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CFTR-Mediated Anion Conductance Regulates Na^+-K^+-Pump Activity in Calu-3 Human Airway Cells

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We studied the role of CFTR in the Na^+-K^+-pump activity of Calu-3 human airway cells. To estimate the Na^+-K^+-pump activity on the basolateral membrane, the ouabain-sensitive component of the short-circuit current (Isc) was measured after permeabilization of the apical membrane with nystatin, a Na^+ ionophore. The Na^+-K^+-pump activity was diminished by a selective CFTR blocker (glybenclamide) or non-specific Cl^- channel inhibitors (NPPB and DPC) but not by outwardly rectifying Cl^- channel blockers (DNDS, DIDS). Augmentation of anion conductance by 8-bromo-cyclic AMP (8Br-cAMP, 1 mM) potentiated the Na^+-K^+-pump activity that was reduced by blocking CFTR or by the replacement of Cl^- with gluconate, a less membrane-permeant anion. The Na^+-K^+-pump activity was unaffected by the replacement of Cl^- with NO_3^- that has equal permeability through the CFTR. These results suggest that the anion movement through the CFTR may contribute to the Na^+-K^+-pump activity in Calu-3 cells by regulating the rate of Na^+ entry.

Regulation of the liquid on the airway surface is important in the formation of low viscosity mucus, thereby maintaining a conductive and aseptic environment in the lung. The amount of the intraluminal fluid is determined by the balance of secretion and absorption regulated by an active ion transport system whose energy is mainly produced by the Na^+-K^+-ATPase pump (Na^+-K^+-pump) (1): the Na^+ gradient produced by the Na^+-K^+-pump is the driving force for several Na^+-coupled ion transporters, such as the Na^+-K^+-$2Cl^-$-cotransporters (2), which are responsible for transepithelial Cl^- transport (3). In addition, the Na^+-K^+-pump itself is the extrusion step across the basolateral membrane for the transepithelial Na^+ transport in various kinds of polarized epithelial cells (4–6). Recently it has been established that the cystic fibrosis transmembrane conductance regulator (CFTR) functions not only as an anion channel but also as a regulator of the Na^+ channel that is the entry step for the transepithelial Na^+ transport (7). However, the effects of CFTR on the Na^+-K^+-pump have not been investigated. Thus we examined the role of CFTR in the Na^+-K^+-pump capacity in a Calu-3 human airway cell line that appeared to have a phenotype very similar to that of lung serous cells (8, 9). In the present study, we applied a non-invasive and non-destructive technique using a modified Ussing chamber to observe the interaction between the Na^+-K^+-pump and CFTR (10–12). We here demonstrate that anion conductance through CFTR contributes to the activity of the Na^+-K^+-pump.

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MATERIALS AND METHODS

Cell culture. Calu-3 cells were purchased frozen (−80°C) from American Type Culture Collection (Rockville, MD) and grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO), 100 μg/ml streptomycin, and 100 U/ml penicillin (GIBCO). The cells were incubated in culture flasks (T75) at 37°C in a humidified incubator with 5% CO_2 in air. When 80–90% confluent, cells were detached with a solution of phosphate-buffered saline (PBS), 0.04% EDTA, and 0.25% trypsin. For short-circuit current (Isc) measurement, cells from the flasks were subcultured at 10^6 cells/cm^2 on snapwell inserts (0.4 μm pore size, 12 mm diameter, polyester; Costar, Cambridge, MA) which had been coated overnight with 0.5 mg/ml human placental collagen type VI (Sigma Chemical Co., St. Louis, MO). One day after seeding the cells on the inserts, the medium bathing the apical side was removed to establish an air interface (13). The cells formed a confluent monolayer in 6 days and were used for experiments in 7–13 days.

Solutions. The normal solutions used in this study contained (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4 at 37°C). For experiments of anion replacement, Cl^- was replaced by gluconate or NO_3^-, and called "gluconate solution" or "NO_3 solution" respectively. HCO_3^--containing solutions without Cl^- (called "HCO_3 solution") consisted of (mM): 115 Na-gluconate, 25 $NaHCO_3$, 5 K-gluconate, 1 Mg-gluconate, 2 Ca-gluconate, 10 glucose, and 10 HEPES. When preparing this solution, the pH was adjusted to 7.4 at 37°C before adding $NaHCO_3$.

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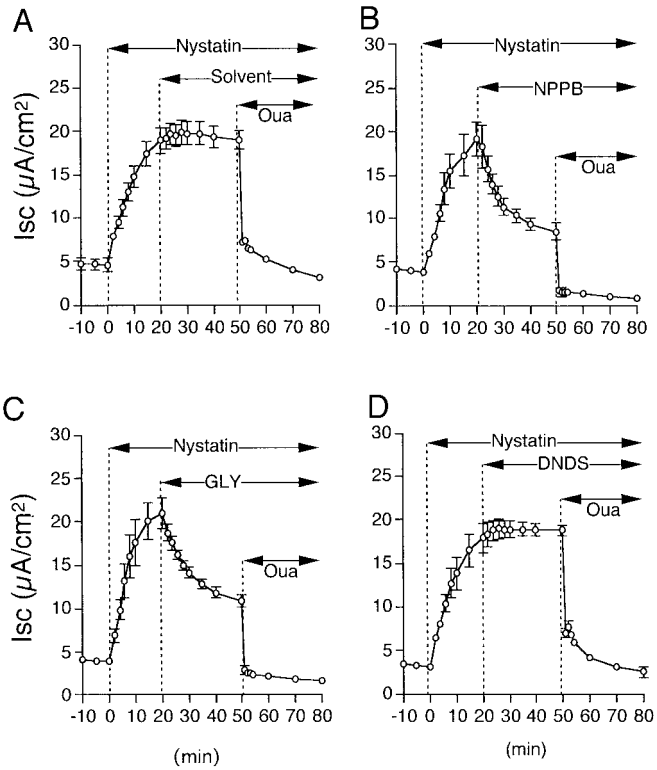

FIG. 1. Effects of Cl^- channel blockers on the ouabain-sensitive Isc. Nystatin (50 μM) applied to the apical solution elicted a rapid increase in Isc (A) that reached the maximum level in 20–30 min followed by a sustained component even when the solvent (dimethylsulfoxide, 0.1%) was applied. Ouabain (Oua, 1 mM) applied in the basolateral solution abolished the Isc. 100 μM NPPB (B) or 300 μM glybenclamide (GLY) (C) applied to the apical and basolateral solution produced a rapid decrease in the nystatin-induced Isc with the result that significantly reduced ouabain-sensitive Isc remained. In contrast, 500 μM DNDS (D) applied to the apical and basolateral solutions did not affect the nystatin-induced and ouabain-sensitive Isc. Data are means ± S.E.M. (n = 4–5).

Drugs. Ouabain, bumetanide, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), diphenylamine-2-carboxylate (DPC), 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were obtained from Research Biochemicals International (Natick, MA). Nystatin, phlorizin, and 8-bromo cyclic AMP (8Br-cAMP) were obtained from Sigma Chemical. All chemicals in the present study except 8-bromo cAMP were dissolved in dimethylsulfoxide (DMSO). 8-bromo cAMP was dissolved in distilled water. Nystatin solution was prepared just before every experiment.

Measurements of short-circuit current (Isc) and anion conductance. The filter inserts with confluent monolayers were mounted in modified Ussing chambers (EasyMount Chamber; Physiologic Instrument, San Diego, CA) with one of the solutions mentioned above. The bath solutions were bubbled with 95% O_2, 5% CO_2 (in the HCO_3 solution) or air (in the other solutions) at 37°C. The monolayers were continuously open-circuited to measure transepithelial potential differences (PD) by a voltage clamp amplifier (VCC MC2; Physiologic Instruments, San Diego, CA). Every 20 s we applied a 2 μA pulse for 0.5 s to the monolayer under open-circuit conditions so that we could calculate transepithelial conductance (Gt) from the change in the PD (ΔPD) using Ohm's law (Gt = 2 μA/ΔPD). When the Isc was measured, the PD was clamped to 0 mV by the amplifier. In accordance with previously reported procedures (10–12), we measured the ouabain (1 mM)-sensitive component of Isc to evaluate Na^+-K^+ pump activity under two conditions: in the presence or absence of 50 μM nystatin applied to the apical side, because the Na^+-K^+ pump activity is dependent on the Na^+ availability in the cytosolic space. Nystatin has been reported to create aqueous pores of about 4 Å radius in the lipid bilayer (14, 15). This pore size selectively enables monovalent cations, water, and small nonelectrolytes to permeate the membrane (14). Treatment with nystatin increases apical Na^+ conductance with the result that Na^+ extrusion by the Na^+-K^+ pump across the basolateral membrane becomes the rate-limiting step of transepithelial Na^+ transport (16): therefore, the treatment with nystatin enables us to measure the capacity of Na^+ extrusion by the Na^+-K^+ pump. In the absence of nystatin, ouabain-sensitive Isc may indicate the partial Na^+-K^+ pump activity stimulated by the limited Na^+ entry, but is thought to reflect the physiological state of the Na^+-K^+ pump activity. In Calu-3 cells, Na^+ entry across the apical membrane is mediated through the Na^+-glucose transporter instead of Na^+ channels. We estimated the Na^+ entry current by phlorizin (200 μM)-sensitive Isc. To assess the apical and basolateral conductance (G_a and G_b) of Cl^-, the NPPB-sensitive component of G_a or G_b was measured after permeabilizing the basolateral or apical membrane respectively with 100 or 50 μM nystatin.

Statistics. Data are expressed as means ± S.E.M. 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

Inhibitory effects of Cl^- channel blockers on the Na^+-K^+-pump activity. Isc increased after adding nystatin (50 μM) to the apical solution in the Calu-3 human airway cell line (Fig. 1). The Isc reached the nearly maximum level about 20 to 30 min after adding nystatin and maintained it for at least 50 min even when the

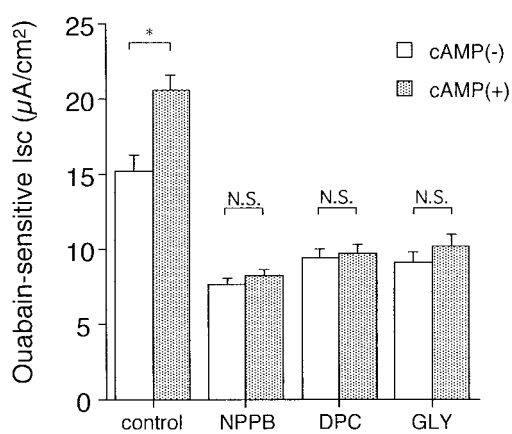

FIG. 2. Effects of several Cl^- channel blockers on the ouabain-sensitive Isc in the presence of 8-bromo-cyclic AMP (cAMP(+)) and its absence (cAMP(−)). In the presence of 1 mM 8-bromo-cyclic AMP applied to the basolateral membrane, the ouabain-sensitive Isc 50 min after the application of nystatin was significantly potentiated (n = 5), but the action was abolished by the application of NPPB (100 μM), DPC (1 mM), or glybenclamide (GLY, 300 μM) applied to the apical and basolateral solutions (n = 4). In these experiments, 8-bromo cyclic AMP, NPPB, DPC or GLY was applied 20 min after adding nystatin (50 μM), and another 30 min later, the ouabain-sensitive Isc was measured. Data are means ± S.E.M. (n = 4–5, $^*P < 0.05$).

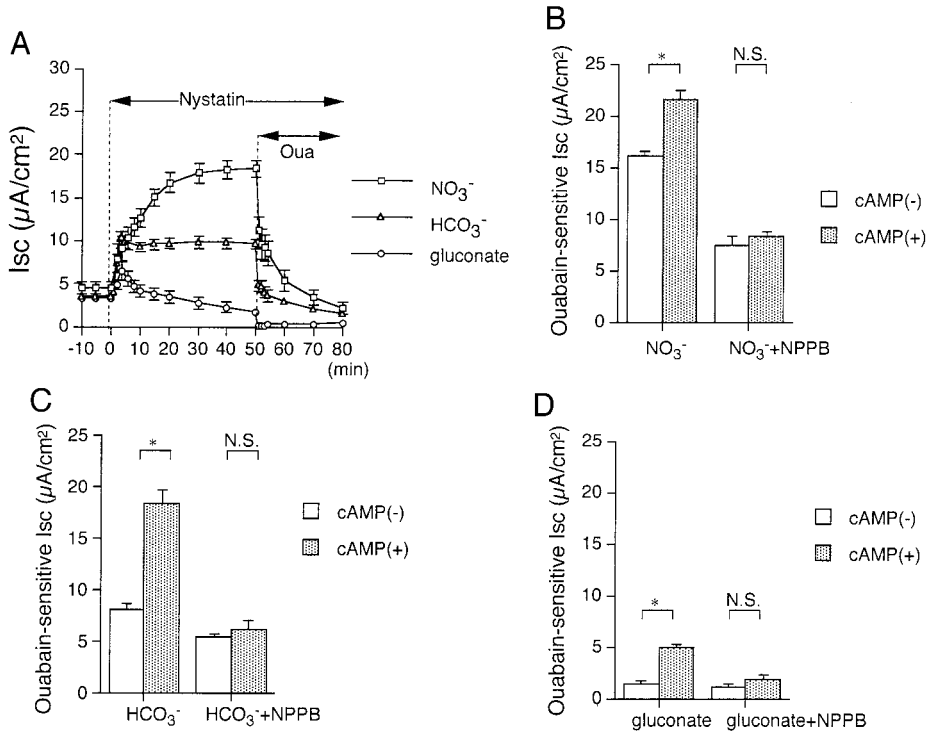

FIG. 3. Effects of anions substituted for Cl^- on the ouabain (Oua)-sensitive Isc in the presence of 8-bromo-cyclic AMP (cAMP(+)) and its absence (cAMP(−)). In the presence of alternative anions (NO_3^-, HCO_3^-, or gluconate) (A), nystatin (50 μM) was applied to the apical solution, and 50 min later the ouabain-sensitive Isc was measured. Application of 8-bromo-cyclic AMP (1 mM) to the basolateral solution potentiated the ouabain-sensitive Isc (B, C, D), which was suppressed by NPPB (100 μM) applied to the apical and basolateral solution. The timing of reagent application was the same as that in Fig. 2. Data are means ± S.E.M. (n = 4–6, $^*P < 0.05$).

solvent (0.1% DMSO) was applied to both the apical and basolateral solutions (Fig. 1A). The application of 1 mM ouabain to the basolateral solution totally abolished the nystatin-generated Isc, as previously had been shown (10). In the absence of any Cl^- channel blocker, the ouabain-sensitive component of the nystatin-generated Isc (called the "pump-current") obtained in the present study was 15.8 ± 0.9 μA/cm^2 (n = 5). When we applied 100 μM NPPB, a nonselective Cl^- channel blocker, to both the apical and basolateral solutions, however, nystatin-induced Isc showed a rapid decrease from 19.1 ± 1.9 to 8.5 ± 1.9 μA/cm^2 in 30 min with the result that significantly few ouabain-sensitive Isc remained (7.7 ± 0.8 μA/cm^2, n = 4, Fig. 1B). Similar results were obtained when 300 μM glybenclamide (a CFTR blocker) or 1 mM DPC (another nonselective Cl^- channel blocker) was applied: the remaining ouabain-sensitive Isc was 9.4 ± 0.6 μA/cm^2 (n = 6) or 9.1 ± 0.9 μA/cm^2 (n = 6), respectively (Figs. 1C and 2). In contrast, DNDS (500 μM) and DIDS (500 μM), ORCC blockers, applied to both sides did not affect the pump current (16.3 ± 1.1 μA/cm^2, Fig. 1D; 15.8 ± 1.0 μA/cm^2, n = 4).

Activation of CFTR and its effect on the Na^+-K^+-pump activity. To activate CFTR, 8-bromo-cyclic AMP (8Br-cAMP, 1 mM) was applied to the basolateral solution, resulting in potentiation of the pump current (20.5 ± 1.0 μA/cm^2, n = 5). In the presence of NPPB (100 μM), DPC (1 mM) or glybenclamide (300 μM), however, 8Br-cAMP failed to potentiate the pump current (8.2 ± 0.4, 9.7 ± 0.6, and 10.2 ± 0.7 μA/cm^2, n = 4, Fig. 2). These results were not significantly different from the suppressed pump current produced by these Cl^- channel blockers in the absence of 8Br-cAMP (7.7 ± 0.8, 9.4 ± 0.6, and 9.1 ± 0.9 μA/cm^2, n = 4).

Effects of replacement of Cl^- on the Na^+-K^+-pump activity. To investigate the role of Cl^- in the Na^+-K^+-pump activity, we replaced Cl^- with NO_3^-, which appeared to have equal permeability through the CFTR in Calu-3 (17). The pump current was unaffected by this replacement both in the absence of 8Br-cAMP (16.1 ± 0.4 μA/cm^2, n = 4) and in its presence (21.5 ± 0.9 μA/cm^2, n = 4, Figs. 3A and 3B). On the other hand, the replacement of all Cl^- with gluconate, a less membrane-permeant anion, greatly diminished the pump current (1.4 ± 0.4 μA/cm^2, n = 5, Fig. 3D), which was partially regained in the HCO_3 solution (8.1 ± 0.6 μA/cm^2, n = 6, Fig. 3C). Even in the gluconate and HCO_3 solution, 8Br-cAMP (1 mM) on the basolateral side augmented the pump current (18.4 ± 1.3 in HCO_3, and 4.9 ± 0.3 μA/cm^2 in gluconate solution, n = 4). Similar to the data in Fig. 2, however, treatment with

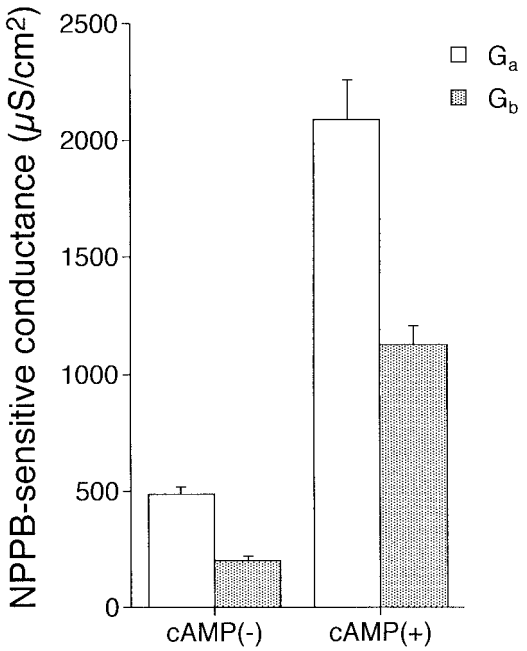

FIG. 4. Estimation of anion conductance of the apical and basolateral membrane in the presence of 8-bromo-cyclic AMP (cAMP(+)) and its absence (cAMP(−)). NPPB (100 μM)-sensitive conductance (anion conductance) was measured after permeabilizing either side of the membrane with 50 (to the apical) or 100 μM nystatin (to the basolateral) solution. The NPPB-sensitive components of the apical and basolateral conductance (G_a and G_b) were elevated by the application of 8-bromo cyclic AMP (1 mM) to the basolateral solution. Data are means ± S.E.M. (n = 5–6).

NPPB (100 μM) abolished the 8Br-cAMP-induced potentiation of the pump current (8.3 ± 0.5 in NO_3, 6.1 ± 0.9 in HCO_3, and 1.9 ± 0.5 μA/cm^2 in gluconate solution, n = 4), resulting in no significant difference from the suppressed pump current produced by NPPB in the absence of 8Br-cAMP (7.4 ± 0.8, 5.5 ± 0.3, and 1.2 ± 0.2 μA/cm^2, n = 4).

Measurement of Cl^- conductance of the apical and basolateral membrane. To estimate anion conductance of the apical or basolateral membrane, NPPB (100 μM)-sensitive conductance was measured after permeabilizing either side of the membrane with nystatin (Fig. 4). Application of nystatin at 100 μM to the basolateral membrane and at 50 μM to the apical membrane enabled us to measure G_a and G_b respectively (2, 12). Basal G_a and G_b had NPPB-sensitive components (485.8 ± 36.1 and 206.9 ± 18.2 μS/cm^2, n = 5–6) which were elevated in the presence of 1 mM 8Br-cAMP (2090.1 ± 167.0 and 1124.7 ± 80.6 μS/cm^2, n = 5–6). Neither DIDS- nor DNDS-sensitive conductance was detected in the presence and absence of 8Br-cAMP.

Ouabain- and phlorizin-sensitive Isc in the absence of nystatin. Finally, we comfirmed that CFTR was implicated in the Na^+-K^+ pump activity even under physiological conditions. The ouabain-sensitive Isc was measured without permeabilization of the cell membrane by nystatin (Fig. 5A). Under these conditions, the ouabain-sensitive Isc is considered to include the component of transepithelial Cl^- current, because Na^+-K^+ ATPase produces the driving force for the Na^+-K^+-2Cl^- cotransporter that contributes to the transepithelial Cl^- transport. Accordingly, we measured the ouabain-sensitive Isc in the presence of bumetanide (50 μM), a blocker of the Na^+-K^+-2Cl^- cotransporter. Under the physiological conditions, 8Br-cAMP (1 mM) applied to the basolateral solution failed to potentiate the ouabain-sensitive Isc (from 2.2 ± 0.2 to 2.6 ± 0.2 μA/cm^2, n = 5). Further, NPPB (100 μM) had no effect on the ouabain-sensitive Isc both in the presence of 8Br-cAMP (2.3 ± 0.1 μA/cm^2, n = 5) and its absence (2.3 ± 0.2 μA/cm^2, n = 5). Under the normal conditions, NPPB and 8br-cAMP also did not affect the

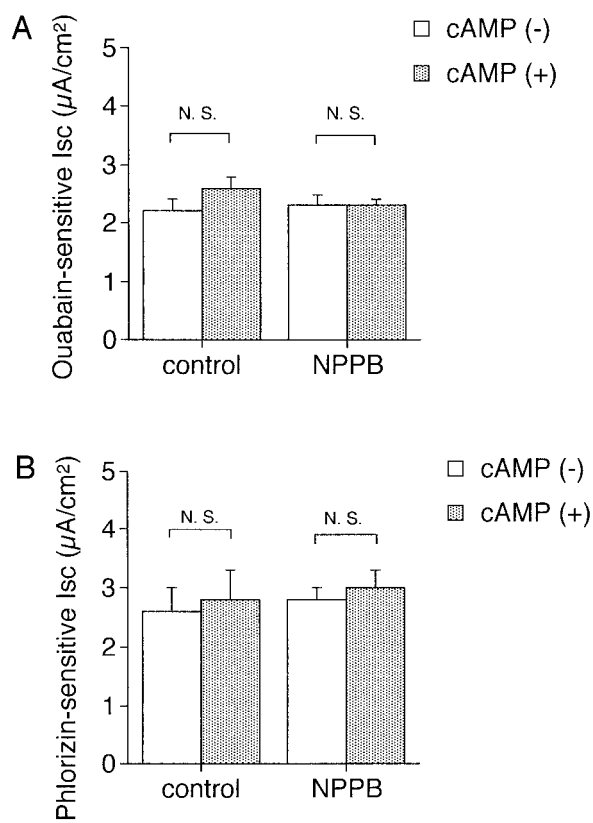

FIG. 5. Effects of NPPB, a Cl^- channel blocker on the ouabain- and phlorizin-sensitive Isc in the presence of 8-bromo-cyclic AMP (cAMP(+)) and its absence (cAMP(−)) under physiological conditions (no nystatin permeabilization). Application of 8-bromo-cyclic AMP (1 mM) in the basolateral solution failed to potentiate the (A) ouabain- and (B) phlorizin-sensitive Isc, and application of NPPB (100 μM) both in the apical and basolateral solutions had no effect on the Isc. In these experiments, 8-bromo-cyclic AMP and NPPB were applied 30 min before adding ouabain (1 mM) or phlorizin (200 μM). Every experiment was pretreated with 50 μM bumetanide. Data are means ± S.E.M. (n = 5–7, *P < 0.05).

phlorizin-sensitive Isc that reflects the Na^+ entry across the apical membrane in Calu-3 cells (Fig. 5B).

DISCUSSION

It has been believed that Na^+-K^+ pump activity is maintained by Na^+, ATP, Mg^{2+} from inside, and by K^+ from outside the basolateral membrane (18). However, we here provide new evidence that CFTR is also one of the regulators to the Na^+-K^+-pump activity in Calu-3 human lung cells. Calu-3 has been reported to possess at least two distinct Cl^- channels: CFTR and ORCCs. CFTR is almost exclusively detected in cell-attached and whole-cell patch clamp studies from confluent cells, and ORCCs are only found in the non-confluent cells or after the tight junctions are disrupted (19). DNDS and DIDS (100–500 μM) have been shown to block ORCCs without any effect on CFTR (20, 21). In the present study using a monolayer of confluent cells, three kinds of Cl^- channel blockers sensitive to CFTR (NPPB, DPC, and glybenclamide) reduced the Na^+-K^+-pump activity, but ORCC blockers (DNDS, DIDS) did not, suggesting that CFTR contributed exclusively to Cl^- conductance to maintain the Na^+-K^+-pump activity in polarized Calu-3 cells. The permeabilized cells of either side of the membrane also revealed that both apical and basolateral membranes seem to have cAMP-stimulated conductance that is insensitive to DNDS and DIDS, although both CFTR and ORCCs are believed to be activated by cAMP (22). These results suggest that ORCCs are unlikely to be present even on the basolateral membrane in the confluent Calu-3 cells.

To further confirm the role of CFTR in the Na^+-K^+-pump activity, we replaced Cl^- with other anions of different permeabilities, since CFTR has been reported to conduct various kinds of anions like NO_3^- and HCO_3^- as well as Cl^- in Calu-3 (17). The permeation ratio of Cl^-:NO_3^-:HCO_3^- has been demonstrated to be 1.0:0.8–1.0:0.15–0.20 (17, 23). We also used gluconate as a less permeant anion through CFTR (12). Under the cAMP-unstimulated condition, the replacement of Cl^- with NO_3^- did not affect the pump current, but substituting gluconate markedly diminished the current. In the HCO_3 solutions, the pump current was smaller than that in the NO_3 solutions and larger than that in gluconate solutions. These results suggest that anion permeability through CFTR would, at least, be the rate-limiter of the Na^+-K^+-pump activity.

Several reports have shown that cAMP modulated the Na^+-K^+-ATPase α-subunit (catalytic subunit) by its phosphorylation and/or its increased numbers on the basolateral membrane in distal lung cells (24, 25). However, we here demonstrated that cAMP would, at least in part, augment the Na^+-K^+-pump activity through an increase in anion conductance through CFTR in the Calu-3 human airway cell line, since the augmentation of the ouabain-sensitive Isc produced by 8Br-cAMP was abolished by blocking CFTR in the solutions containing CFTR-permeant anions in the presence of nystatin.

Movement of monovalent cations (Na^+, K^+) and Cl^- across the membrane to couple with each other maintains intracellular electrical neutrality and regulates cell volume (26, 27, 28). In the present study, the rate of Na^+ entry may be limited by the rate at which Cl^- can enter the cells in the presence of nystatin that allows only monovalent cations to pass. Namely, when the anion conductance through CFTR increases, the rate of Na^+ entry is thought to be up-regulated, allowing the Na^+-K^+ pump to extrude Na^+ at a high rate. For the same reason, when the anion conductance is reduced by the CFTR inhibitors and less permeant anions, the Na^+ entry rate is thought to fall, resulting in a decrease in Na^+ extrusion by the Na^+-K^+ pump. On the other hand, the Cl^- entry seems unlikely to be the rate-limiter of Na^+ entry under physiological conditions in Calu-3 cells because both blockade and activation of CFTR had no effect on the ouabain- and phlorizin-sensitive Isc in the absence of nystatin. In several epithelia possessing Na^+ permeant channels, however, it has been shown that an increase in Na^+ transport induced by hyposomlality or forskolin was inhibited by Cl^- channel blockers without nystatin permeabilization (12, 27). Therefore, it may suggests that Cl^- movement through CFTR is crucial for the potentiation of the Na^+ absorption.

It is generally known that apical CFTR greatly contributes to Cl^- secretion in the mucosal and submucosal gland cells of the lung. The basolateral membrane in various kinds of epithelial cells is also reported to have anion conductance (12, 29). Permeabilizing either side of the membrane in the present study suggested that the basolateral membrane may also have anion pathways predominantly through CFTR in the Calu-3 cell line, consequently being involved in the Na^+-K^+ pump activity.

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