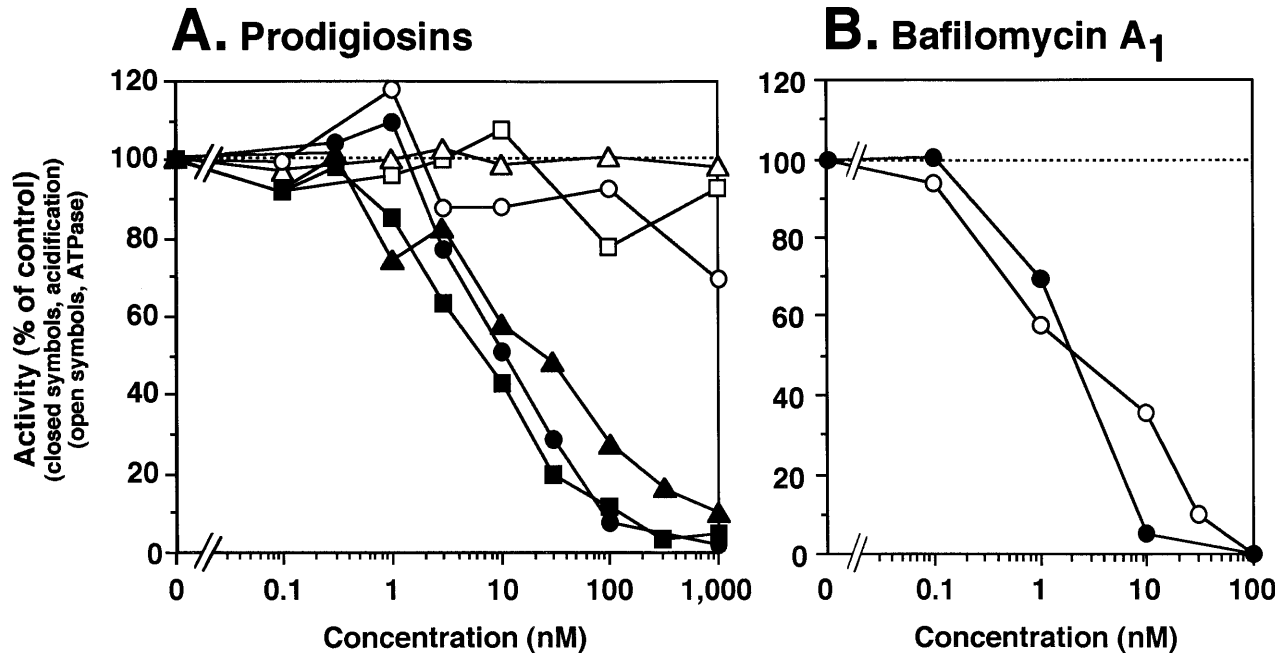

Figure 3 Effects of prodigiosins on the lysosomal acidification (closed symbols) and the bafilomycin A_1-sensitive ATPase activities (open symbols)

Acidification of lysosomes was measured using dextranosomes loaded with FD as described in the Experimental section in buffers [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/0.2 M sucrose/0.5 mM $MgCl_2$] with or without the indicated concentrations of inhibitors (1% DMSO as solvent control) added 1 min before the addition of 1 mM Na_2ATP. Bafilomycin A_1-sensitive ATPase activity of lysosomes was measured using TMG solubilized with n-octyl β-D-thioglucoside [35,36] as described in the Experimental section in buffers with or without the indicated concentrations of inhibitors (1% DMSO as solvent control). The ATPase reaction was started by the addition of enzyme and the mixture was incubated for 40 min at 30 °C. (**A**) Prodigiosins: ■, □, prodigiosin; ○, ●, metacycloprodigiosin; ▲, △, prodigiosin 25-C. (**B**) Bafilomycin A_1.

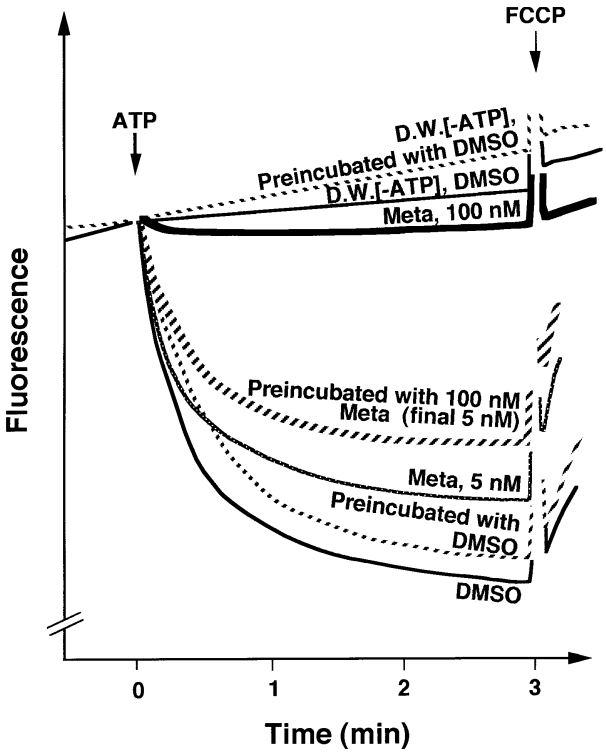

Figure 4 Reversibility of the effect of metacycloprodigiosin on the lysosomal H^+ pump activity

Lysosomes loaded with FD were added to 100 μl of the preincubation mixture [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/0.2 M sucrose/0.5 mM $MgCl_2$, with or without the indicated concentration of metacycloprodigiosin (Meta) (1% DMSO as solvent control)] and preincubated at 37 °C for 2 min. A 1.9 ml portion of the dilution buffer [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/0.2 M sucrose/0.5 mM $MgCl_2$] was added before the addition of 1 mM Na_2ATP. D.W., distilled water.

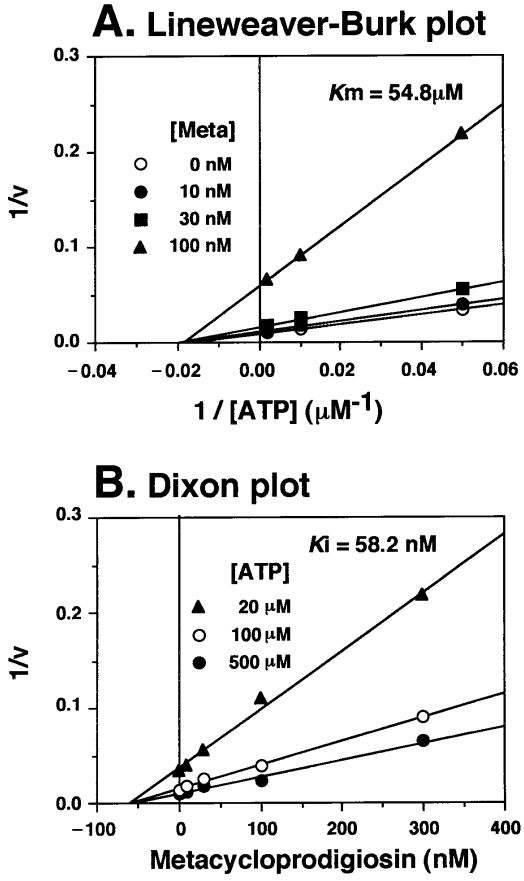

Figure 5 Kinetics of the inhibition of lysosomal acidification by metacycloprodigiosin

Acidification activity was measured as described in the Experimental section in buffers with or without the indicated concentrations of metacycloprodigiosin (Meta) (1% DMSO as solvent control) added 1 min before the addition of various concentrations of Na_2ATP and the results were analysed by (**A**) a Lineweaver-Burk or (**B**) a Dixon plot.

Prodigiosins inhibit acidification of proteoliposomes reconstituted from lysosomal V-ATPase

To obtain more direct evidence that the target molecules of prodigiosins are not anion channels, we measured their activity on a reconstituted lysosomal H^+ pump that was devoid of chloride channels [28]. In this system, reconstituted proteoliposomes were sonified to remove chloride channels. As shown in Figure 8, even in this system intraliposomal acidification was strongly (even at lower concentrations, most probably due to the very low concentrations of target molecules in the assay system) inhibited by prodigiosin 25-C. This result clearly shows that the target of prodigiosins is not anion channels.

Prodigiosins do not inhibit ATP-dependent formation of $\Delta\psi$

Figure 9 shows $\Delta\psi$ formation as detected by the fluorescence quenching of diS-C_3-(5). Unlike ordinary inhibitors of H^+ pump (like bafilomycin and FCCP; Figure 9B), prodigiosins did not affect the formation of $\Delta\psi$ (inside-positive) driven by V-ATPase at concentrations ranging from 10 pM to 1 μM (Figure 9A).

Prodigiosins induce a quick reversal of acidified lysosomal pH requiring Cl^-

The fact that prodigiosins do not inhibit $\Delta\psi$ formation by lysosomal V-ATPase suggests that prodigiosins functionally (1)

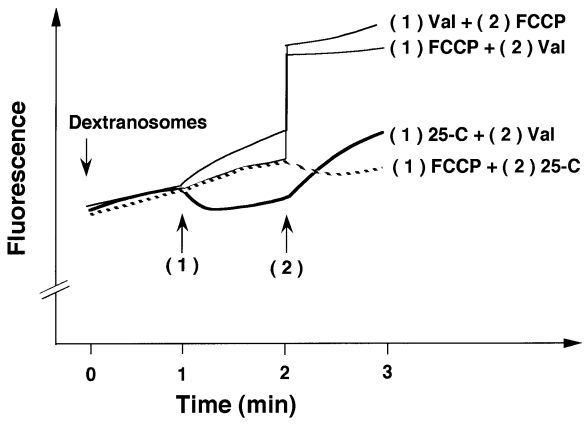

Figure 6 Prodigiosin 25-C does not show ionophoric activity for H^+ or K^+

Lysosomes loaded with FD were added to 2 ml of incubation buffer [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/0.2 M sucrose/0.5 mM $MgCl_2$] and incubated at 37 °C and, at the indicated time points, FCCP (2.5 μM), valinomycin (Val, 2.5 μM) or prodigiosin 25-C (25-C, 250 nM) was added to the incubation mixture. Solvent control (1% ethanol for the ionophores and 1% DMSO for prodigiosin 25-C) did not show any detectable effect on lysosomal pH.

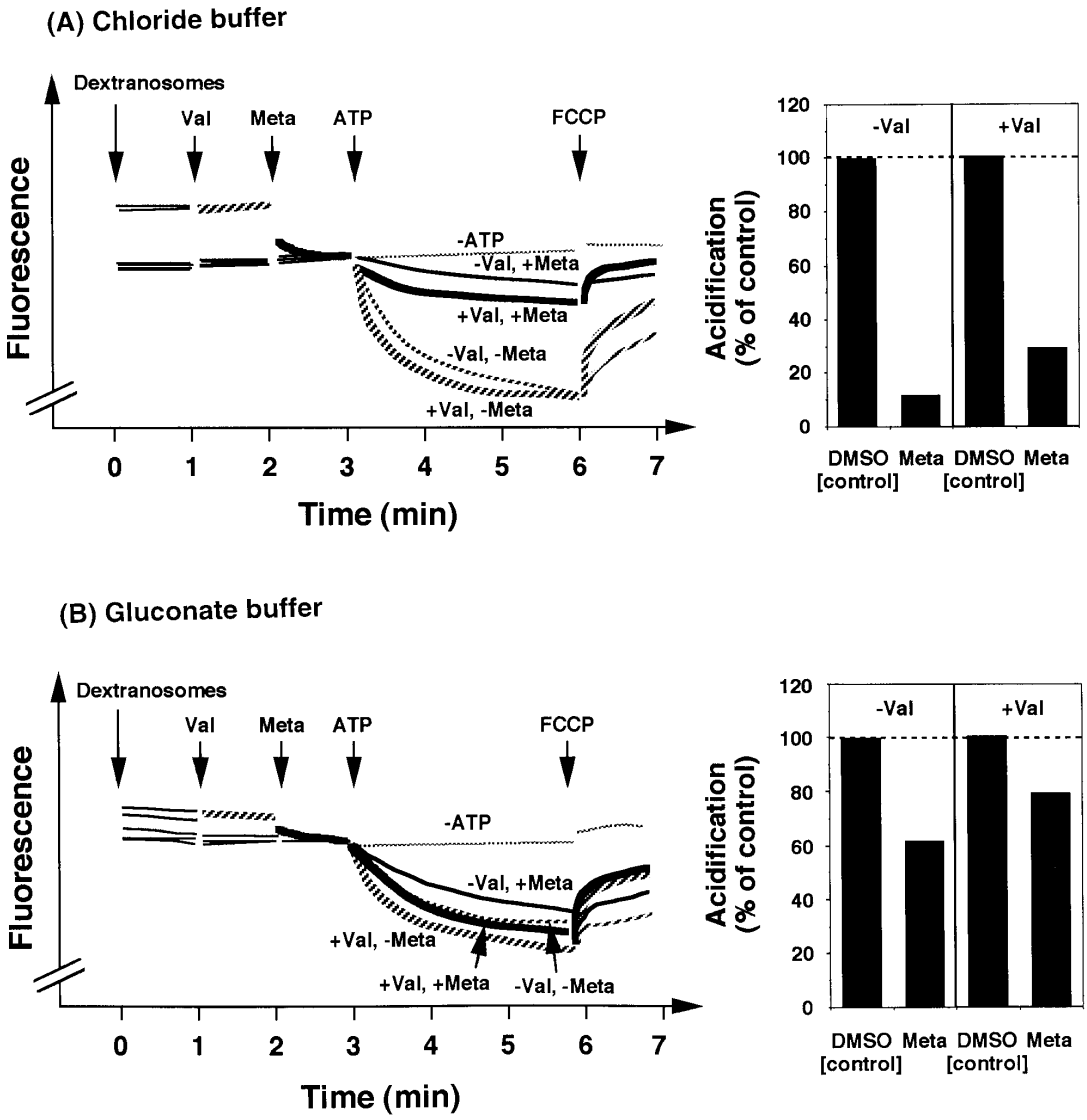

Figure 7 Effects of metacycloprodigiosin and anion on the lysosomal acidification in the presence and absence of valinomycin

Lysosomes loaded with FD were incubated in (**A**) chloride (KCl/NaCl) buffer or (**B**) gluconate (potassium/sodium gluconate) buffer, where the concentration ratio of K^+ and Na^+ was adjusted so that addition of valinomycin did not cause significant change in the rate of internal pH change and the acidification activity was measured as described in Figure 2 in the presence or absence of valinomycin. ATP, 1 mM MgATP; Meta, 1 μM metacycloprodigiosin; Val, 5 μM valinomycin; FCCP, 2.5 μM FCCP. The solvent control (1% DMSO) did not show any detectable effect on lysosomal pH. Compare, between chloride and gluconate buffers, the fluorescence quenching (acidification) just after the addition of metacycloprodigiosin and of ATP.

inhibit transformation of $\Delta\psi$ (driven by V-ATPase) into ΔpH [41], (2) change the ion specificity of the ATPase from H^+ (or OH^-) to other cations [such as Na^+ (V- or F-)ATPase and respiratory Na^+ pump] [42] or (3) change the ion specificity of the ATPase from H^+ (or OH^-) to another anion (like the relationship between halorhodopsin and bacteriorhodopsin [43] or OH^-/Cl^- exchangers [38]).

To test these possibilities, lysosomal acidification was measured, first of all, in buffers containing different cation or anion species, namely in K^+ or Na^+ as cation (KCl or NaCl) and Cl^- or gluconate$^-$ as anion (KCl or potassium gluconate). Their ability to inhibit lysosomal acidification was obtained only with Cl^- as anions (Figure 7), but was expressed irrespective of the cation species [see the dotted lines in Figures 10B(1) and 10B(2)].

Furthermore, prodigiosins quickly reversed the acidification of lysosomal pH driven by V-ATPase (Figure 10): when prodigiosins were added after the completion of intralysosomal acidification, they promptly raised the pH, the rate of reversal being much more rapid than that with bafilomycin A_1 and similar to that with TBT or of NH_4Cl (Figure 10A; for prodigiosin 25-C). The effective concentrations for 50% fluorescence recovery in 10 s were 0.8, 0.8 and 4 nmol/mg of protein for prodigiosin, metacycloprodigiosin and prodigiosin 25-C respectively as compared with 8 nmol/mg of protein for TBT. In Figure 10(B) we examined the ion requirement of the reversal of lysosomal pH: quick reversal was obtained irrespective of the cation species (K^+ or Na^+) [Figures 10B(1) and 10B(2)], but required Cl^- and did not occur in gluconate buffer [Figure 10B(3)]. Furthermore, the prodigiosin-induced reversal of intralysosomal pH was not affected by the H^+-channel blocker, bafilomycin A_1 [44,45] or *N*,*N*′-dicyclohexylcarbodi-imide ('DCCD') (results not shown), suggesting that the H^+ channel of V-ATPase did not participate

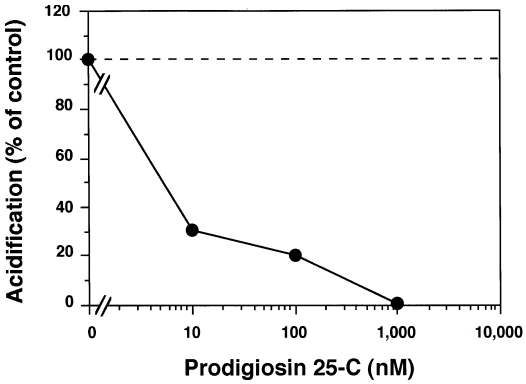

Figure 8 Effect of prodigiosin 25-C on the ATP-dependent acidification of proteoliposomes reconstituted from lysosomal H^+-ATPase

Reconstituted proteoliposomes (see the Experimental section) were incubated at 37 °C in an assay mixture [20 mM Hepes/TMAH (pH 7.5)/0.15 M KCl/0.5 mM $MgCl_2$/1 μM Acridine Orange) containing the indicated concentration of prodigiosin 25-C (1% DMSO as solvent control). ATP-dependent acidification was monitored fluorimetrically as described in the Experimental section.

in this pH reversal [Figure 10B(1)]. These results suggest that the prodigiosins act to promote co-transport of H^+ with Cl^- (or exchange of OH^- with Cl^-).

Prodigiosins, like TBT, acidify lysosomes, requiring Cl^-

In fact, as shown in Figure 11, prodigiosins, like TBT, accelerated lysosomal acidification (H^+ uptake, equivalent to OH^- extrusion) in chloride buffer but not in gluconate buffer, which was further amplified by the addition of membrane-impermeant acidic buffer (Mes/TMAH, pH 6.0). These results suggest that prodigiosins functionally promote the co-transportation of H^+ and Cl^- (or exchange of OH^- with Cl^-).

Prodigiosins, like TBT, induce Cl^--dependent swelling of liposomes and erythrocytes

We tested whether prodigiosins have such ionophoric activities on liposomal and erythrocyte membranes that are devoid of H^+-pump proteins, using a well-established method to detect Cl^--dependent vesicular swelling in iso-osmotic ammonium salt solution. In this method, anion permeability is estimated by vesicular swelling in iso-osmotic ammonium salt of the anions of interest [38]. As shown in Figure 12, metacycloprodigiosin (Figure 12A), like TBT (Figure 12B), reduced the absorbance of liposomes in 0.2 M NH_4Cl (but not in ammonium gluconate). All the prodigiosins showed such an activity (Figure 12C). Figure 12(D) shows the difference in the rate of absorbance decrease between NH_4Cl and ammonium gluconate. The effect of prodigiosin 25-C is relatively weak and that of TBT the strongest. Figure 13 represents the results of erythrocytes swelling, showing essentially the same effects, expressed by the dose-dependent time courses of all the prodigiosins used. The effect of prodigiosin 25-C is stronger than in liposomes, but still slow, and that of TBT is comparable with the effects of prodigiosins.

DISCUSSION

H^+/Cl^- symport (OH^-/Cl^- exchange)-promoting activity itself is not new: organotin compounds (for example, trialkyltins like TBT) are well known OH^-/Cl^- exchangers [46,47] and have been extensively used as such reagents [48]. However, in contrast with prodigiosins, organotin compounds also bind to the proteolipids of F- and V-type H^+-ATPases [49,50] and inhibit their catalytic activity [29,51]. Probably owing to this direct effect on ATPases, TBT, like bafilomycin, gradually increased the lysosomal pH, even after the rapid increase, owing to its ionophoric effect (Figure 10). In this respect prodigiosins may be a group of new H^+/Cl^- symporters (OH^-/Cl^- exchangers) with no inhibitory effect on ATP hydrolysis. Furthermore, prodigiosins may combine with Cl^- electrostatically at, or close to, the protonated nitrogen atom in the pyrrole ring and form lipophilic ion-pairs to

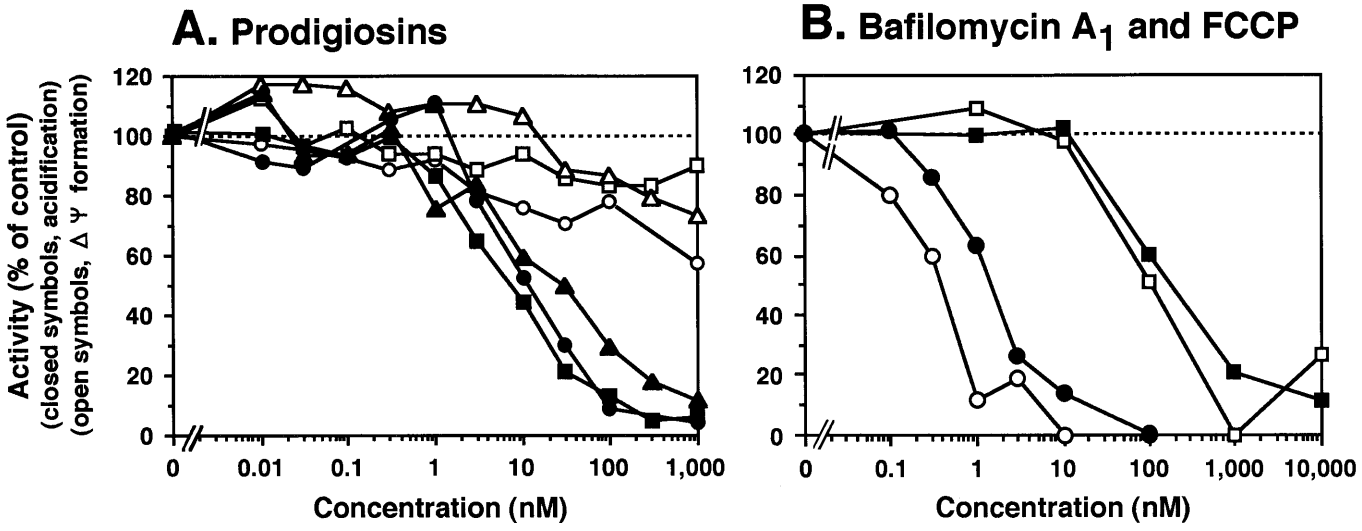

Figure 9 Effect of (A) prodigiosins as compared with (B) bafilomycin A_1 and FCCP on the ATP-dependent formation of membrane potential

Lysosomes loaded with FD were added to 2 ml of the incubation mixture [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/0.2 M sucrose/0.5 mM $MgCl_2$/0.5 μg/ml diS-C_3-(5)]. After addition of the indicated concentration of prodigiosin 25-C, metacycloprodigiosin, prodigiosin, bafilomycin A_1 (1% DMSO as solvent control) or FCCP (1% ethanol as solvent control), Na_2ATP was added at 1 mM and the fluorescence quenching of FD and diS-C_3-(5) was monitored as described in the Experimental section. (**A**) Prodigiosins: □, ■, prodigiosin; ○, ●, metacycloprodigiosin; △, ▲, prodigiosin 25-C. (**B**) Bafilomycin A_1 (○, ●) and FCCP (□, ■). Closed symbols, acidification; open symbols, $\Delta\psi$ formation.

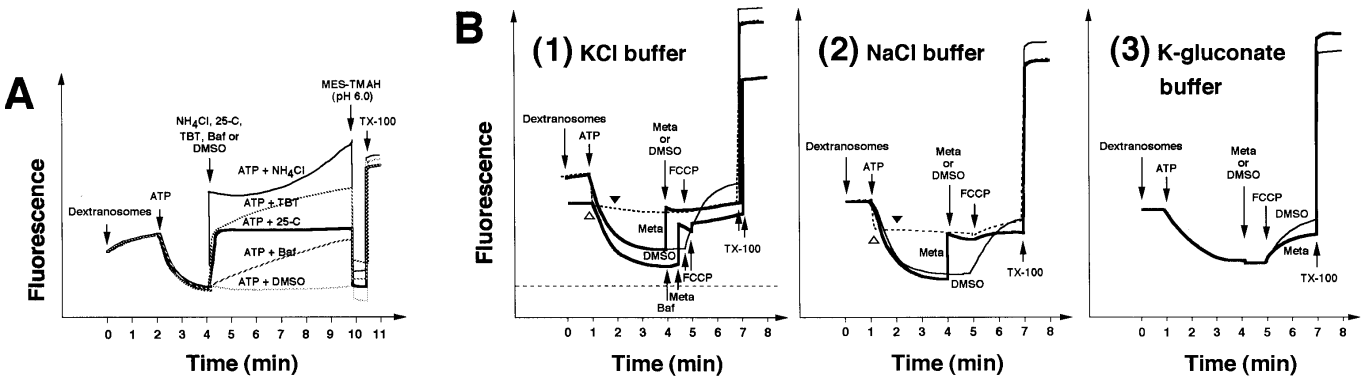

Figure 10 Cl^--dependent quick reversal of acidified intralysosomal pH by metacycloprodigiosin

(**A**) Prodigiosin 25-C rapidly reverses acidification of lysosomal pH. Lysosomes (dextranosomes) containing FD were incubated at 37 °C in an incubation mixture [20 mM Hepes/TMAH (pH 7.5)/0.1 M KCl/ 0.2 M sucrose/0.5 mM $MgCl_2$] and the FD fluorescence was recorded continuously as described in Figure 2. At 2 min after the addition of ATP, prodigiosin 25-C, bafilomycin A_1, DMSO (as solvent control), TBT or NH_4Cl were added. ATP, 1 mM Na_2ATP; Baf, 100 nM bafilomycin A_1; TBT, 1 μM TBT; NH_4Cl, 40 mM NH_4Cl; 25-C, 1 μM prodigiosin 25-C; MES-TMAH, Mes/TMAH, pH 6.0, 50 mM; TX-100, 0.1% TX-100. (**B**) Ion-dependency of the prodigiosin-induced reversal of lysosomal pH: (1) KCl buffer; (2) NaCl buffer and (3) potassium gluconate buffer. Lysosomes (dextranosomes) containing FD were incubated at 37 °C in an incubation mixture [20 mM Hepes/TMAH (pH 7.5)/0.2 M sucrose/0.5 mM Mg^{2+} (as $MgCl_2$ for Cl-buffers or $MgSO_4$ for gluconate buffer)] containing 0.1 M KCl, NaCl or potassium gluconate and the FD fluorescence was recorded continuously as described in Figure 2. At the indicated time points, ATP (1 min), metacycloprodigiosin/DMSO (as solvent control) (at 4 min), bafilomycin A_1 (at 4 min), followed by metacycloprodigiosin (at 4.5 min), FCCP (at 5.0 min) and TX-100 (at 7 min) were added, except for the broken lines, where lysosomes were added with metacycloprodigiosin (△) before the addition of ATP (▼) to show cation-independency of the acidification inhibitory effect of metacycloprodigiosin. ATP, 1 mM MgATP; Meta, 1 μM metacycloprodigiosin; FCCP, 2.5 μM FCCP; Baf, 100 nM bafilomycin A_1; TX-100, 0.1% TX-100.

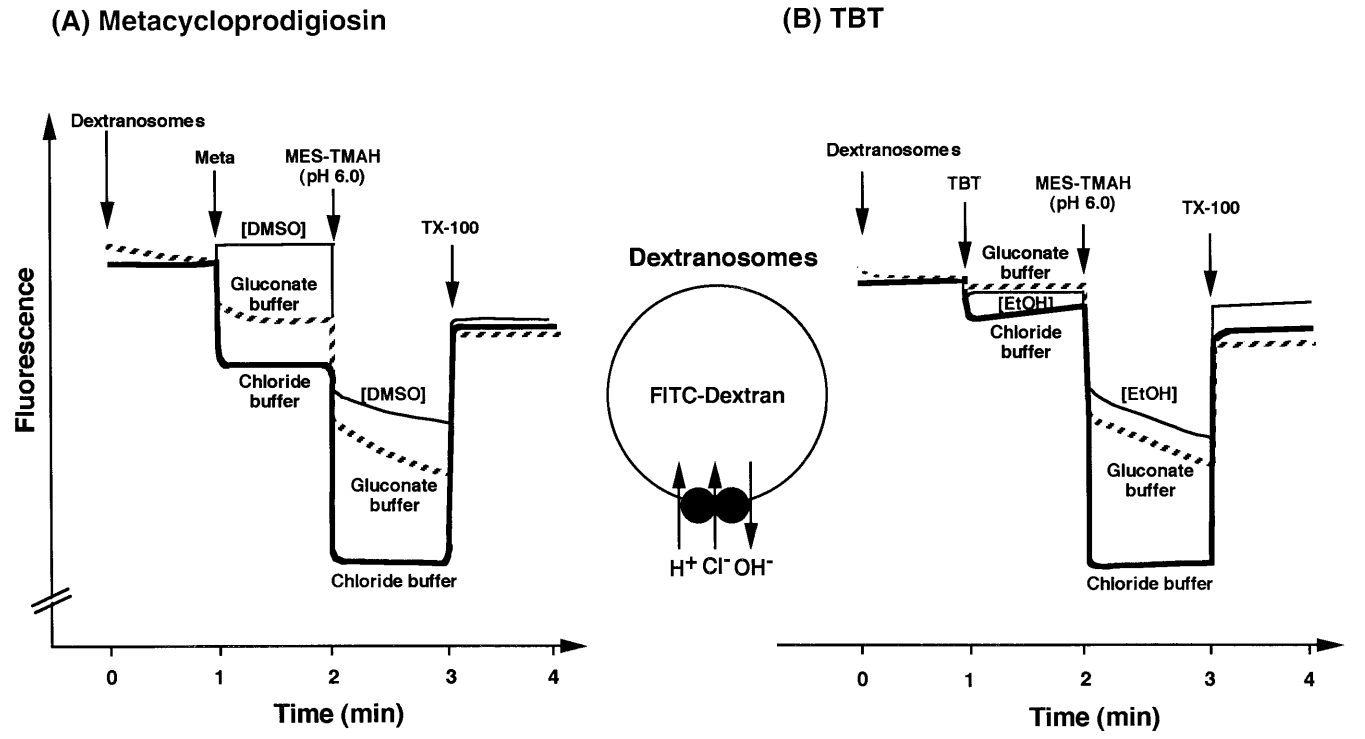

Figure 11 (A) Metacycloprodigiosin, like (B) TBT, promotes Cl^--dependent lysosomal acidification driven by acidic external pH

Lysosomes (dextranosomes) containing FD were incubated at 37 °C in an incubation mixture [20 mM Hepes/TMAH (pH 7.5)/0.2 M sucrose] containing 0.1 M KCl or potassium gluconate, and the FD fluorescence was continuously recorded as described in Figure 2. Meta, 1 μM metacycloprodigiosin; TBT, 1 μM TBT; MES-TMAH, Mes/TMAH, pH 6.0, 50 mM; TX-100, 0.1% TX-100; [DMSO] and [EtoH], traces of 1% DMSO (solvent control for metacycloprodigiosin) and 1% ethanol (solvent control for TBT) in KCl buffer respectively; continuous lines, KCl buffer; broken lines, potassium gluconate buffer.

facilitate H^+-coupled transmembrane transport of Cl^-. In this sense prodigiosins are H^+/Cl^- symporters rather than OH^-/Cl^- exchangers. The much higher effect of TBT on liposomes (Figures 10 and 11) might also be related to the differences between TBT and prodigiosins in their mode of action and/or in the permeability properties (in respect of liposomes and organellar

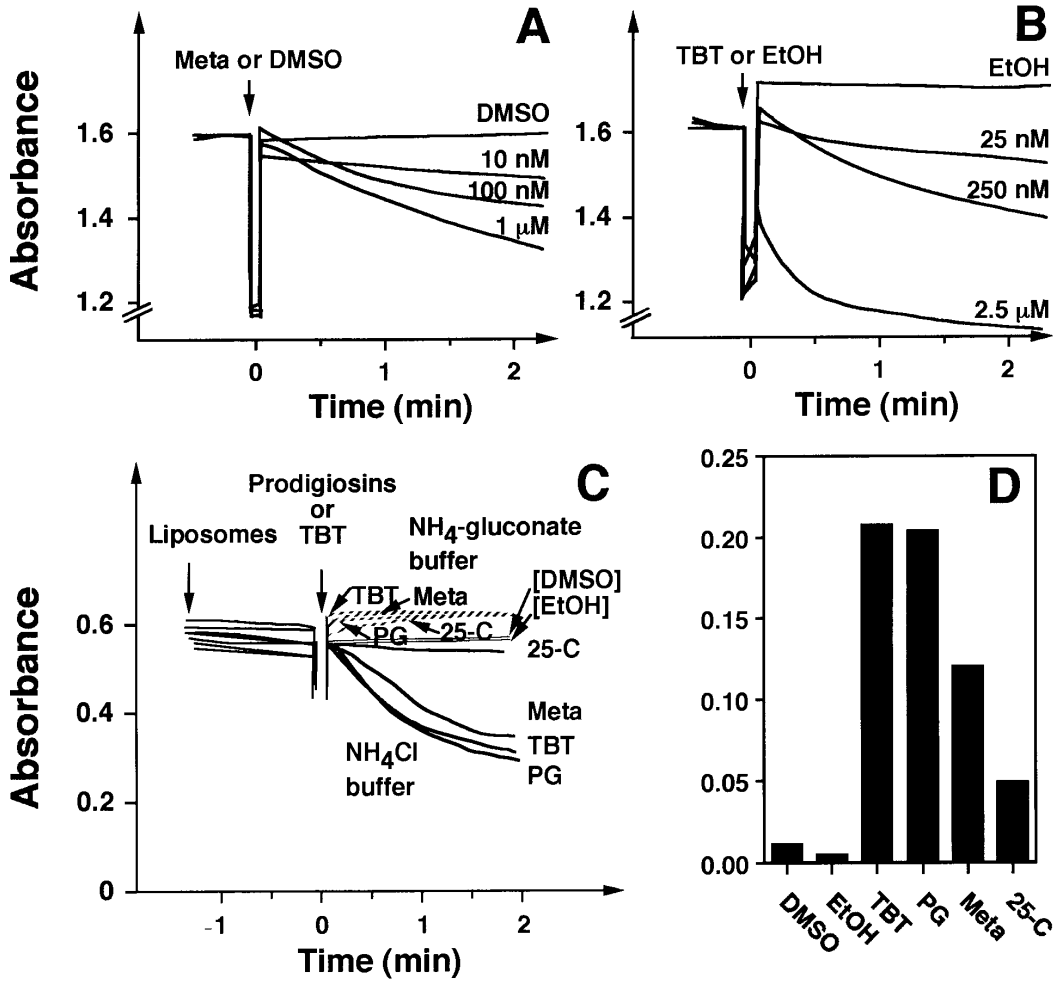

Figure 12 Cl^--dependent swelling of liposomes by prodigiosins

Asolectin liposomes prepared in 2 mM Hepes/TMAH (pH 7.5)/0.25 M sucrose were added at 1 mg/ml to 0.2 M ammonium salt (pH 7.0) of either chloride (NH_4Cl) or gluconate (ammonium gluconate) and the A_{550} was monitored. (**A**) Dose–response of metacycloprodigiosin in NH_4Cl buffer. (**B**) Dose–response of TBT in NH_4Cl buffer. (**C**) Cl^--dependent swelling of liposomes by various prodigiosins: 25-C, 1 μM prodigiosin 25-C; Meta, 1 μM metacycloprodigiosin; PG, 1 μM prodigiosin; TBT, 100 nM TBT; [DMSO], 1% DMSO; [EtOH], 1% ethanol. Continuous lines, NH_4Cl buffer; broken lines, ammonium gluconate buffer. (**D**) Cl^--dependent swelling of liposomes by prodigiosins and TBT. The differences in absorbance change (in 1 min) between NH_4Cl and ammonium gluconate buffers (Figure 12C) are shown for each treatment. DMSO and ethanol were taken as solvent controls for prodigiosins and TBT respectively. 25-C, 1 μM prodigiosin 25-C; Meta, 1 μM metacycloprodigiosin; PG, 1 μM prodigiosin; TBT, 100 nM TBT; DMSO, 1% DMSO; EtOH, 1% ethanol.

membranes). Recently, several other types of H^+/Cl^- symporters (OH^-/Cl^- exchangers) have been reported: thallium [52], bepridil [53], Hg^{2+} and Cu^+ [54], cryptate [55] and sapphyrin [56]. However, their effects on ATPase activities are not known.

Our findings may also provide insights into the mechanism of action of prodigiosins on cellular functions [14]: why do prodigiosins cause mitochondrial swelling? Why do they not affect the cellular ATP content in spite of their inhibitory effect on mitochondrial F-ATPase [20]? Actually, these phenomena themselves constitute evidence for the H^+/Cl^- symport activity of prodigiosins. If they actually act as H^+/Cl^- symporters, they should not affect the formation of $\Delta\psi$ (the main form of protonmotive force in mitochondria) or the ATP synthesis in mitochondria. Indeed, OH^-/Cl^- exchangers like organotins (for example, TBT) do not affect the $\Delta\psi$ formation of cytochrome *c* oxidase [48] or the formation of the proton-motive force (nor, probably, ATP formation) driven by respiration, and have been used as such a reagent [57]. On the other hand, the mechanism of swelling of Golgi apparatus by prodigiosins may be related to their general weak basic nature, because weak bases, including chloroquine, first accumulate within, and cause swelling of, the Golgi apparatus [58]. The Golgi apparatus, rather than lysosomes, swell possibly because Golgi membranes are less permeable to K^+ [59], resulting in more Cl^- uptake than K^+ extrusion. However, it might also be related to their ionophoric activity, as acidic ionophores (nigericin and X-537A) also induce swelling of Golgi apparatus but not of lysosomes [60], although acidic ionophores induce osmotic lysis of lysosomes *in vitro* [33].

As prodigiosins inhibit the vacuolar acidification *in vivo* driven by V-ATPase(s) without markedly affecting the cellular ATP level [14], they should also act *in vivo* as selective inhibitors of V-ATPase(s). Therefore they may affect every aspect of cellular functions involving V-ATPase(s) [2,5–8]. Indeed, we have already reported the immunosuppressive activity of prodigiosin 25-C [15–19,61]. We also found these antibiotics inhibit bone resorption [20], secretion, cell differentiation and apoptosis (results not shown). Furthermore, we can expect prodigiosin-specific reactions, because the drugs do not inhibit $\Delta\psi$ formation: for

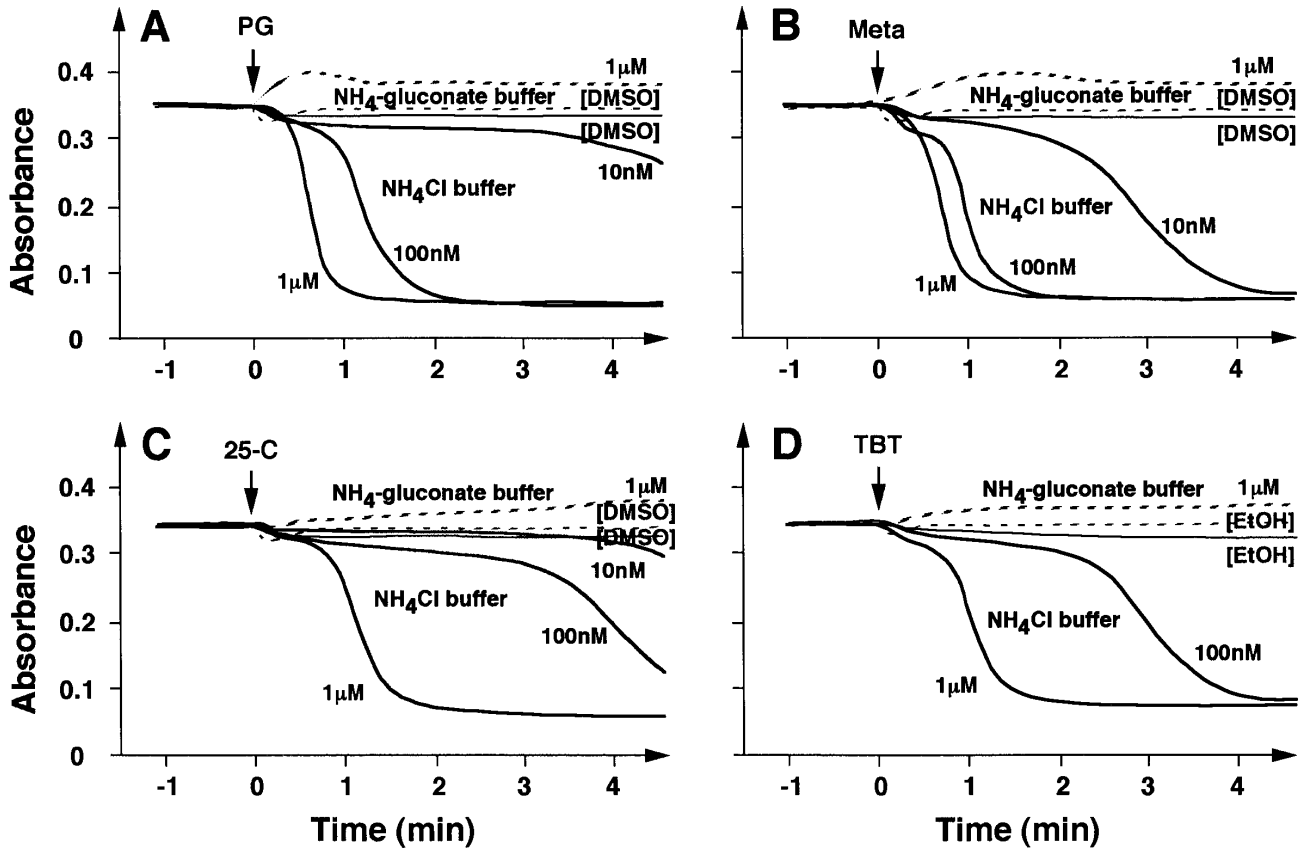

Figure 13 Cl^--dependent swelling of erythrocytes by prodigiosins

Human erythrocytes prepared in 0.15 M NaCl (or sodium gluconate) containing 1 mM acetazolamide were loaded at 0.033% (v/v) in 0.2 M ammonium salt (pH 7.0) containing 1 mM acetazolamide of either chloride (NH_4Cl) or gluconate (ammonium gluconate) and the A_{550} was monitored. (**A**) Prodigiosin; (**B**) metacycloprodigiosin; (**C**) Prodigiosin 25-C; (**D**) TBT; PG, prodigiosin; Meta, metacycloprodigiosin; 25-C, prodigiosin 25-C; [DMSO], 1% DMSO (solvent control for prodigiosins); [EtOH], 1% ethanol (solvent control for TBT). Continuous lines, NH_4Cl buffer; broken lines, ammonium gluconate buffer.

example, inhibition of ΔpH-dependent dopamine uptake, but not $\Delta\psi$-dependent glutamate uptake, into synaptic vesicles [62]. Prodigiosins are also promising drugs for chemotherapy of malarial and tubercular infections [63]. Like bafilomycin A_1, a potent selective inhibitor of V-ATPase that suppresses the growth of pancreatic tumours transplanted into nude mice causing apoptosis [64], prodigiosins are also promising anticancer agents and have been reported to suppress the growth of neoplastic Chinese-hamster ovary cells [65].

In conclusion, we found that prodigiosins are the fourth vacuolar pH perturbators after (1) weak bases, (2) acidic ionophores and (3) a group of potent selective inhibitors of V-ATPase (antibiotic bafilomycins, concanamycins and destruxins). Among these perturbators, both weak bases and acidic ionophores increase vacuolar pH, converting ΔpH into $\Delta\psi$ and induce vacuolar swelling [33], while prodigiosin-group antibiotics affect ΔpH but not $\Delta\psi$, and the potent selective inhibitors of V-ATPase affect both the ΔpH and $\Delta\psi$ created by V-ATPase. Proper application of these probes to various aspects of cellular functions will accelerate the clarification of vacuolar functions as well as ΔpH-dependent reactions within cells.

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