Biochemical and Biophysical Research Communications **260,** 13–16 (1999)
Article ID bbrc.1999.0861, available online at http://www.idealibrary.com on IDEAL®

Tetracyclines Reduce Na^+/K^+ Pump Capacity in Calu-3 Human Airway Cells

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Received April 21, 1999

We studied the effect of tetracyclines on the Na^+/K^+ pump activity in Calu-3, a human airway cell line. To estimate Na^+/K^+ pump capacity on the basolateral membrane, an ouabain-sensitive component of the short-circuit current (Isc) was measured in the presence of nystatin, an ionophore of Na^+. The application of ouabain (1 mM) to the basolateral solution completely inhibited the Isc generated by adding nystatin (50 μM) to the apical solution. Tetracycline (TC), minocycline (MC), or demethylchlortetracycline (DC) at 0.5 mM applied to the apical but not to the basolateral solution also decreased the nystatin-induced Isc. Neither phlorizin- nor diphenylamine-2-carboxylic acid-sensitive Isc was affected by TC, MC, or DC. These results indicate that tetracyclines may permeate only through the apical membrane with the result that the Na^+/K^+ pump's capacity for Na^+ extrusion should be suppressed without a decrease in Cl^- transport. © 1999 Academic Press

Active ion transport mechanisms regulate the balance of secretion and reabsorption that determines the amount of liquid on the airway surface. Patients with cyctic fibrosis (CF) have a genetic defect in the cyctic fibrosis transmembrane regulator (CFTR) that abnormally decreases Cl^- secretion and consequently increases Na^+ absorption to synergistically cause airway dehydration and result in chronic respiratory infection (1, 2). Inhibition of Na^+ absorption has potential to alleviate this symptom in CF patients. Recently tetracycline (TC) has been reported to inhibit amiloride-sensitive Na^+ absorption in sheep tracheal epithelia only when it is applied from the mucosal side (3). However, the mechanisms underlying this effect are unfortunately still unknown. For transepithelial Na^+ transport, Na^+ extrusion by the Na^+/K^+ pump from inside to outside the cell across the basolateral membrane is as crucial as Na^+ entry across the apical membrane. Thus we examined the effect of TC and its derivatives on Na^+ absorption, focusing especially on the Na^+/K^+ pump, in Calu-3 human airway cells which have many similarities to submucosal gland serous cells (4, 5). We here demonstrate that tetracyclines applied to the apical side inhibit Na^+/K^+ pump capacity without affecting Cl^- secretion.

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MATERIALS AND METHODS

Calu-3 cells were purchased frozen (−80°C) from American Type Culture Collection (Rockville, MD) and grown in T_{75} tissue culture flasks at 37°C in a humidified incubator with 5% CO_2 in air. We used a 1:1 mixture of Dulbecco's Modified Eagle Medium and F12 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO), 100 g/ml streptomycin and 100 U/ml penicillin (GIBCO) for the culture medium. For short-circuit current (Isc) measurement, cells from the flasks were subcultured by the air interface method (6) at 10^6 cells/cm^2 onto human placental collagen-coated Costar Snapwell inserts (0.4 μm pore size, 12 mm diameter, polyester; Costar, Cambridge, MA) for 7–13 days. The filter inserts with confluent monolayers were mounted in modified Ussing chambers (EasyMount Chamber; Physiologic Instrument, San Diego, CA) with a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES with pH of 7.4 at 37°C. The Isc was measured by a voltage clamp amplifier (VCC MC2; Physiologic Instrument, San Diego, CA). Tetracycline (TC), minocycline (MC), and demethylchlortetracycline (DC) were kindly provided by Wyeth Lederle Japan Inc. Ouabain and diphenylamine-2-carboxylic acid (DPC) were obtained from Research Biochemicals International (Natick, MA). Nystatin and phlorizin were obtained from Sigma Chemical (St. Louis, MO). All chemicals in the present study were dissolved in dimethylsulfoxide (DMSO). Data are expressed as means ± S.E. with the number of preparations used (n). Statistical difference was determined by Student's *t* test or one-way ANOVA. Values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Transepithelial Na^+ current is thought to pass through two membrane barriers: the apical and basolateral membranes. When amiloride-sensitive Isc is inhibited by a certain agent, there are three possible explainations of this phenomenon, i.e., the agent inhib-

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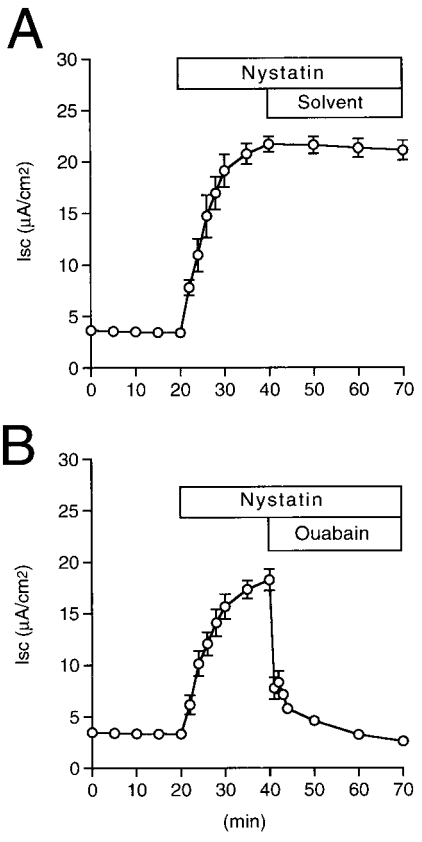

FIG. 1. Effects of ouabain (1 mM) and the solvent on the nystatin-induced short circuit current (Isc). (A) 50 μM nystatin, a Na^+ ionophore, elicted a rapid increase in Isc and reached the maximum level within 20 min followed by a sustained component (n = 4). Dimethylsulfoxide (0.1%), the ouabain solvent, applied in the basolateral solution did not affect the Isc, (B) whereas 1 mM ouabain applied in the basolateral solution 20 min after adding nystatin produced a quick decrease in Isc to below the basal current (n = 6).

its amiloride-sensitive Na^+-permeant channels on the apical membrane, the Na^+/K^+ pump on the basolateral membrane, or both. TC has been demonstrated to inhibit amiloride-sensitive Isc in airway epithelium although its chemical structure has no similarity to that of amiloride (3). This fact motivated us to examine the mechanisms underlying the inhibitory effect of TC on Na^+ absorption. First we measured the Na^+/K^+ pump-generated current by applying 50 μM nystatin (an ionophore of Na^+) to the apical solution in the Calu-3 human airway cell line, according to previously reported procedures (7, 8, 9). This treatment increases the Na^+ conductance of the apical membrane, and raises intracellular Na^+ concentration to the extent that Na^+/K^+ pump becomes the rate-limiting step of transepithelial Na^+ transport. Figure 1 shows the action of ouabain, a Na^+/K^+ pump blocker, applied to the basolateral solution in the presence of nystatin. The Isc reached the nearly maximum level about 20 min after adding nystatin and was maintained for at least 50 min even when the solvent (0.1% DMSO) was applied to the basolateral solution (Fig. 1A). In contrast, the addition of 1 mM ouabain to the basolateral solution 20 min after application of nystatin almost immediately reduced the Isc to below the basal level (Fig. 1B), implying that the nystatin-induced Isc mainly consists of Na^+/K^+ pump-generated current (pump current). The ouabain-sensitive pump current obtained in the present study a was 15.7 $\pm$ 1.2 $\mu A/cm^2$ (n = 6). Because TC (1 mM) inhibited sodium absorption only when it was applied to the mucosal side in sheep tracheal epithelia (3), we applied TC (0.5 mM) to the apical solution 20 min after producing the nystatin-induced current (Fig. 2A). The Isc showed a rapid decrease from 17.7 $\pm$ 0.9 to 5.3 $\pm$ 0.6 $\mu A/cm^2$, (n = 5, in 30 min) in response to TC with the result that significantly few ouabain-sensitive Isc remained (3.9 $\pm$ 0.6 $\mu A/cm^2$, n = 5). In contrast, basolateral application of TC did not affect the ouabain-sensitive Isc (18.0 $\pm$ 1.2 $\mu A/cm^2$, n = 4, Fig. 2B). To eliminate the effects of the solvent on the pump current, 0.1% DMSO was applied to the apical solution, where it had no influence (Fig. 2C). When either TC (0.5 mM) or hydrochloric acid (HCl, 0.5 mN) was added in the solution adjusted to pH 7.40, the pH was equally changed to 7.33 (n = 4). To rule out the possibility that the acidification might affect the pump current, HCl (0.5 mN) was applied to the apical solution, also resulting in no effect (Fig. 2D). Since TC has

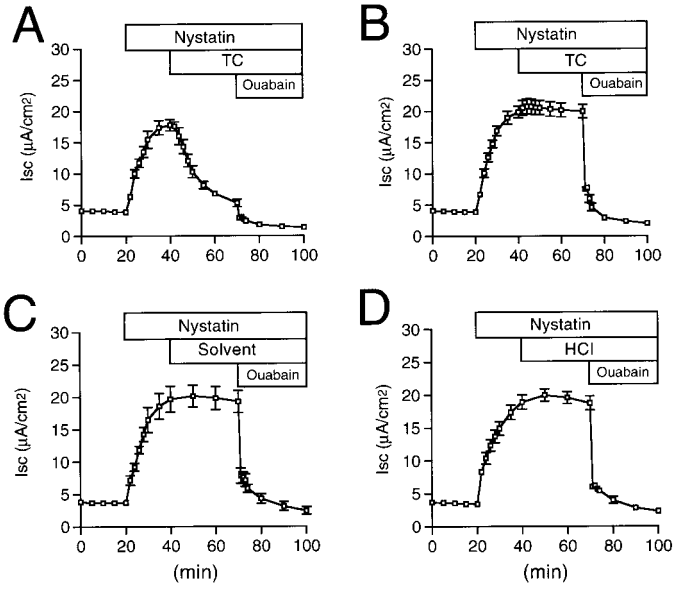

FIG. 2. Effects of tetracycline (TC), the solvent, and hydrochloric acid (HCl) on the nystatin-induced short circuit current (Isc). These reagents were applied 20 min after adding nystatin (50 μM). (A) TC applied in the apical solution produced a rapid decrease in the nystatin-induced Isc with the result that significantly reduced ouabain-sensitive Isc remained (n = 5). (B) In contrast, TC applied in the basolateral solution did not affect the nystatin-induced and ouabain-sensitive Isc (n = 4). (C) Dimethylsulfoxide (0.1%), the ouabain solvent, and (D) HCl (0.5 mN) applied in the apical solution also had no significant effects on the Isc (n = 4, respectively).

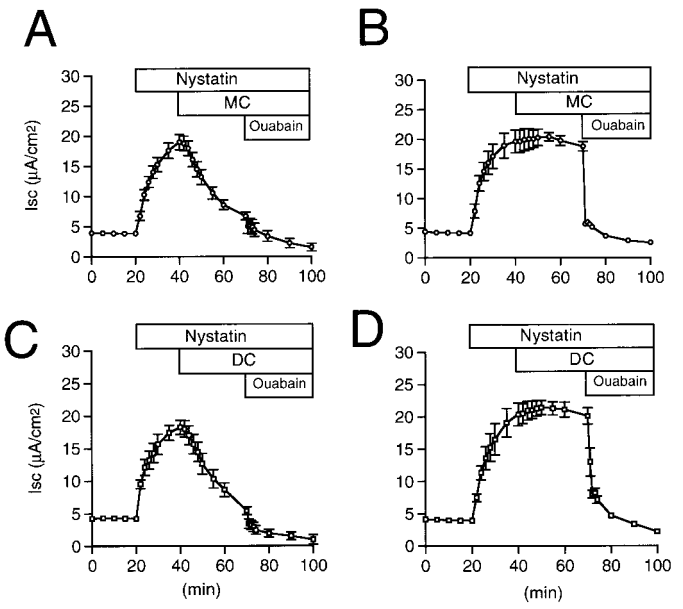

FIG. 3. Effects of minocycline (MC) and demethylchlortetracycline (DC) on the nystatin-induced short circuit current (Isc). Both were applied 20 min after adding nystatin (50 μM). (A) MC and (C) DC at 0.5 mM applied in the apical solution produced a rapid decrease in the nystatin-induced Isc so that only significantly reduced ouabain-sensitive Isc remained, (B, D) whereas both applied in the basolateral solution had no significant effect on the Isc (n = 4, respectively).

several derivatives which are structually analogous, we next examined the effects of minocycline (MC) and demethylchlortetracycline (DC) on the pump current (Fig. 3). Both derivatives inhibited nystatin-induced current, consequently suppressing ouabain-sensitive Isc (5.2 $\pm$ 0.3 in MC and 4.5 $\pm$ 0.6 μA/cm^2 in DC, n = 4), only when applied to apical side (Fig. 3A and C). These results suggest that TC and its derivatives with similar chemical structures seem likely to permeate only the apical membrane, resulting in an inhibition of Na^+/K^+ pump capacity from the cytosolic side.

Nystatin has been reported to create aqueous pores of about 4 Å radius in the lipid bilayer (10, 11). The pore size selectively enables monovalent cations, water, and small nonelectrolytes to permeate the membrane (10); thus tetracyclines, whose molecular weight is 444.44 in TC, 529.97 in MC, and 501.32 in DC, presumably could not pass through pores made by nystatin. Considering this, there seems likely to be a difference of properties between apical and basolateral membranes in terms of tetracyclines' permeability.

In recent investigations, tetracyclines were found to possess anti-inflammatory (12), anti-enzymatic (13, 14), anti-proliferative (15), anti-osteopenic (16, 17) and immunomodulating effects (18) unrelated to their antimicrobial effects. The mechanisms underlying these effects are believed to be based on the chelating ability of their divalent cations (19, 20, 21), especially Ca^{2+}, causing a decrease in cytosolic Ca^{2+} (16, 17). Although lowering extracellular Ca^{2+} concentration caused by Ca^{2+} chelation should make the gap junctions leaky, membrane resistance was not changed by TC (from 471.5 $\pm$ 16.6 to 597.4 $\pm$ 20.5 μcm^2, n = 5), MC (from 437.2 $\pm$ 73.0 to 553.4 $\pm$ 98.5 μcm^2, n = 5), and DC (from 457.4 $\pm$ 41.6 to 591.2 $\pm$ 48.2 μcm^2, n = 5) administered for at least 30 min in the present study, suggesting that extracellular Ca^{2+} would not be chelated as much as affecting tight junctions. If, however, tetracyclines do decrease in cytosolic Ca^{2+} concentration presumably due to their previously repored (16, 17) chelating effect, there is every possibility that the reduction of intracellular free divalent cations affects the Na^+/K^+ pump. Na^+/K^+ ATPase is composed of α and β units, and the α subunit (a catalytic subunit) contains the binding sites for Na^+, ATP, and Mg^{2+} facing the cytosolic side and for K^+ and ouabain facing

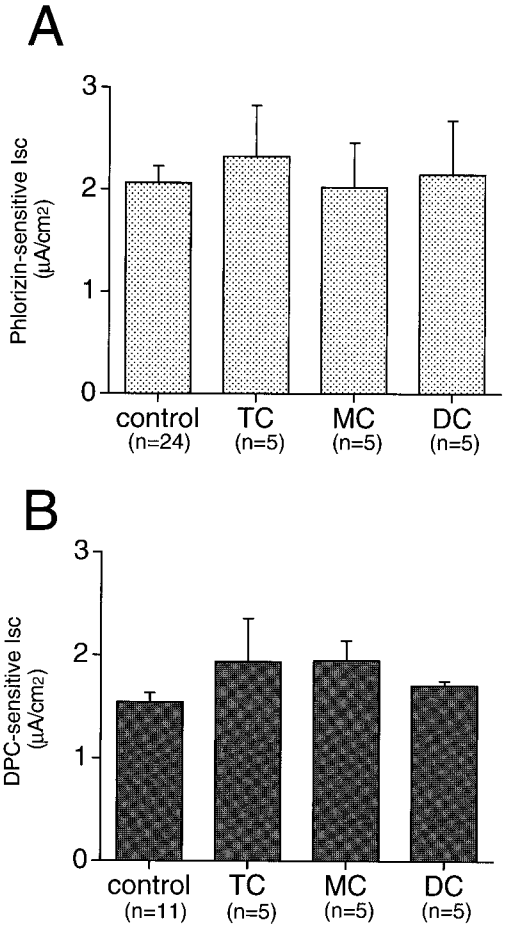

FIG. 4. Effects of tetracycline (TC), minocycline (MC), and demethylchlortetracycline (DC) on (A) phlorizin- and (B) diphenylamine-2-carboxylic acid (DPC)-sensitive short circuit current (Isc). These Isc were measured 30 min after the application of TC, MC, and DC at 0.5 mM to the apical side and were evaluated as the difference in the Isc from before to 10 min after the application of phlorizin (200 μM) or DPC (500 μM) to the apical solution. No significant differences were found in any comparison of the values.

the outside of the cell (22, 23). Mg^{2+} is crucial for catalyzing ATP hydrolysis in Na^+/K^+ATPase. Thus one of the presumable mechanisms underlying inhibition of the Na^+/K^+ pump produced by tetracyclines is Mg^{2+} chelation. Since, however, possible alternative mechanisms have not been demonstrated in previous reports or in the present study, further investigations will be necessary to clarify them.

In the present study, we unfortunately could not observe the effect of tetracyclines on Na^+ permeant channels because of their absence in Calu-3 (24). Instead, we examined the effects of tetracyclines on the Na^+/glucose cotransporter (Fig. 4A), which contributes to Na^+ influx across the apical membrane in Calu-3 (24, 25). The ionic current produced by the Na^+/glucose cotransporter was estimated as phlorizin-sensitive Isc. Exposure to TC, MC, or DC (0.5 mM) for 30 min in the apical solution produced no significant effect in the transporter phlorizin-sensitive Isc (2.3 ± 0.5 in TC, 2.0 ± 0.4 in MC, 2.1 ± 0.5 $\mu A/cm^2$ in DC, $n = 6$), compared with the control (2.1 ± 0.2 $\mu A/cm^2$, $n = 12$). These values are all significantly lower than the suppressed ouabain-sensitive Isc after application of tetracyclines, shown in Figs. 2A and 3A and C, suggesting that the Na^+/glucose cotransporter should still be a rate-limiting step in the transepithelial Na^+ transport even after the Na^+/K^+ pump is inhibited by tetracyclines.

To examine the effects of tetracyclines on the Cl^- transport (Fig. 4B), we used diphenylamine-2-carboxylate (DPC, 500 μM), a Cl^- channel blocker, applied with the apical solution. DPC-sensitive Isc also was unaffected by TC (1.9 ± 0.4 $\mu A/cm^2$, $n = 5$), MC (1.9 ± 0.2 $\mu A/cm^2$, $n = 5$), and DC (1.7 ± 0.04 $\mu A/cm^2$, $n = 5$), compared with the control (1.5 ± 0.1 $\mu A/cm^2$, $n = 12$). These results are consistent with the evidence that Calu-3 does not have Ca^{2+}-dependent Cl^- channels but CFTR (5, 26), supposing the effects of tetracycline would be due to Ca^{2+} chelation.

Taken together, in a Calu-3 human airway cell line possessing cellular polarity, tetracyclines seem likely to affect the Na^+/K^+ pump capacity from the cytosolic side after permeating only the apical but not basolateral membrane without inhibiting the CFTR and the Na^+/glucose tranporter. The actions may be attributed to their Mg^{2+} chelation although this concept has not proved in the present study.

ACKNOWLEDGMENT

We are very grateful to Mr. K. Kinoshita (Wyeth Lederle Japan Inc.) for kindly providing tetracyclines and information regarding these drugs.

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