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Physiological and morphological effects of alendronate on rabbit esophageal epithelium

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Dobrucali, A., N. A. Tobey, M. S. Awayda, C. Argote, S. Abdulnour-Nakhoul, W. Shao, and R. C. Orlando. Physiological and morphological effects of alendronate on rabbit esophageal epithelium. *Am J Physiol Gastrointest Liver Physiol* 283: G576–G586, 2002. First published April 24, 2002; 10.1152/ajpgi.00014.2002.—Alendronate, an aminobisphosphonate, produces as a side effect a topical (pill induced) esophagitis. To gain insight into this phenomenon, we assessed the effects of luminal alendronate on both esophageal epithelial structure and function. Sections of rabbit esophageal epithelium were exposed to luminal alendronate at neutral or acidic pH while mounted in Ussing chambers to monitor transmural electrical potential difference (PD), short-circuit current (I_{sc}), and resistance (R). Morphological changes were sought by light microscopy in hematoxylin and eosin-stained sections. Impedance analysis was used for localization of alendronate-induced effects on ion transport. Luminal, but not serosal, alendronate (pH 6.9–7.2), increased PD and I_{sc} in a dose- and time-dependent manner, with little change in R and mild edema of surface cell layers. The changes in I_{sc} (and PD) were reversible with drug washout and could be prevented either by inhibition of Na,K-ATPase activity with serosal ouabain or by inhibition of apical Na channels with luminal acidification to pH 2.0 with HCl. An effect on apical Na channel activity was also supported by impedance analysis. Luminal alendronate at acidic pH was more damaging than either alendronate at neutral pH or acidic pH alone. These data suggest that alendronate stimulates net ion (Na) transport in esophageal epithelium by increasing apical membrane sodium channel activity and that this occurs with limited morphological change and no alteration in barrier function. Also alendronate is far more damaging at acidic than at neutral pH, suggesting its association with esophagitis requires gastric acid for expression. This expression may occur either by potentiation between the damaging effects of (refluxed) gastric acid and drug or by acid-induced conversion of the drug to a more toxic form.

biphosphonates; esophagitis; transepithelial ion transport; Ussing chamber; sodium channels

ALENDRONATE, AN AMINOBISPHOSPHONATE, is a potent inhibitor of osteoclast-mediated bone resorption, which makes this an effective agent for the treatment of osteoporosis (3, 7, 10, 13). Although generally well tolerated, an early important side effect of taking alendronate 10 mg once a day (alendronate is now also available in 70-mg tablets for treatment once weekly) was the development of erosive esophagitis (1, 2, 9, 12, 14, 19). Indeed, in some cases, the esophagitis was severe enough to require hospitalization due to epithelial ulceration and healing with stricture formation (5, 9, 11, 12). On the basis of clinical history (patients ingesting medication with little liquid or reclining shortly after taking the medication) and endoscopic observations of the pattern of damage, initial reports concluded that alendronate damaged the esophagus by direct (luminal) contact with the esophageal epithelium, resulting in a form of "pill-induced" esophagitis. To gain insight into this phenomenon, therefore, we assessed the effects of luminal alendronate on both esophageal epithelial structure and function and did this at both neutral and acidic pH, the latter to take into account the potential in humans for refluxed gastric acid to alter the potential for alendronate toxicity. The stratified squamous epithelium from the rabbit esophagus was chosen for these studies because it is *1*) structurally and functionally similar to that of the human esophageal epithelium, *2*) well characterized in terms of its transport and barrier physiology, and *3*) devoid of submucosal glands and is ideally suited for investigation of squamous epithelial structure/function alone when mounted in Ussing chambers (18, 20, 22). Also noteworthy is that the concentration range of alendronate selected for study, 1–10 mg/ml, was by necessity empiric because pill-induced esophagitis principally occurs because of adherence of ingested tablets to the epithelial surface, an occurrence that would create, as the tablets dissolve, high concentrations of chemical in a localized area of epithelium.

METHODS

Male New Zealand White rabbits weighing 8–9 lb were killed by administration of an intravenous overdose of pentobarbital sodium (60 mg/ml). The esophagus was dissected

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free, opened lengthwise, and pinned mucosal surface down in a paraffin tray containing ice-cold oxygenated normal Ringer solution. The submucosa and muscularis propria were grasped with hemostats, lifted up, and dissected free of the underlying mucosa with a scalpel. This process left attached to the paraffin tray a sheet of tissue consisting of stratified squamous epithelium and a small amount of underlying connective tissue. From this tissue, four sections were cut and mounted as flat sheets between Lucite half-chambers with an aperture of 1.13 cm^2, which permitted contact with separate bathing solutions for the luminal and serosal sides of the tissue.

Tissues mounted in chambers were bathed initially with normal Ringer solution composed of the following (in mM): 140 Na^+, 119.8 Cl^-, 5.2 K^+, 25 HCO_3^-, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 2.4 HPO_4^{-2}, 0.4 $H_2PO_4^{-2}$, with osmolality 268 mosmol/kgH_2O and pH 7.4 when gassed with 95% O2–5% CO_2 at 37°C. Luminal and serosal solutions were connected to calomel and Ag-AgCl electrodes by Ringer agar bridges for measurements of potential difference (PD) and short-circuit current (I_{sc}) with a voltage clamp (World Precision Instruments, Sarasota, FL). Tissues were continuously short circuited, except for 5–10 s when the open circuit PD was read. Tissue electrical resistance (R), a marker of epithelial permeability, was calculated from the PD and I_{sc} using Ohm's law (R = PD/I_{sc}). Tissues were equilibrated in Ringer for 45 min before examination to allow stabilization of electrical parameters. After equilibration, tissues having $R > 1{,}000$ Ω/cm^2 were paired by R (R within 25%) for comparative studies between those treated with alendronate and untreated controls.

Alendronate solutions (1–10 mg/ml) were prepared by pulverizing to a powder with mortar and pestle commercially available 10-mg tablets of alendronate (Fosamax; Merck, West Point, PA). The powder was dissolved in 10 ml of normal Ringer solution, yielding solutions whose pH ranged from pH 6.9 to 7.2. For alendronate exposure, after equilibration in 10 ml normal Ringer solution, the luminal bathing solution was drained and substituted for 10 ml normal Ringer containing the desired concentration of alendronate. The control was handled in a similar manner, except that the luminal solution was replaced with normal Ringer solution, which was titrated with 0.1 N HCl, when necessary, to match the pH of the alendronate solution. After initial titration with HCl, pH of all solutions remained constant. In some experiments, the effect of pretreatment with alendronate on the ability of the esophageal epithelium to resist injury by acid was tested by acidification of the luminal bath to pH 2.0 with 3 N HCl. Injury was assessed by monitoring R and by fixing tissues with 10% formaldehyde for histology. Tissue sections were cut and subsequently stained with hematoxylin and eosin for evaluation by light microscopy. Morphological injury, which was evaluated by an observer with no knowledge of treatment groups was scored as follows: 0, normal; 1, inter/intracellular edema; 2, patchy necrosis; 3, diffuse necrosis; 4, transmural necrosis (ulceration).

Impedance analysis. This was carried out as previously described by Van Driessche et al. (23). Briefly, tissues were mounted in modified Ussing chambers and clamped to 0 mV using a low noise four-electrode clamp with agar bridges connected by low-resistance Ag/AgCl electrodes. Impedance was continuously measured at five frequencies: 128 Hz, 343 Hz, 1.0 kHz, 4.1 kHz, and 16.4 kHz. The direct current (DC) conductance (g_m) was measured using a low-frequency (0.25 Hz) signal, and this value was used to calculate the capacitance (C_m) at each of the individual frequencies. Every 10 min an impedance spectrum was measured at 99 frequencies ranging from 1 Hz to 16 kHz.

Junction potentials were measured for all solutions according to a method modified following that of Read and Fordtran (15). Inasmuch as these values were found to be equal or less than +1.1 mV, no correction was made for them in the presentation of results.

All chemicals were obtained from Sigma (St. Louis, MO), except where otherwise indicated. Ouabain, 10^{-4} M, was prepared by dissolving in a small volume of DMSO.

Statistics. Statistical significance was determined using Student's t-test for parametric data (electrical parameters),

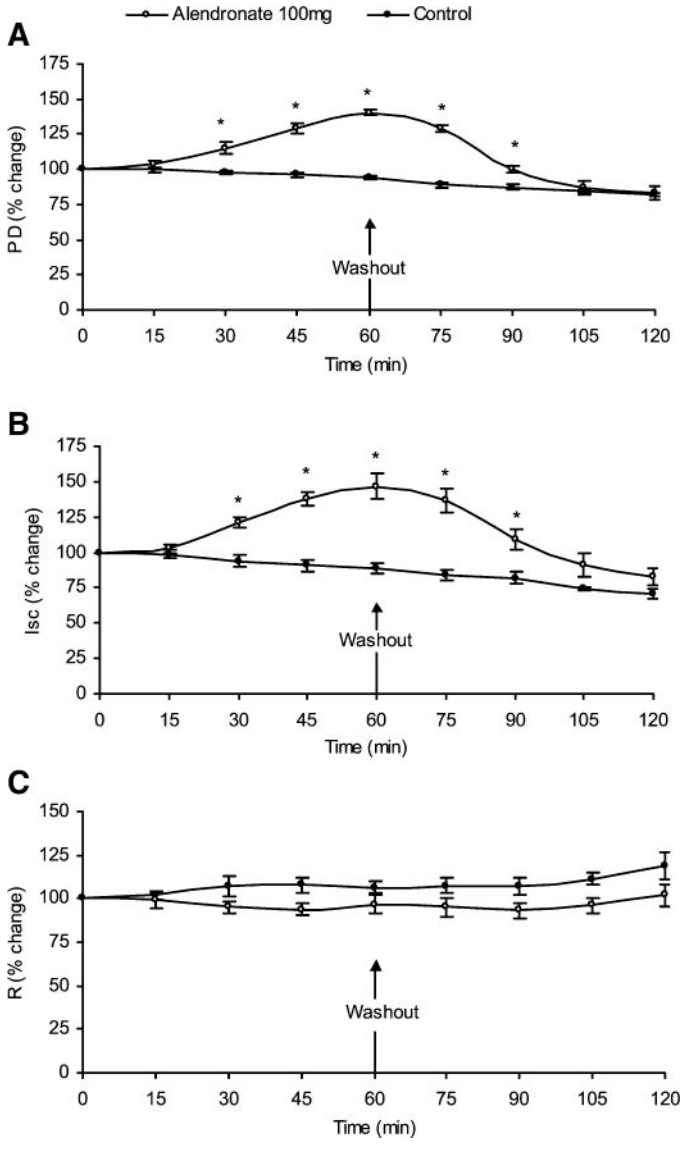


Fig. 1. Effect of luminal alendronate, 10 mg/ml, on the potential difference (PD; A), short-circuit current (I_{sc}; B), and electrical resistance (R; C) of rabbit esophageal epithelium mounted in Ussing chamber in normal Ringer solution, pH 6.9, for 1 h. Control tissues are exposed to normal Ringer at pH 6.9. At 60 min after exposure to alendronate, the luminal bathing solutions were removed for both alendronate-treated and control tissues and replaced with normal Ringer solution (washout). Data are expressed as percentage of initial values before alendronate exposure. Values are means ± SE; $n = 4$ for each group; $^*P < 0.05$ compared with controls. Note: absolute values for PD, I_{sc}, and R for each group were similar before treatment and were −12.8 ± 1.4 mV and −17.4 ± 1.4 mV, 10.5 ± 2.6 μAmp/cm^2 and 11 ± 1.2 μAmp/cm^2, and 1,649 ± 227 Ω/cm^2 and 1,869 ± 234 Ω/cm^2 for alendronate and controls, respectively.

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the Mann-Whitney *U* test for nonparametric data (morphological injury scores), and the regression coefficient analysis for dose-response studies. All data were reported as the means ± SE, and $P < 0.05$ was accepted as indicating a statistically significant difference among groups.

All studies were approved by the Animal Welfare Committee.

RESULTS

To determine if alendronate had any effect on the barrier or ion transport properties of esophageal epithelium, rabbit esophageal epithelium was initially exposed to a high concentration (10 mg/ml) of alendronate in normal Ringer solution while mounted in Ussing chambers for 1 h. The results, as shown in Fig. 1, illustrate that acute exposure to alendronate increases PD and I_{sc} with time while having little effect on R. The PD increased by 54 ± 6% and I_{sc} increased by 64 ± 7% at 1 h vs. the control, which PD decreased by 10 ± 3% and I_{sc} decreased by 15 ± 2% at 1 h (n = 4/group, $P < 0.01$). This increase translates into an increase of 5 ± 0.2 mV and 5.5 ± 2 μAmps/cm^2 in alendronate-treated tissues. Moreover, this effect was reversible because removal of alendronate by replacement of the luminal bath with normal Ringer solution results in complete reversibility by 45 min. At the end of the washout period (1 h post-alendronate removal), tissues were fixed and examined morphologically. The histology of alendronate-exposed tissues showed minimal changes with some membrane "wrinkling" and mild edema of the surface cell layers when compared with normal Ringer-exposed controls (Fig. 2, *A* and *B*), and this was reflected in scores for alendronate-treated tissues of 1.2 ± 0.5 vs. Ringer-exposed controls of 0.3 ± 0.2 (n = 4/group, $P < 0.01$).

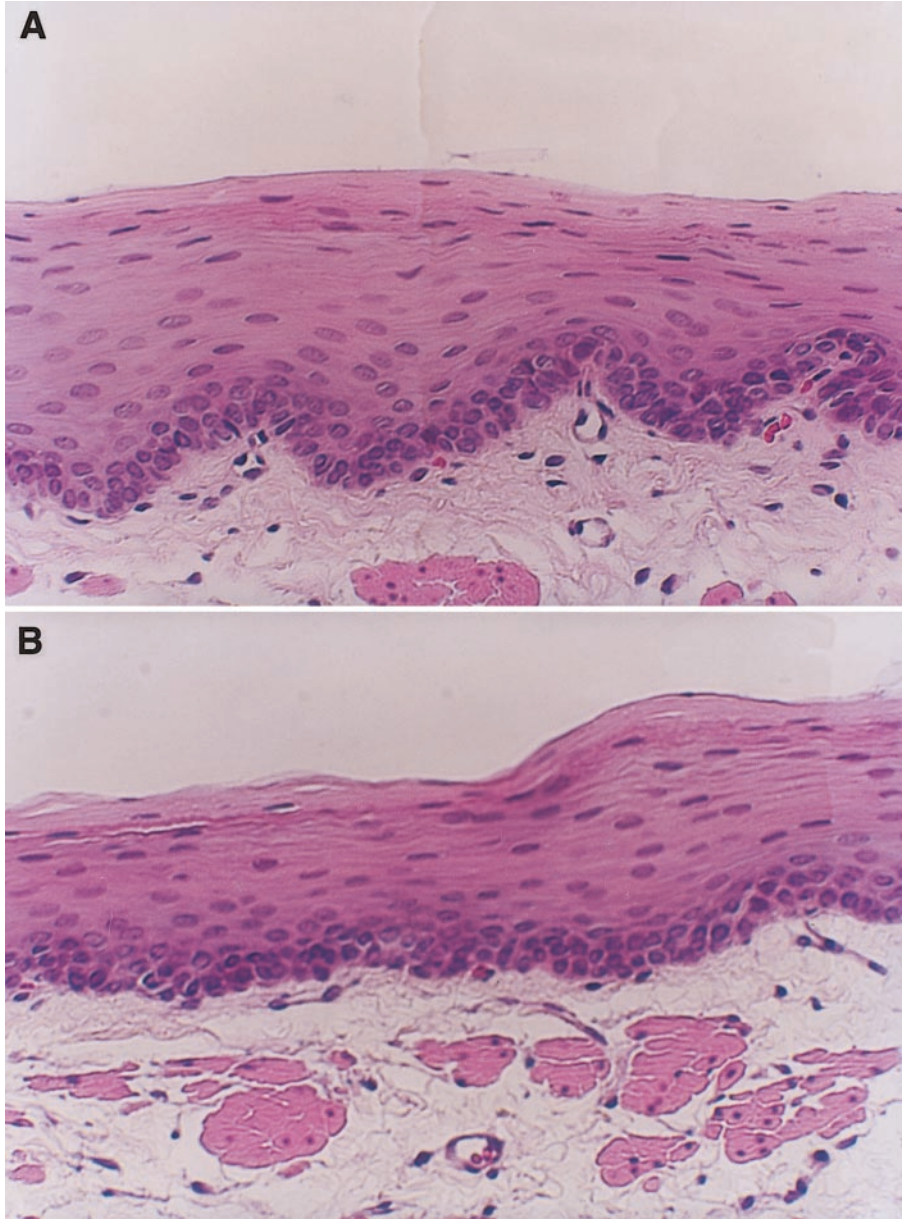


Fig. 2. Representative light micrographs of rabbit esophageal epithelium exposed to normal Ringer solution, pH 6.9 (*A*), or alendronate, 10 mg/ml, in normal Ringer solution, pH 6.9 (*B*), for 1 h. Note that the alendronate-exposed tissues exhibit minor changes in the surface layers of epithelium with some "wrinkling" of the membranes and mild edema. Hematoxylin and eosin, ×150.

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Notably, alendronate exists commercially as a sodium salt, and when dissolved in normal Ringer solution, this salt liberates Na ions such that it increased the luminal Na concentration by 20 meq/l. To determine whether the effect of alendronate observed above was the result of the higher Na concentration, tissues were exposed to normal Ringer solution or to normal Ringer solution to which 20 meq/l of Na was added in the form of Na isothionate. The results of this experiment establish that the additional Na in the luminal bath has no effect on the electrical parameters (PD, I_{sc}, R) of the tissue as illustrated for the I_{sc} in Fig. 3. This suggests that the stimulation of ion transport in alendronate-exposed tissues was a direct effect of the agent on the epithelium. Moreover, and suggesting that the effect of alendronate is a drug class effect, luminal exposure to risedronate, 10 mg/ml, also resulted in stimulation of the I_{sc}; however, the magnitude of the current rise, 17 ± 4.6% (n = 4), above basal levels was significantly lower than that for alendronate, 64 ± 7% (n = 4, $P < 0.05$).

The dose at which alendronate exerted its effects on PD and I_{sc} was subsequently evaluated by exposing rabbit esophageal epithelium in Ussing chambers to varying concentrations of alendronate for 1 h. In Fig. 4, the results of these experiments are depicted as a plot of the concentration of alendronate vs. percent change in PD, I_{sc}, and R at 1 h. Note the strong linear relationship for the increase in PD and I_{sc} over the entire dose range up to 10 mg/ml with $R^2 > 0.93$ for each parameter.

To address the possible mechanism by which alendronate stimulates I_{sc} (and so PD) across the esophageal epithelium, tissues were pretreated with serosal ouabain, 10^{-4} M, for 30 min to block active transport before exposure to luminal alendronate, 10 mg/ml. As shown in Fig. 5, ouabain progressively reduced PD and I_{sc} with time such that by 30 min both parameters were significantly lower than untreated normal Ringer-exposed controls. When alendronate was then added to the luminal bath, PD and I_{sc} increased in the untreated tissues, whereas treatment of the tissues with ouabain prevented the increase in both I_{sc} and PD. This suggests that alendronate's ability to increase PD and I_{sc} was a direct effect on net (transcellular) ion transport rather than an effect on the paracellular pathway.

To better understand the site of action of alendronate, tissues were also exposed to alendronate serosally in doses of 10, 5, and 3 mg/ml. Unlike luminal alendronate, however, the results revealed a prompt dose-dependent decline in I_{sc} (and PD). For 10 mg/ml, I_{sc} declined by 38%; for 5 mg/ml, by 11%; and for 3 mg/ml, by 13% within 15 min, and these reductions were completely reversible inasmuch as replacement of the alendronate solution with normal Ringer resulted

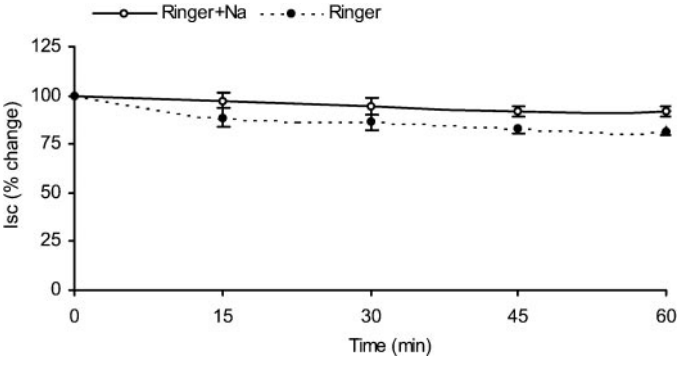


Fig. 3. Effect on the I_{sc} of rabbit esophageal epithelium mounted in Ussing chambers of luminal addition of 20 meq/l of Na in the form of Na isothionate to normal Ringer solution, pH 6.9. Controls contain normal Ringer solution, pH 6.9, alone. Values are means ± SE; n = 4 for each group. Note that the I_{sc} of esophageal epithelium was unaffected by the additional Na in the luminal bath. (Note: absolute values for I_{sc} before treatment were 12.6 ± 0.8 and 12 ± 1.5 μAmp/cm^2 for Na isothionate-added Ringer and regular Ringer, respectively.)

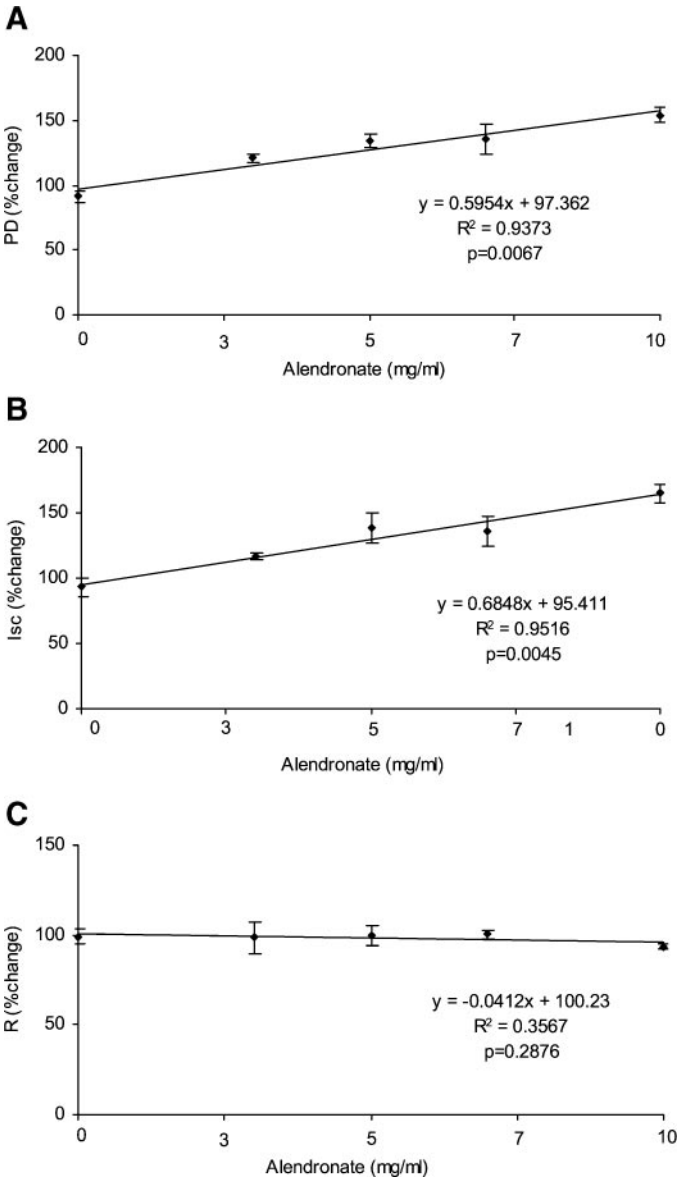


Fig. 4. A strong linear relationship is shown between the dose of luminal alendronate and its effects on the PD (*A*) and I_{sc} (*B*) of rabbit esophageal epithelium mounted in Ussing chambers. There is no effect of varying dose on electrical R (*C*). The values plotted represent those taken after 1 h of exposure to the agent.

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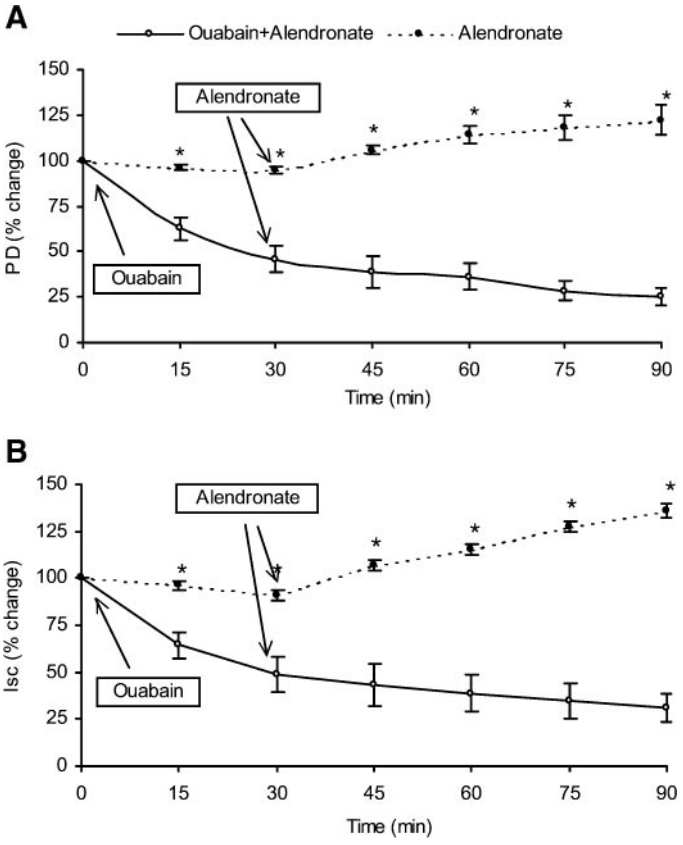


Fig. 5. Effect of pretreatment with serosal ouabain, 10^{-4} M, on the ability of luminal alendronate, 10 mg/ml, to increase the PD (*A*) and I_{sc} (*B*) of rabbit esophageal epithelium mounted in Ussing chambers in normal Ringer solution, pH 6.9, for 1 h. Alendronate treatment was initiated at 30 min after ouabain treatment. Control tissues were exposed to luminal alendronate only at 30 min. Note that pretreatment with ouabain abolished the increase in PD and I_{sc} associated with the addition of alendronate to the luminal bath. Data are expressed as percentage of initial values. Values are means ± SE; $n = 4$ for each group; $^*P < 0.05$ compared with controls. (Note: absolute PD and I_{sc} values before treatment were −15.9 ± 4.4 and −19.3 ± 4.7 mV and 13.6 ± 2.8 and 17.6 ± 4.3 μAmp/cm² for tissue pretreated with ouabain and control, respectively.)

in return of the I_{sc} (and PD) to baseline values within 15 min (data not shown). (Note: serosal pH 6.9 in the absence of alendronate has no effect on current.) Because these results suggest that alendronate's luminal action was not related to absorption and action on the basolateral membrane, attention was focused on alendronate's effect on apical membrane sodium channels in this actively sodium-transporting tissue. This was done by first exposing the esophageal epithelium to alendronate, 10 mg/ml, to produce an increase in I_{sc} and PD, and then titrating the luminal bath with 3 N HCl to reach a pH of 2.0. The luminal bath was acidified to pH 2.0 as a means of inhibiting Na transport through apical membrane Na channels (see DISCUSSION and Refs. 18 and 21). As illustrated in Fig. 6, luminal acidification almost completely abolished the alendronate-induced increase in PD and I_{sc}. [Note: in the absence of acidification, alendronate-treated tissues exhibit continued elevations in I_{sc} and PD over baseline and these elevations continue to slowly increase over the hour of these experiments. Also luminal acidification to pH 2.0 alone has no significant effect on PD, I_{sc}, or R or on tissue histology in esophageal epithelium. This is evident by comparison of luminal acidification to pH 2.0 for 1 h (60- to 120-min time period in Ussing chamber) in Fig. 6 with that of nonacidified controls exposed to normal Ringer solution for 1 h (over the same 60- to 120-min time period) in Fig. 1]. Further luminal acidification to pH 2.0 produced a similar inhibitory effect on I_{sc} (and PD; data not shown) over the entire dose range up to 10 mg/ml (Fig. 7). Additional evidence to support the increase in PD and I_{sc} by alendronate was due to stimulation of active Na transport; tissues were exposed to alendronate, 10 mg/ml, for 1 h in Cl-free bathing solution (both luminal and serosal). (Note: Na-free experiments cannot be done technically because alendronate releases Na from its salt.) Similar to alendronate in Cl-containing Ringer solution, alendronate in Cl-free solution produced sim-

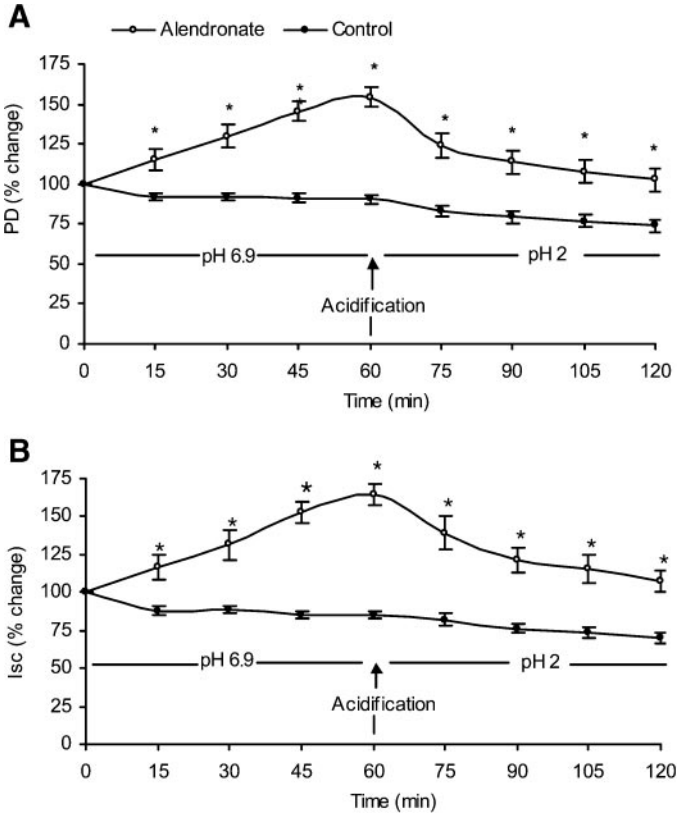


Fig. 6. Effect of luminal acidification with HCl, pH 2.0, on the increase in PD (*A*) and I_{sc} (*B*) produced by luminal alendronate, 10 mg/ml, in rabbit esophageal epithelium mounted in Ussing chambers in normal Ringer solution, pH 6.9, for 1 h. Luminal acidification was initiated at 60 min after alendronate treatment. Control tissues were only exposed to normal Ringer solution before luminal acidification at 60 min. Note that luminal acidification at pH 2.0 has little if any effect on PD or I_{sc} in controls (see Fig. 1 for comparison to normal Ringer alone from 60 to 120 min) but substantially reduces the increase in PD and I_{sc} produced by exposure to alendronate. Data are expressed as percentage of initial values. Values are means ± SE; $n = 7$ for each group; $^*P < 0.05$ compared with controls. (Note: absolute PD and I_{sc} values for alendronate and control group before treatment were −12.1 ± 0.9 and −10.1 ± 0.6 mV and 13.5 ± 1.8 and 12.4 ± 0.6 μAmp/cm², respectively.)

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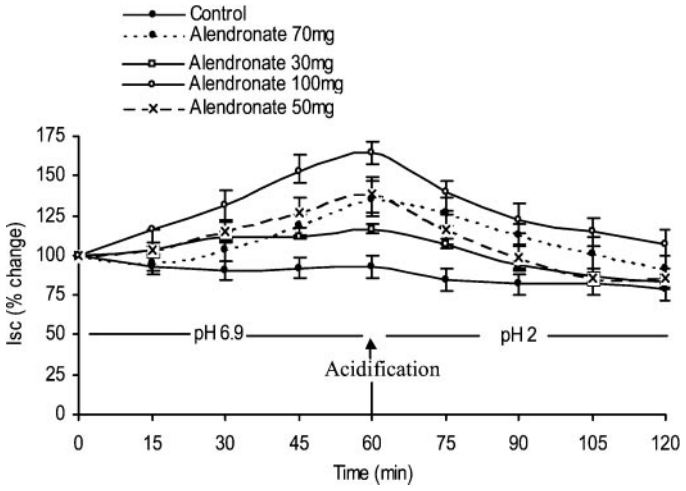


Fig. 7. Effect of luminal acidification with HCl, pH 2.0, on the increase in PD and I_{sc} produced by varying doses of luminal alendronate, 3–10 mg/ml, in rabbit esophageal epithelium mounted in Ussing chambers in normal Ringer solution for 1 h. Luminal acidification was initiated at 60 min after alendronate treatment. Control tissues were only exposed to normal Ringer solution before luminal acidification at 60 min. Note that luminal acidification at pH 2.0 has little if any effect on PD or I_{sc} in controls (see Fig. 1 for comparison to normal Ringer alone from 60 to 120 min) but reduces the increase in PD and I_{sc} produced by exposure to all doses of alendronate. Data are expressed as percentage of initial values. Values are means ± SE; $n = 5–7$ for each group; $^*P < 0.05$ compared with controls. (Note: absolute values for I_{sc} before treatment and at 60 min were 10.8 ± 1.4. and 10 ± 1.6 μAmp/cm^2 for control; 10.6 ± 1 and 12.4 ± 1.3 μAmp/cm^2 for alendronate, 3 mg/ml; 9 ± 1.1 and 12.2 ± 1.4 μAmp/cm^2 for alendronate, 5 mg/ml; 8.2 ± 0.8 and 10.8 ± 0.8 μAmp/cm^2 for alendronate, 7 mg/ml; and 13.5 ± 1.8 and 22 ± 2.8 μAmp/cm^2 for alendronate, 10 mg/ml.)

ilar increases in I_{sc} (61 ± 10% over controls) and PD (46 ± 1.6% over controls) ($n = 3$).

To determine the effects of alendronate on transepithelial properties we used impedance analysis. As shown in Fig. 8, alendronate, 0.67 mg/ml, stimulated both conductance and the I_{sc}. This stimulation was reversible, and both parameters returned to control after washout of alendronate. The increase of conductance was in the range of 10% and was smaller than the ~45% increase of I_{sc}. As the transepithelial conductance of rabbit esophageal epithelia is dominated by the paracellular pathway (20), the smaller change of conductance vs. current rules out appreciable changes of the paracellular pathway and indicates that the likely target of alendronate is the cellular apical and basolateral membrane resistance. This is also consistent with the lack of detectable changes of the high-frequency capacitance (see Ref. 23 and Fig. 8). To demonstrate the effects of low-dose alendronate on the apical membrane, impedance spectra were also collected (Fig. 9). Under these conditions, the time constant of the apical and basolateral membranes were sufficiently different from each other to permit resolution of the relative contributions of each membrane to the transepithelial impedance. In these experiments, a small decrease of transepithelial resistance with alendronate (leftward shift of the impedance spectrum) was noted. Although the absolute value of the apical and basolateral resistances cannot be determined without a priori knowledge of the resistance of the paracellular pathway, the relative contribution of each of the membrane resistances could be assessed. Therefore, the

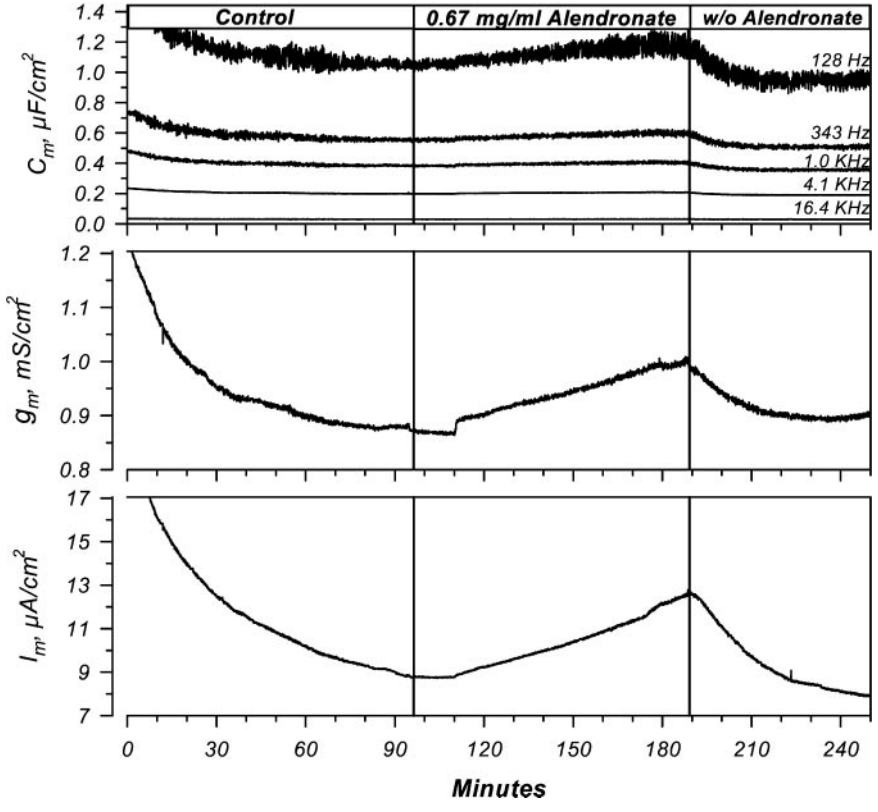


Fig. 8. Time course of the effects of alendronate on transepithelial impedance. After a 90-min control period, alendronate was added to the apical side at 0.67 mg/ml. This caused a stimulation of both membrane conductance (g_m) and short-circuit current (I_m). The values of the capacitance (C_m) calculated at low frequencies is highly dependent on the direct current resistance. Therefore changes at 128 Hz and other low frequencies represent changes of resistance rather than changes of capacitance and therefore membrane area. At high frequency, the equivalent capacitance is the sum of both membrane capacitances and is highly affected by the resistance of the lateral intercellular spaces (23). As the capacitance at 4.1 and 16.4 kHz is essentially unaffected by alendronate, we interpret this finding in support of appreciable effects on the paracellular pathway ($n = 5$).

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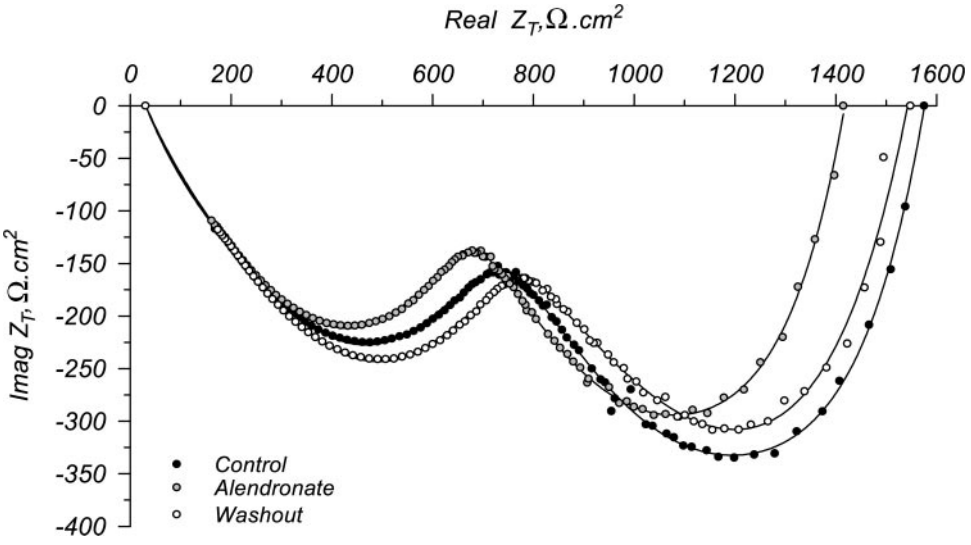


Fig. 9. Nyquist plot of the transepithelial impedance in rabbit esophageal epithelia and the effects of alendronate. The real and imaginary components of the impedance (Z_T) were calculated and plotted in the Nyquist format. In this representation, a biological membrane consisting of a resistor parallel with a capacitor yields a semi-circle and an epithelium consisting of 2 series membranes results in 2 semi-circles. Owing to lower capacitance, the apical membrane impedance is dominant at high frequency (*left*), whereas the basolateral membrane is dominant at low frequencies. As seen in this figure, the decrease of resistance (0 frequency extrapolation) is predominantly due to a decrease of the apical (high frequency) semi-circle, providing evidence for effects of alendronate on the apical membrane and presumably the apical Na conductive pathway ($n = 5$).

decrease of impedance was found to be predominantly due to a decrease of apical membrane impedance and more specifically its DC resistance (Fig. 9, *left*). These data support the concept that the changes of I_{sc} are mediated via effects on the apical membrane.

To determine the effects of a high dose of alendronate on tissue impedance, tissues were exposed to 10 mg/ml of alendronate. As shown in Fig. 10, luminal alendronate caused a marked stimulation of the I_{sc}. This stimulation was similar in magnitude to that observed with 0.67 mg/ml, indicating potential saturation of the response of Na transport. The changes of conductance were also similar with the high dose of alendronate. Moreover, addition of basolateral alendronate, as shown in Fig. 10, reversed the effects on transport but further potentiated the stimulation of conductance. These effects indicate the potential for a complex response at the higher doses, which may involve separate effects on both apical and basolateral membranes.

To address the effects of high dose alendronate on both membranes, impedance spectral analysis was also carried out. As shown in Fig. 11, alendronate caused a large increase of apical resistance (R_a) and a compensatory decrease of basolateral resistance (R_{bl}), such that the total resistance was decreased as observed in

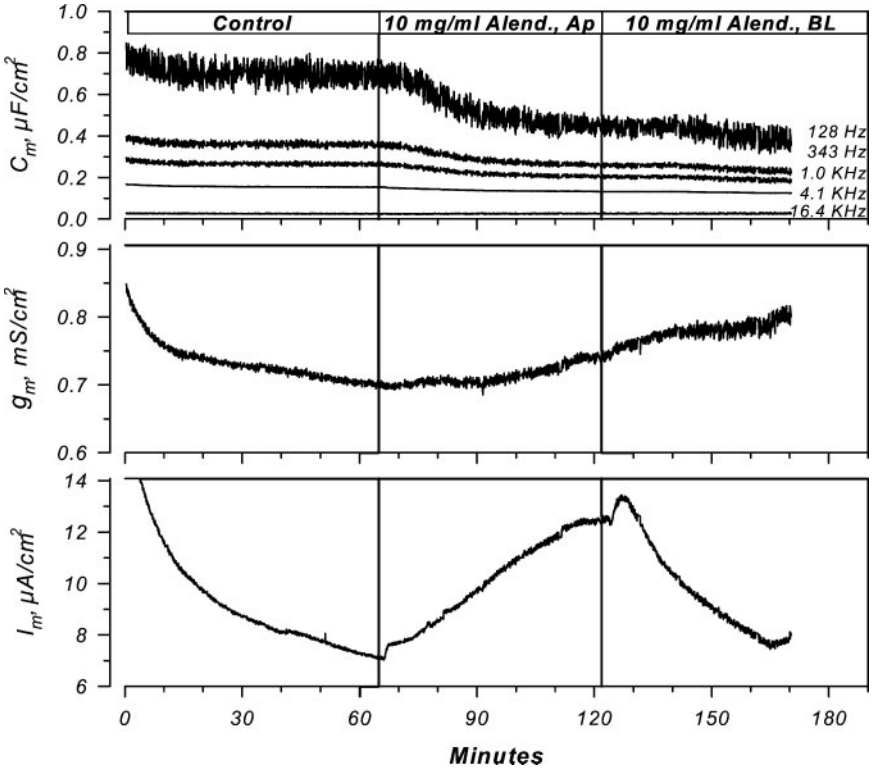


Fig. 10. Time course of a high dose of alendronate (10 mg/ml). Apical (Ap) addition of alendronate increases both g_m and I_m. The changes of current were, however, larger than those of conductance. Addition of alendronate to the basolateral solution (BL) caused a small further increase of conductance. However, the stimulation observed with apical addition was reversed and the I_m returned to control prealendronate levels.

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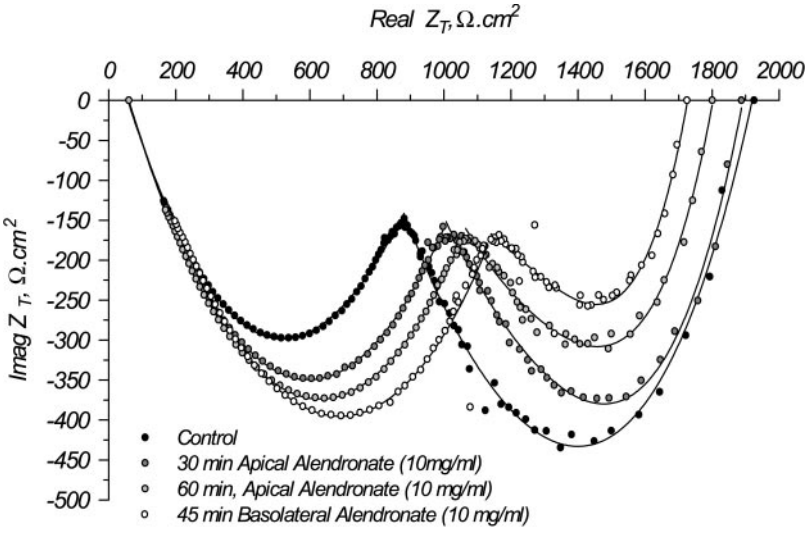


Fig. 11. Nyquist plot of high dose alendronate, 10 mg/ml, on both apical and basolateral membranes. Impedance (Z_T) spectra showed that the addition of luminal alendronate caused a small initial decrease of apical resistance similar to that observed with 0.67 mg/ml alendronate (Figs. 8 and 9). However, this was followed by an increase of apical resistance (see data at 30 and 60 min). The overall resistance was decreased as shown in Fig. 10, owing to the additional effects on the basolateral membrane resulting in a large decrease of its resistance. These data indicate that at higher doses alendronate affects both the apical and basolateral membranes.

Fig. 10. The increase of R_a was initially (within the first 10 min) preceded by a small decrease as observed with the lower doses of alendronate (data not shown). Furthermore, addition of sequential intermediate doses of alendronate (2, 4, 6 mg/ml) indicated that the sustained decrease of R_a with 0.67 mg/ml was also observed at 2 mg/ml but not at the higher doses (data not shown). These data are consistent with the interpretation that low doses of alendronate, up to 2 mg/ml, stimulate the apical conductance, whereas higher doses block it. This is, however, not reflected as inhibition of transport with high dose luminal alendronate, as these changes are accompanied by stimulation of basolateral conductance. This decrease in R_{bl} is likely due to increased basolateral K conductance, which in turn increases the electrical driving force for apical Na entry into the cells. Notably, addition of basolateral alendronate inhibits the I_{sc} despite large stimulation of the K channels. This is due, as shown in Fig. 11, to severe inhibition of apical (Na channel) conductance.

In another set of experiments, the morphological effects of luminal alendronate, 10 mg/ml, for 1 h were compared at acidic (HCl, pH 2) and neutral pH (Ringer, pH 6.9) in Ussing chamber-mounted tissue sections from the same rabbit. Alendronate at neutral pH again, as noted in Fig. 2, produced only minor changes in histology. Similarly, luminal acidity, pH 2, had little effect on esophageal morphology. In contrast, luminal alendronate at pH 2 resulted in significant injury as evidenced by the presence of diffuse edema and cell necrosis (Figs. 12 and 13, *A* and *B*). In addition, pretreatment with ouabain for 30 min to inhibit active Na transport before exposure to alendronate, 10 mg/ml, plus HCl, pH 2.0, for 1 h failed to alter the ability of this combination to produce morphological injury (morphological injury score was 2.16 ± 0.3, $n = 3$).

DISCUSSION

In the present study we observed that luminal alendronate at neutral pH has little effect on the morphology or barrier function of the esophageal epithelium. However, interestingly, it produced a dose-dependent stimulatory effect on active ion transport (increase in I_{sc}), active ion transport in rabbit esophageal epithelium being principally the result of active Na absorption (Na absorption is ~85% of I_{sc}; Ref. 16). Support for an effect of alendronate on Na absorption is provided in our studies by inhibition of this stimulatory effect on I_{sc} by ouabain, an Na,K-ATPase inhibitor, or by luminal acidification, the latter a maneuver previously shown by our lab to inhibit apical membrane Na channels in this tissue (16, 21) and by the fact that alendronate produced similar increases in I_{sc} and PD in Cl-free Ringer solution. Furthermore, because serosal alendronate did not increase I_{sc} (actually decreased I_{sc}), the

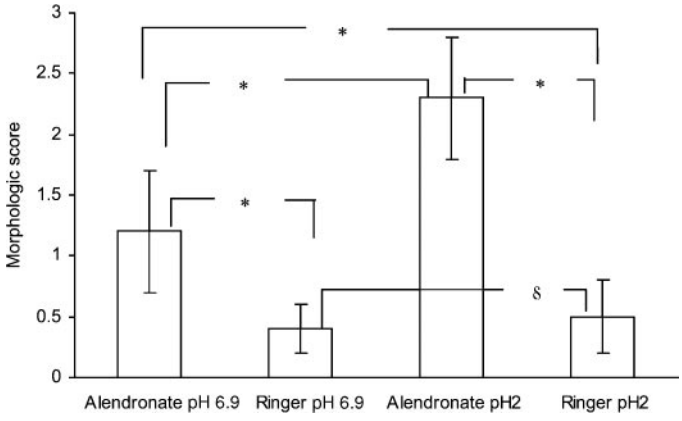


Fig. 12. Difference was significant between the morphological scores of the tissues treated for 1 h with luminal acidification, HCl pH 2.0, alone ($n = 7$), or luminal acidification plus alendronate, 10 mg/ml ($n = 7$) (morphological scores were 0.6 ± 0.3 and 2.3 ± 0.5, respectively, $P < 0.05$). The addition of alendronate to the acidic bathing solution produces significantly greater damage than acidification alone. Also we detected that the tissues exposed to alendronate at pH 6.9 ($n = 4$) were significantly more damaged than tissues exposed to Ringer alone at pH 2 (morphological scores were 1.2 ± 0.5 and 0.6 ± 0.3, respectively, $P < 0.05$). There was no significant difference between morphological scores of tissues exposed luminally to Ringer at pH 6.9 ($n = 4$) and pH 2 (morphological scores were 0.4 ± 0.2 and 0.6 ± 0.3, respectively, $P > 0.05$). *$P < 0.05$; §$P > 0.05$.

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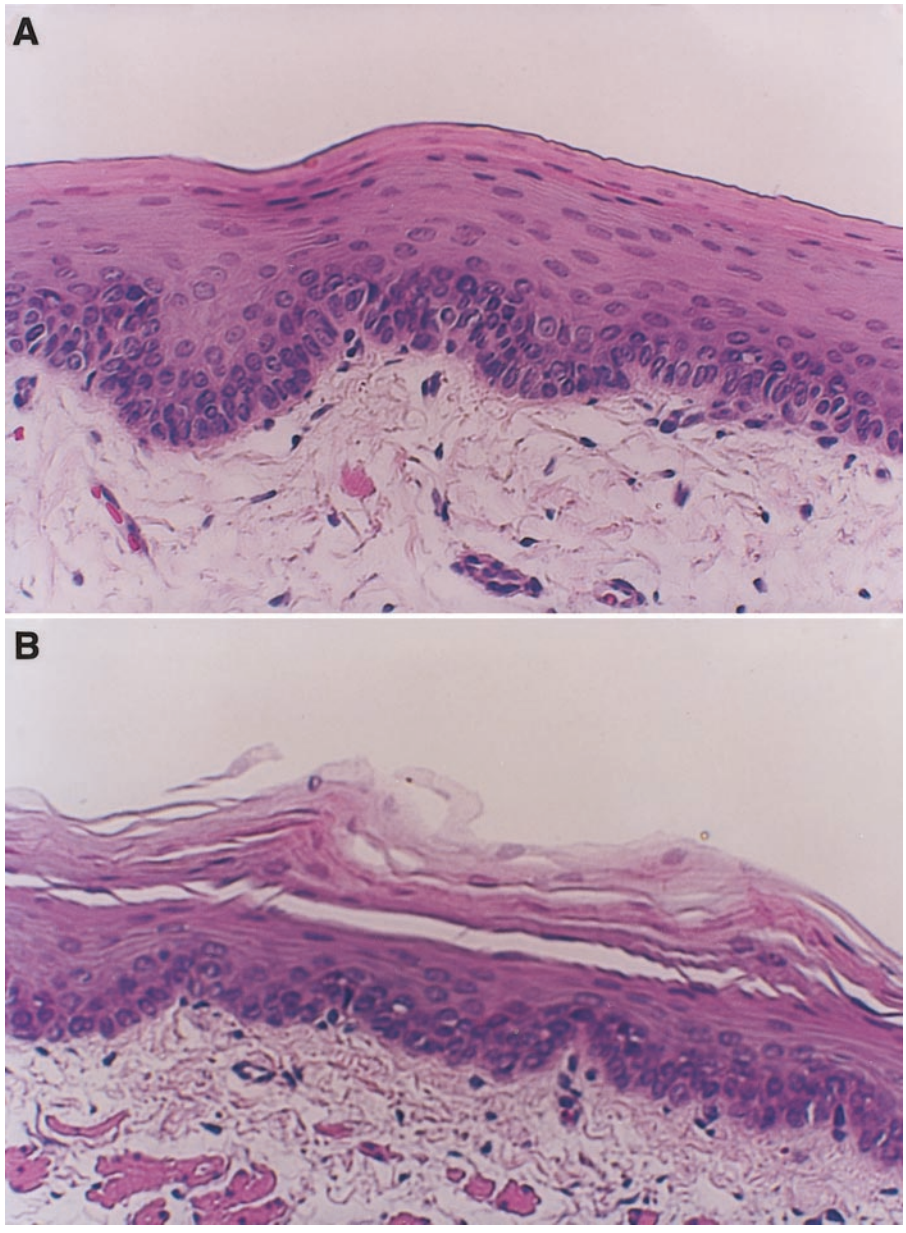


Fig. 13. Representative light micrographs of rabbit esophageal epithelium exposed for 1 h to HCl-acidified Ringer solution, pH 2.0 (*A*), or to HCl-acidified Ringer solution containing alendronate, 10 mg/ml (*B*). Tissues exposed to HCl, pH 2.0, show no evidence of significant morphological damage (see Fig. 2 for comparison to normal Ringer exposure), whereas tissues exposed to acid plus alendronate show both diffuse edema and cell necrosis. Hematoxylin and eosin, ×150.

action of luminal alendronate was unlikely to be mediated by its absorption and action on the squamous cell basolateral membranes. Additional support for the conclusion that alendronate stimulates active transport in esophageal epithelium by an action on the apical membrane was obtained using impedance analysis. In these experiments, luminal alendronate, up to 2 mg/ml, reversibly stimulated both I_{sc} and conductance, with the effect on I_{sc} being much greater than that on conductance. Because conductance is dominated by the paracellular pathway in rabbit epithelium (20), this difference coupled with a lack of change in capacitance at high frequency (Fig. 8) mitigates against an appreciable change in paracellular permeability and in favor of a change in membrane permeability (23). Furthermore, based on time constants, the impedance spectra indicated that the primary effect of alendronate was on the apical membrane. These data, together, suggest that alendronate at low dose stimulates active (Na) transport in esophageal epithelium by an effect on the apical membrane Na channels. It remains unclear, however, whether this effect is a direct effect on the Na channel itself or mediated indirectly by changing apical membrane fluidity (4, 6). At higher doses, 4–10 mg/ml, similar to the lower dose, alendronate stimulated I_{sc}. However, this was only briefly associated with a decrease in apical resistance because the higher doses then rapidly increased apical resistance. Nonetheless, I_{sc} increased at the higher dose because of coincident increases in basolateral conductance, as shown by the decrease in R_{bl} in Fig. 10. This is most likely a reflection of stimulation of basolateral K conductance, which hyperpolarizes the cells and increases the apical driving force for Na entry. The

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only previous reports of an effect of a bisphosphonate on ion transport have been in osteoclasts. In these cells it was shown that alendronate and tiludronate can inhibit Na-independent H^+ extrusion that occurs via a vacuolar H^+-ATPase (8, 24).

Although luminal alendronate at neutral pH had a significant effect on esophageal epithelial ion transport, it had minimal effects even at high concentration on esophageal morphology. This argues against the fact that a topical action by the drug as ingested (at neutral pH) was alone responsible for pill-induced esophagitis. A similar conclusion was reached by Peter and colleagues (17) based on experiments perfusing alendronate (0.2 mg/ml) in vivo into the esophagus of dogs for 5 days. Moreover, they observed that unlike neutral perfusions with alendronate, esophageal perfusion with alendronate at acidic pH (pH 2) resulted in significant esophageal pathology as characterized by marked ulcerative esophagitis. Our findings are consistent with these observations in that luminal alendronate for 1 h at an acidic pH (pH 2) that was itself nondamaging resulted in significant edema and cell necrosis. This indicates that alendronate's potential for esophageal injury is expressed on exposure to gastric acid. Furthermore, this injury appears to be unrelated to the ability of alendronate to stimulate active Na transport because acid pH 2.0 is known to inhibit Na absorption via the apical membrane Na channel in esophageal epithelium and prior treatment with ouabain to inhibit active Na transport failed to alter the extent of tissue injury induced by alendronate plus acid. In humans in vivo this might occur with adherence of a pill containing alendronate to the esophageal epithelium as a result of patients ingesting medication with little liquid or reclining shortly after taking the medication. Damage then occurs when the pill-containing esophagus is subsequently bathed by gastric acid through either physiological, or in some patients with coincident gastroesophageal reflux disease, pathologic reflux. Alternatively, alendronate tablets dissolving in acidic gastric juice in the stomach could, through physiological or pathologic reflux, reenter and damage the esophagus at acid pH. [Note: This latter possibility seems less likely because renewed attention on the proper means for taking the medication (follow medication with full glass of water and remain upright for at least one-half hour) appears to have greatly diminished the frequency of this clinical problem (personal observations).] The mechanism for the toxicity of alendronate under these conditions is unclear but Peter and colleagues showed that it was not specific to aminobisphosphonates such as alendronate or risedronate because similar injury in their model could be produced by perfusion of a non-aminobisphosphonate (tiludronate) at acid pH. Moreover, they suggested that the damage by alendronate may be due to conversion of the medication at acid pH to a more toxic form (17). Indeed alendronate exists as a monosodium salt at pH >3.5, whereas at pH <3.5 it is primarily in a free acid form that is known to be more irritating to mucosa (1, 17). The development of pill-induced esophagitis could also in some circumstances result from the potentiation between alendronate and acid such that two mildly injurious processes synergize to produce severe esophagitis.

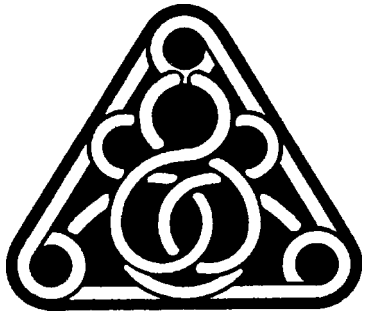
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