

Endogenous ATP release inhibits electrogenic Na^+ absorption and stimulates Cl^- secretion in MDCK cells

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Received: 7 December 2006 / Accepted: 2 February 2007 / Published online: 8 March 2007
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Abstract Our previous studies with a line of Madin-Darby canine kidney (MDCK) cells (FL-MDCK) transfected with FLAG-labeled α , β , and γ subunits of epithelial Na^+ channel (ENaC) showed that, although most of the short-circuit current (I_{sc}) was amiloride sensitive (AS- I_{sc}), there was also an amiloride-insensitive component (NS- I_{sc}) due to Cl^- secretion (Morris and Schafer, *J Gen Physiol* 120:71–85, 2002). In the present studies, we observed a progressive increase in NS- I_{sc} and a corresponding decrease in AS- I_{sc} during experiments. There was a significant negative correlation between AS- I_{sc} and NS- I_{sc} both in the presence and absence of treatment with cyclic adenosine monophosphate (cAMP). NS- I_{sc} could be attributed to both cystic fibrosis transmembrane conductance regulator (CFTR) and a 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS)-sensitive Ca^{2+} -activated Cl^- channel (CaCC). Continuous perfusion of both sides of the Ussing chamber with fresh rather than recirculated bathing solutions, or addition of hexokinase (6 U/ml), prevented the time-dependent changes and increased AS- I_{sc} by 40–60%, with a proportional decrease in NS- I_{sc} . Addition of 100 μM adenosine

triphosphate (ATP) in the presence of luminal amiloride produced a transient four-fold increase in NS- I_{sc} that was followed by a sustained increase of 50–60% above the basal level. ATP release from the monolayers, measured by bioluminescence, was found to occur across the apical but not the basolateral membrane, and the apical release was tripled by cAMP treatment. These data show that constitutive apical ATP release, which occurs under both basal and cAMP-stimulated conditions, underlies the time-dependent rise in Cl^- secretion and the proportional fall in ENaC-mediated Na^+ absorption in FL-MDCK cells. Thus, endogenous ATP release can introduce a significant confounding variable in experiments with this and similar epithelial cells, and it may underlie at least some of the observed interaction between Cl^- secretion and Na^+ absorption.

Keywords ATP · CFTR · CLC · Calcium-activated Cl^- channel · ENaC · Purinergic effects

Introduction

Madin-Darby canine kidney (MDCK, type I) cells grown as epithelial monolayers have been used extensively as a model of the mammalian collecting duct (CD). These monolayers exhibit many characteristics of the CD, including a high transepithelial resistance and amiloride-sensitive Na^+ reabsorption that is mediated by the epithelial Na^+ channel (ENaC) and stimulated by agonists that increase intracellular cyclic adenosine monophosphate (cAMP) [1, 2]. Similar cell lines that are derived from the CD, including mouse cortical CD (M-1) and inner medullary CD (mIMCD-K2) cells [3–5] and A6 cells from the *Xenopus* distal nephron [6, 7] share these characteristics. However, in contrast to the CD, all of these cell lines also have Cl^- channels in their apical membranes, and,

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when studied under short-circuit conditions, exhibit Cl^- secretion, which is observed as an amiloride-insensitive component of the short-circuit current ($\text{NS-}I_{\text{sc}}$) that is also stimulated by cAMP [2, 3, 5–8].

We previously developed a line of MDCK cells (FL-MDCK) that had been retrovirally transfected with rat ENaC subunits containing the FLAG epitope in their extracellular loops [2]. Monolayers of these cells had higher rates of ENaC-mediated Na^+ absorption, measured as the amiloride-sensitive short-circuit current ($\text{AS-}I_{\text{sc}}$), than did the original MDCK cell line. Our previous studies with FL-MDCK monolayers in Dulbecco's modified Eagle's medium (DMEM) showed that cAMP treatment produced a rapid transient peak in the total short-circuit current (I_{sc}) within 5 min, followed by a broad peak that decayed over 20 min [2]. This biphasic response to cAMP treatment has also been reported in A6 and M-1 cultures and has been attributed to rapid stimulation of Cl^- secretion mediated by cystic fibrosis transmembrane conductance regulator (CFTR) followed by a slower activation of ENaC [6–9]. In FL-MDCK cells, the biphasic response to cAMP treatment was followed by a decay of I_{sc} , which reflected a decrease in $\text{AS-}I_{\text{sc}}$ [2]. Morris and Schafer [2] found that this late fall in $\text{AS-}I_{\text{sc}}$ in FL-MDCK monolayer was prevented by the omission of Cl^- from the bathing solution and attributed the effect to Cl^- secretion via CFTR, which has subsequently been demonstrated to be present in this cell line [10].

A large body of evidence indicates that CFTR is a cAMP-regulated Cl^- channel as well as a conductance regulator, which is colocalized with ENaC in airway, colonic, and other epithelial tissues [11, 12]. It has been proposed that when cAMP activates CFTR, ENaC is inhibited, and this inhibitory effect of CFTR has been hypothesized to explain the pathophysiology of cystic fibrosis in airway epithelia [13, 14]. In *Xenopus* oocytes expressing ENaC, coexpression of CFTR reduces the ENaC-mediated Na^+ current [15], an effect that might be attributed to a direct effect of CFTR on ENaC activity. However, Kunzelman and his collaborators [16, 17] have shown that the inhibitory effect of CFTR can be mimicked by coexpression of other anion channels or treatment with amphotericin B and is explained by an increase in intracellular Cl^- ($[\text{Cl}^-]_i$). We have shown that an increase in $[\text{Cl}^-]_i$ also inhibits ENaC in FL-MDCK cells; however, this effect cannot explain the effect of Cl^- secretion on Na^+ absorption with cAMP treatment, because stimulation of Cl^- secretion by cAMP results in a fall rather than a rise in $[\text{Cl}^-]_i$ [10]. Moreover, in the *Xenopus* oocyte expression system, CFTR activation inhibits ENaC at a continuous holding potential at which CFTR mediates inward currents corresponding to Cl^- efflux [18]. Under these conditions, activation of CFTR will not result in an increase in $[\text{Cl}^-]_i$. Therefore, a change in $[\text{Cl}^-]_i$ is unlikely to fully explain the observed reciprocal regulation of ENaC and CFTR.

In the present studies, we show that adenosine triphosphate (ATP) accumulation in the apical solution produces a progressive stimulation of $\text{NS-}I_{\text{sc}}$ and a corresponding decrease in $\text{AS-}I_{\text{sc}}$ when FL-MDCK cells are studied in Ussing chambers. The possibility of constitutive ATP release raises an important issue when interpreting changes in ion transport in experiments such as these. More important in the present context, ATP release can explain at least part of the inverse relationship between Cl^- secretion and Na^+ reabsorption, and, as suggested by Wilson et al. [19], it may be involved in the conversion of CD cells from absorptive to secretory in polycystic kidney disease.

Materials and methods

Cell culture

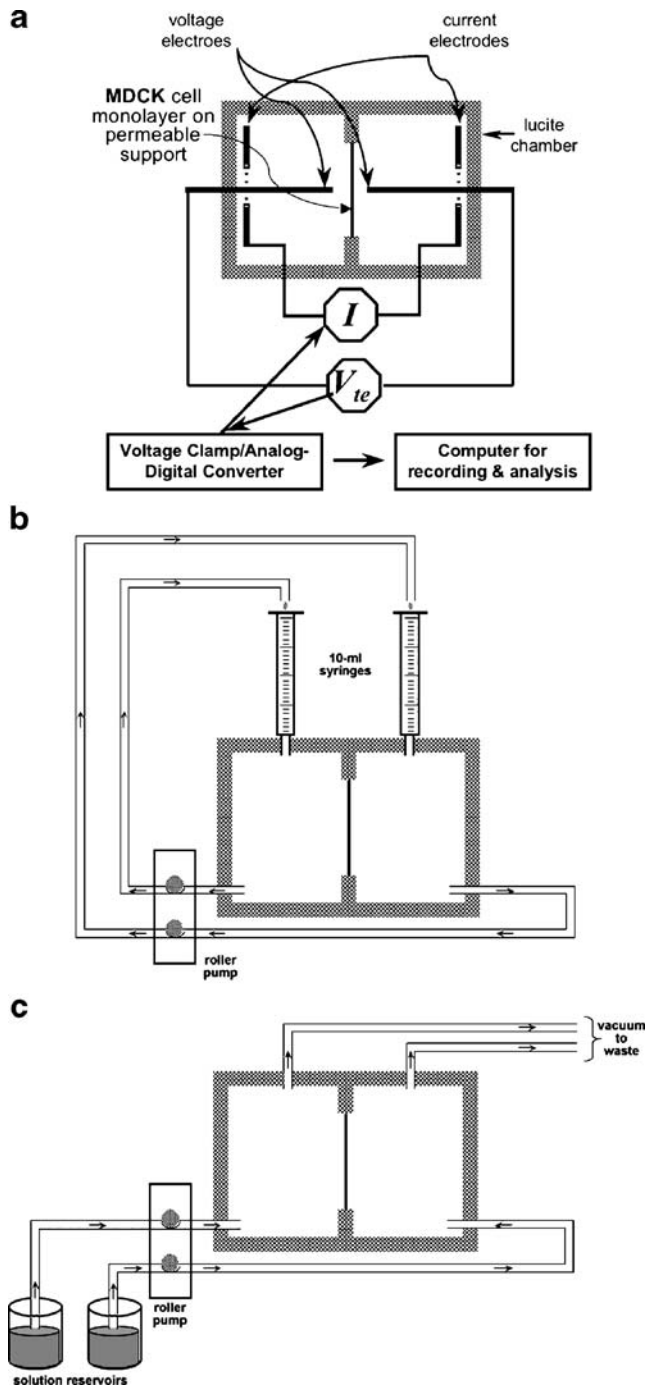
The FL-MDCK cells used were a clone of the type-1 MDCK line that had been retrovirally transfected with flagged rat α -, β -, and γ ENaC by Morris and Schafer [2]. The cells used were from passages 5–35 and were cultured in T-75 flasks at 37°C in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4), 1% Pen/strep-fungizone, and the selection antibiotics G418 (800 $\mu\text{g}/\text{ml}$), hygromycin (300 $\mu\text{g}/\text{ml}$), and puromycin (5 $\mu\text{g}/\text{ml}$). For transepithelial transport studies, cells were seeded onto 24-mm Transwell inserts (Costar; Catalog No. 3412) at a density of $\sim 10^5$ cells/ cm^2 . For bioluminescence assays, cells were seeded on 12-mm collagen-coated Millicell inserts (Millipore; Catalog No. PHIP 012 50) at the same density. After seeding, cells were fed daily with DMEM containing FBS and HEPES but without selection antibiotics for 5–7 days. Before use in the experiments, the cell monolayers were induced with 1 μM dexamethasone plus 2 mM Na^+ butyrate in the culture medium overnight.

Ussing chamber experiments

FL-MDCK monolayers were carefully cut from the plastic insets and were mounted in Ussing-type chambers. In most experiments, both sides of the monolayers were bathed with Krebs-Ringer bicarbonate (KRB) solution, which contained (in mM): 113 NaCl, 1.2 Na_2HPO_4 , 25 NaHCO_3 , 1.1 CaCl_2 , 1.2 MgCl_2 , 4.5 KCl, and 10 glucose. The KRB solutions were continuously gassed with a mixture of 95% O_2 -5% CO_2 to give a pH of 7.40 at 37°C. As shown by the schematic diagram in Fig. 1a, transepithelial short-circuit currents (I_{sc} , $\mu\text{A}/\text{cm}^2$) and voltages (V_{te}) were measured and conductances were evaluated every 30 s by measuring the current deflections induced by 4-s symmetrical voltage pulses

of ± 2 mV, as described previously [2, 10]. In most experiments, KRB was continuously recirculated on both sides of the epithelium from 10-ml reservoirs (Fig. 1b); however, in the experiments involving “fresh perfusion,” as shown in Fig. 1c, both sides of the monolayers were continuously perfused with fresh rather than recirculated KRB. In those experiments involving cAMP treatment, 100 μ M 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) plus 100 μ M isobutylmethylxanthine (IBMX) were added to both the apical

and basolateral solutions. $AS-I_{sc}$ was defined as the change in I_{sc} produced by the addition of 10 μ M amiloride to the apical solution, and the remaining I_{sc} was defined as $NS-I_{sc}$. Because we observed time-dependent changes in $AS-I_{sc}$ and $NS-I_{sc}$ in the voltage clamp experiments, we conducted additional experiments in which the monolayers were left under open-circuit conditions during the course of the experiment, and the open-circuit transepithelial voltage (V_{te} , mV) was measured continuously except for intermittent voltage clamping to 0 mV and ± 2 mV for a total of 4 s every 30 s to obtain I_{sc} and conductance measurements.



Bioluminescence detection of ATP release

ATP released from the MDCK monolayers was measured as described by Taylor et al. [20]. Briefly, culture medium was removed from the inserts, and the cell monolayers were washed three times with phosphate-buffered saline (PBS) solution to remove any FBS present in the culture medium. Opti-minimal essential medium (MEM) with 2 mg/ml luciferin-luciferase reagent (Sigma) was added to the apical or basolateral side of the monolayer. Each insert with cells contained 200 μ l of solution, and the average light signal was measured for 10-s, nonintegrated photon collection periods with a TD-20/20 luminometer (Turner Designs; Promega). Treatment with cAMP was applied by adding 1 μ l of 20 mM CPT-cAMP and 20 mM IBMX stock solution to the 200 μ l apical solution of the inserts (final concentration 100 μ M for both), and control experiments were done with addition of the same volume of vehicle alone. The light output was measured for at least 2 min, and the average was taken for the calculation of ATP release.

Fig. 1 **a** Schematic diagram of the electrophysiological circuitry. The Ussing chamber is constructed of two lucite half-chambers, which are clamped together with the Madin-Darby canine kidney (MDCK) cell monolayer (on a Transwell membrane) between them. Voltage-sensing electrodes are positioned close to the center and on either side of the monolayer. Current passing electrodes, made from silver disks with a central hole to accommodate the voltage electrodes, are placed at the back of each half chamber and parallel to the monolayer. The transepithelial voltage (V_{te}) is measured by the voltage clamp/analog to digital converter (called “the clamp”). When no current is being passed, the voltage recorded is called the “open circuit” V_{te} . To short-circuit the epithelial monolayer, the clamp passes a current (I) that is sufficient to drive V_{te} to zero. The current measured in this situation is called the short-circuit current (I_{sc}). **b** In experiments in which the Krebs-Ringer bicarbonate solutions (KRB) in the Ussing chambers were recirculated, 10-ml syringes were used as reservoirs, and a roller pump was used to continuously move the KRB from each half-chamber to its respective reservoir. **c** In experiments in which fresh KRB solutions were continuously perfused, large beakers served as the solution reservoirs from which KRB was constantly infused into each half-chamber by a roller pump and constantly removed by suction to a waste reservoir. In both the recirculation and fresh perfusion methods, the solutions in the reservoirs (10-ml syringes or beakers, respectively) were constantly bubbled with a 95% O_2 -5% CO_2 gas mixture

Individual batches of luciferin-luciferase reagent were tested for relative sensitivity by measuring the light output response to serial dilutions of an ATP stock solution with 2 mg/ml luciferin-luciferase reagent in Opti-MEM.

Data analysis and statistics

StatView for Macintosh (SAS Institute Inc.) was used for statistical analyses. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) with Bonferroni/Dunn post hoc testing for multiple comparisons and by paired or nonpaired t -tests, as appropriate, for single comparisons. Time dependence of transport was evaluated by linear regression analysis; the R value and significance of the slope are given. Correlation analysis was used to compare AS- I_{sc} and NS- I_{sc} and calculate a correlation coefficient, r .

Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Results

Time-dependent changes in I_{sc}

The I_{sc} in FL-MDCK cells was quite high and variable in the first 5 min after recording was begun using continuous short-circuiting. In six experiments, 10 μ M amiloride added to the apical solution in the initial 5 min decreased I_{sc} by $91 \pm 2\%$, indicating that most of the current was due to ENaC-mediated Na^+ absorption. The initial high I_{sc} subsequently declined to a nadir at ~ 15 min (Fig. 2a and b). Addition of amiloride at this nadir (Fig. 2a) almost completely inhibited I_{sc} ; however, when the experiment was continued beyond the nadir, amiloride was less effective in reducing I_{sc} (Fig. 2b). In 35 experiments such as those in Fig. 2a and b, the time interval between the nadir and amiloride addition to the apical solution was varied from 0 to 45 min. In these experiments, the average I_{sc} at the nadir was $15.2 \pm 0.5 \mu\text{A}/\text{cm}^2$. Regression analysis showed that NS- I_{sc} increased significantly ($R = 0.76$, $P < .001$) and AS- I_{sc} decreased significantly ($R = 0.53$, $P < .002$) as a function of the time between the nadir and the addition of amiloride. The same high initial I_{sc} and the time-dependent increase of NS- I_{sc} were also observed in experiments using the intermittent short-circuit technique (see “Materials and methods”), which indicated that neither effect is produced by prolonged short-circuiting of the epithelium (data not shown).

As shown in Fig. 3, the effect of cAMP on I_{sc} also depended on whether the treatment occurred early (at the nadir of I_{sc} , Fig. 3a) or late (~ 40 min after the nadir,

Fig. 3b). With both early and late cAMP addition, I_{sc} increased immediately, reaching a transient peak within 5 min; however, the magnitude of this initial peak was exaggerated with late cAMP treatment (Fig. 3b). After the initial response, I_{sc} showed a secondary broader increase that decayed more rapidly after late (Fig. 3b) than after early (Fig. 3a) cAMP treatment. In 29 experiments such as those in Fig. 3, the time interval between the nadir in I_{sc} and cAMP treatment was varied from 2 to 58 min, and 10 μ M amiloride was added ~ 30 min after cAMP treatment. Regression analysis showed a significant increase in NS- I_{sc} with the time between the nadir and cAMP treatment ($R = 0.60$, $P < .002$).

Further examination of the data from these experiments (see Fig. 4) revealed a significant negative correlation

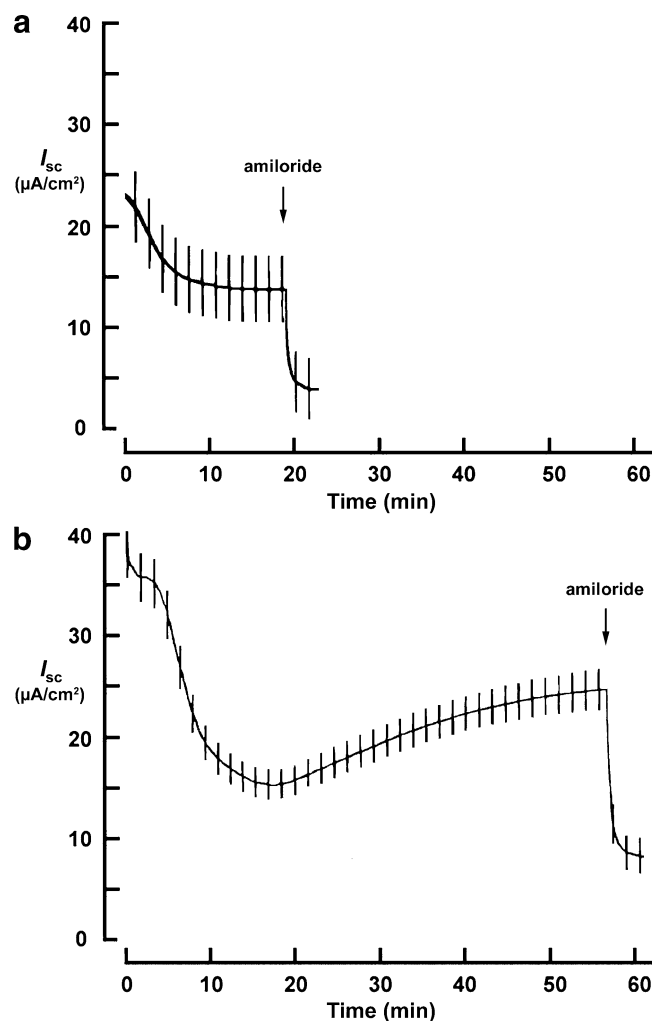


Fig. 2 Time course of the short-circuit current (I_{sc}) and response to amiloride. I_{sc} was measured across monolayers of FL-Madin-Darby canine kidney (FL-MDCK) cells induced overnight with 2 mM Na butyrate and 1 μM dexamethasone. Amiloride (10 μM) was added to the apical solution either early (a) or late (b) at the point indicated by the arrow. The transepithelial conductance (G_{te}) is proportional to the height of the deflections in I_{sc} produced by transiently clamping the voltage to ± 2 mV, as described in “Materials and methods”

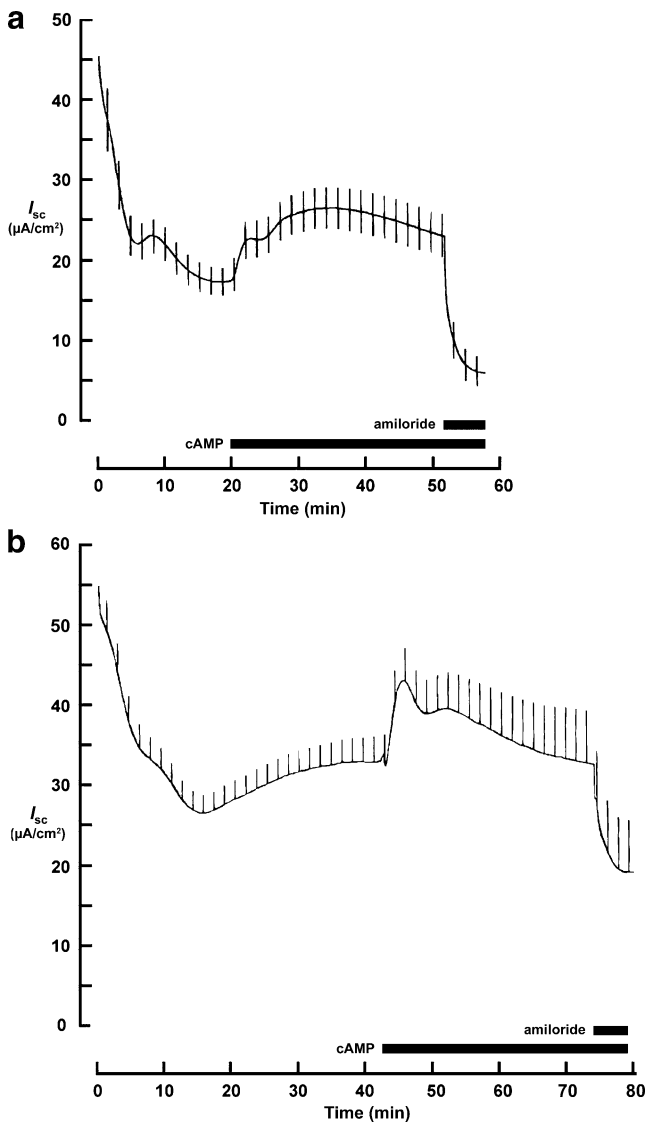


Fig. 3 Time course of I_{sc} response to cyclic adenosine monophosphate (cAMP) treatment. A mixture of 100 μM 8-(4-chlorophenylthio) (CPT)-cAMP plus 100 μM isobutylmethylxanthine (IBMX) was added as indicated by the lower bar, either early (a) or late (b) to both sides of the monolayers, followed by 10 μM amiloride addition to the apical solution only (upper bar)

between AS- I_{sc} and NS- I_{sc} , both with and without cAMP treatment. In other words, the time-dependent increase in NS- I_{sc} observed in both sets of experiments was correlated with a corresponding decrease in AS- I_{sc} . As expected, the average AS- I_{sc} was significantly greater in those experiments with cAMP treatment ($20.2 \pm 1.1 \mu\text{A}/\text{cm}^2$, $n=29$) than in those without (12.8 ± 0.6 , $n=35$), but the correlation with NS- I_{sc} was not significantly different between the two groups.

Effect of cAMP and intracellular Ca^{2+} on NS- I_{sc}

In the experiments shown in Fig. 5, NS- I_{sc} was measured throughout the experiments in the continuous presence of

10 μM amiloride in the apical solution. Treatment with cAMP produced an initial rapid peak in NS- I_{sc} , followed by a sustained plateau. We then tested the effects of two inhibitors of Cl^- transport pathways: glibenclamide, which is a well-established inhibitor of CFTR; and 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS), which is a less selective inhibitor of Cl^- channels including the Ca^{2+} -activated chloride channel (CaCC) and the chloride channel (CLC) family of Cl^- channels but not CFTR. (See the Discussion for more detail about these inhibitors and references to their specificity.) Addition of 200 μM glibenclamide followed by 300 μM DIDS to the apical solution ~20 min after cAMP treatment significantly decreased NS- I_{sc} to or below the magnitude prior to cAMP treatment (Fig. 5a & b). In other experiments, addition of apical DIDS before cAMP treatment reduced the late plateau but not the initial peak of NS- I_{sc} (Fig. 5c and e), whereas apical glibenclamide significantly decreased the initial peak, with no effect on the late plateau (Fig. 5d & e). These data suggest that at least two types of Cl^- channels are stimulated by cAMP. One is sensitive to glibenclamide and

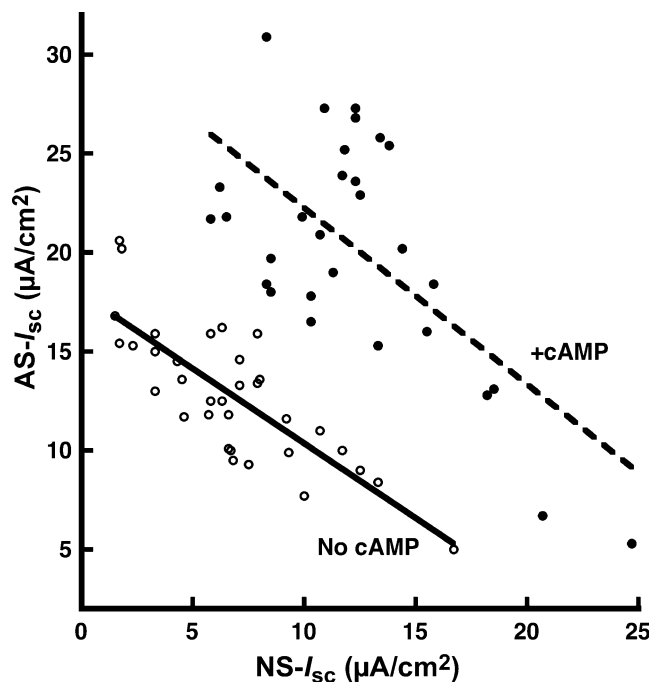


Fig. 4 Negative correlation between the amiloride-sensitive component of the short-circuit current (I_{sc}) (AS- I_{sc}) and the nonamiloride-sensitive component (NS- I_{sc}) in the absence and presence of cyclic adenosine monophosphate (cAMP). The 35 experiments in the first group (open circles, solid line) were conducted using the same protocol as the representative experiments in Fig. 1 with no cAMP addition. The 29 experiments in the second group (solid dots, dashed line) followed the protocol of the representative experiments in Fig. 2, with the addition of 100 μM 8-(4-chlorophenylthio) (CPT)-cAMP plus 100 μM isobutylmethylxanthine (IBMX) ~30 min before the addition of amiloride. In both groups, there was a significant correlation between AS- I_{sc} and NS- I_{sc} : -cAMP group, $r=0.79$, $P < .001$; +cAMP group, $r=0.65$, $P < .001$

responds to cAMP quickly while the other is sensitive to DIDS and responds to cAMP slowly.

The Cl^- channel that was quickly activated by cAMP and inhibited by glibenclamide has been attributed to CFTR, the expression and function of which has been identified by us in this same cell line [10]. Because cAMP increases intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in MDCK cells [21], we examined the hypothesis that CaCC contributes to the fraction of NS- I_{sc} that is inhibited by DIDS by using thapsigargin to produce a modest but sustained elevation of $[\text{Ca}^{2+}]_i$ [22, 23]. (Thapsigargin is known to elevate intracellular Ca^{2+} by releasing it from the endoplasmic reticulum [22].) In the experiments shown in Fig. 6, NS- I_{sc} was measured in the continuous presence of luminal amiloride. The addition of 1 μM thapsigargin to both sides of the monolayer produced a transient spike followed by a

sustained increase in NS- I_{sc} , most of which was sensitive to DIDS (Fig. 6a). When 300 μM DIDS was added to apical solution before thapsigargin, it almost completely blocked the response of NS- I_{sc} to thapsigargin (Fig. 6b).

Effect of Ussing chamber perfusion on time-dependent changes in I_{sc}

In all of the experiments presented up to this point, the KRB solutions that bathed the MDCK monolayers were recirculated as shown in Fig. 1b. To examine whether the time-dependent changes in AS- I_{sc} and NS- I_{sc} might be caused by the accumulation of a secretagogue or metabolite in these recirculated solutions, we conducted paired experiments in which one Ussing chamber was perfused with recirculated KRB solutions and the other chamber was

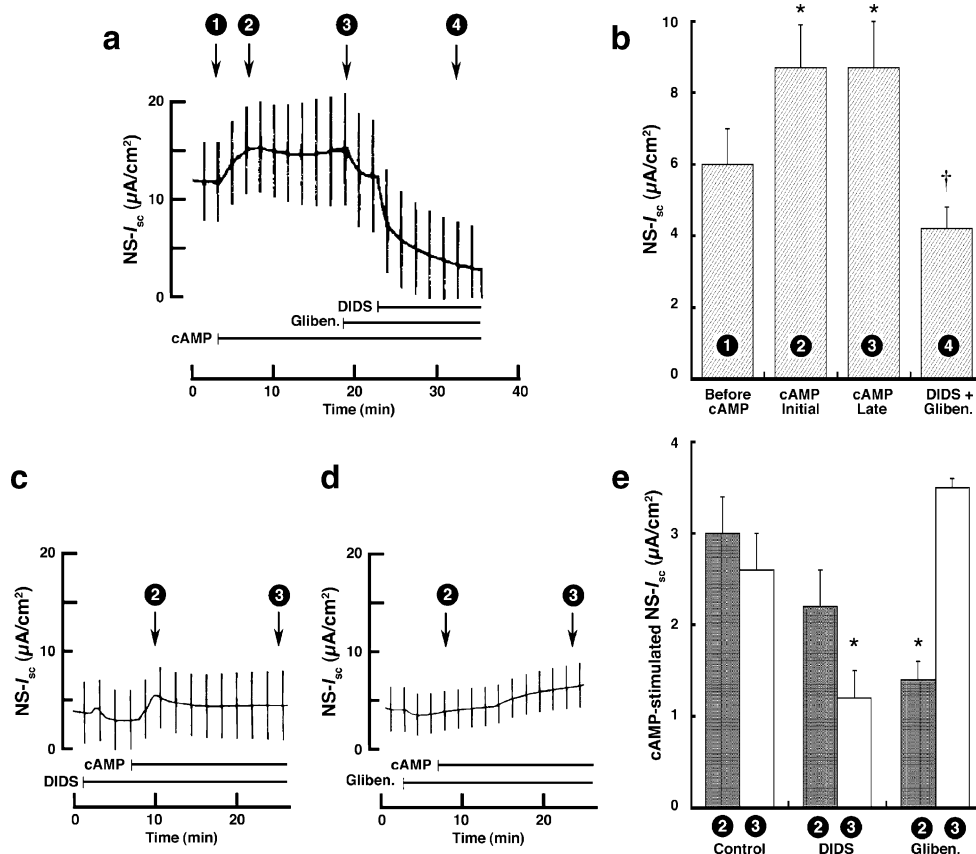


Fig. 5 Effects of glibenclamide and 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS) on the nonamiloride-sensitive component (NS- I_{sc}). In these experiments, 10 μM amiloride was continuously present in the apical solution. **a** Effect of cyclic adenosine monophosphate (cAMP) treatment [100 μM cAMP plus 100 μM isobutylmethylxanthine (IBMX), lowest bar] on NS- I_{sc} . The middle and upper bars indicate the time of addition of 300 μM DIDS and 200 μM glibenclamide (Gliben.) to the apical solution. **b** Summary of 14 experiments such as that in **a**. The mean NS- I_{sc} values immediately before cAMP treatment (point 1 in **a**), 3–5 min ("initial", point 2) and 15–20 min ("late", point 3, just before DIDS or glibenclamide addition) after cAMP treatment, and after DIDS plus glibenclamide

(point 4). * Significantly different from value before cAMP treatment, $P < .001$; † significantly different from the initial and late NS- I_{sc} , $P < .001$. **c** Effect of 300 μM apical DIDS added before cAMP treatment. **d** Effect of 200 μM apical glibenclamide added before cAMP treatment. **e** Mean values of the change in NS- I_{sc} (the cAMP-stimulated NS- I_{sc}) produced by cAMP treatment in the initial (point 2 in **a**, **c**, and **d**) or late (point 3) response period in three sets of experiments: with no inhibitor (control, from summary of 15 experiments in **b**), DIDS before cAMP (as in **c**, $n=6$), and glibenclamide before cAMP (as in **d**, $n=5$). * Significant difference compared with the corresponding control value (calculated from the data in **b**), and between initial and late response to cAMP, $P < .001$

continuously perfused with fresh KRB solutions, as shown in Fig. 1c. The representative control experiment shown in Fig. 7a was conducted in the usual way: the apical and basolateral KRB solutions were recirculated through both sides of the Ussing chamber from 10-ml reservoirs. In the paired experiment in Fig. 7b, we continuously perfused fresh KRB on both sides of the Ussing chamber. In both the recirculation and fresh perfusion experiments, cAMP treatment produced an initial transient spike of I_{sc} lasting less than 6 min, followed by a broader peak; however, I_{sc} decayed more rapidly in the recirculation experiment than it did in the fresh perfusion experiment. In four sets of paired experiments summarized in Fig. 7c, $AS-I_{sc}$ was greater ($39.8 \pm 1.1 \mu A/cm^2$ vs. $25.2 \pm 3.3 \mu A/cm^2$, $P < 0.01$) and $NS-I_{sc}$ was less ($2.8 \pm 0.2 \mu A/cm^2$ vs. $7.7 \pm 1.6 \mu A/cm^2$, $P < 0.01$) with fresh perfusion than in the recirculation group, suggesting that some substance, which inhibited $AS-I_{sc}$ and stimulated $NS-I_{sc}$, was accumulating in the recirculated bathing solution.

Effect of extracellular ATP on time-dependent changes in I_{sc}

We hypothesized that ATP was released by the FL-MDCK cells and accumulated in the recirculated solutions. Extracellular nucleotides are known to act as autocrine and paracrine agents that affect Na^+ absorption and Cl^- secretion, and ATP has been shown to increase intracellular $[Ca^{2+}]$ [24, 25], which might augment the Ca^{2+} -activated component of $NS-I_{sc}$ as well as inhibit ENaC activity. As shown in Fig. 8a, in the presence of amiloride, the addition of 100 μM ATP to the apical solution increased $NS-I_{sc}$, and, as shown in Fig. 8b, this effect was almost completely abolished when the ATP was added together with hexokinase as an ATP scavenger [26]. (These experiments were conducted using the recirculated KRB method.) In five experiments such as that in Fig. 8a, basal $NS-I_{sc}$ (just before ATP addition) averaged $4.1 \pm 0.1 \mu A/cm^2$. With the addition of ATP, $NS-I_{sc}$ reached a peak value of $15.4 \pm 2.2 \mu A/cm^2$ ($P < 0.004$) within 2–3 min, and then declined gradually to a plateau of $5.9 \pm 0.3 \mu A/cm^2$, which was still significantly larger than the basal level ($P < 0.01$). Addition of 300 μM DIDS to the apical solution before the addition of ATP had no effect on the peak amplitude ($14.6 \pm 0.7 \mu A/cm^2$, $n=3$), but decreased the plateau value to $4.5 \pm 0.2 \mu A/cm^2$ ($n=3$), which was not significantly different from the basal value.

To test whether endogenous ATP production by the epithelium might be involved in the time-dependent increase of $NS-I_{sc}$ and decrease of $AS-I_{sc}$, we examined the effect of hydrolyzing extracellular ATP with hexokinase on the time course of $NS-I_{sc}$ and $AS-I_{sc}$. Paired experiments without or with hexokinase were conducted as shown, respectively, in Fig. 9a and b, both using the recirculated KRB method. With hexokinase in the bathing solutions, the response of I_{sc}

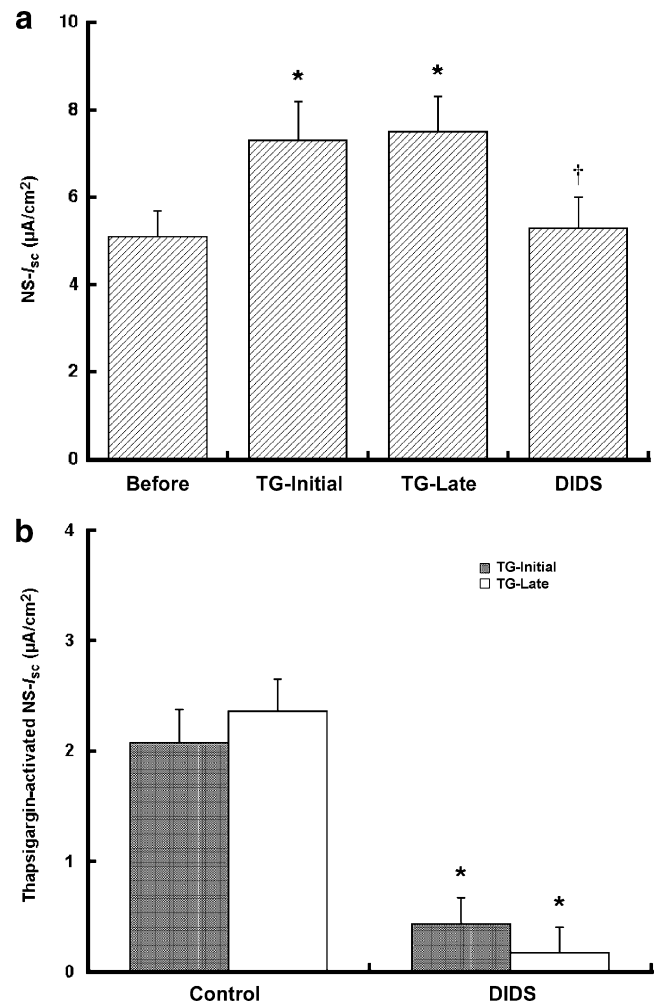
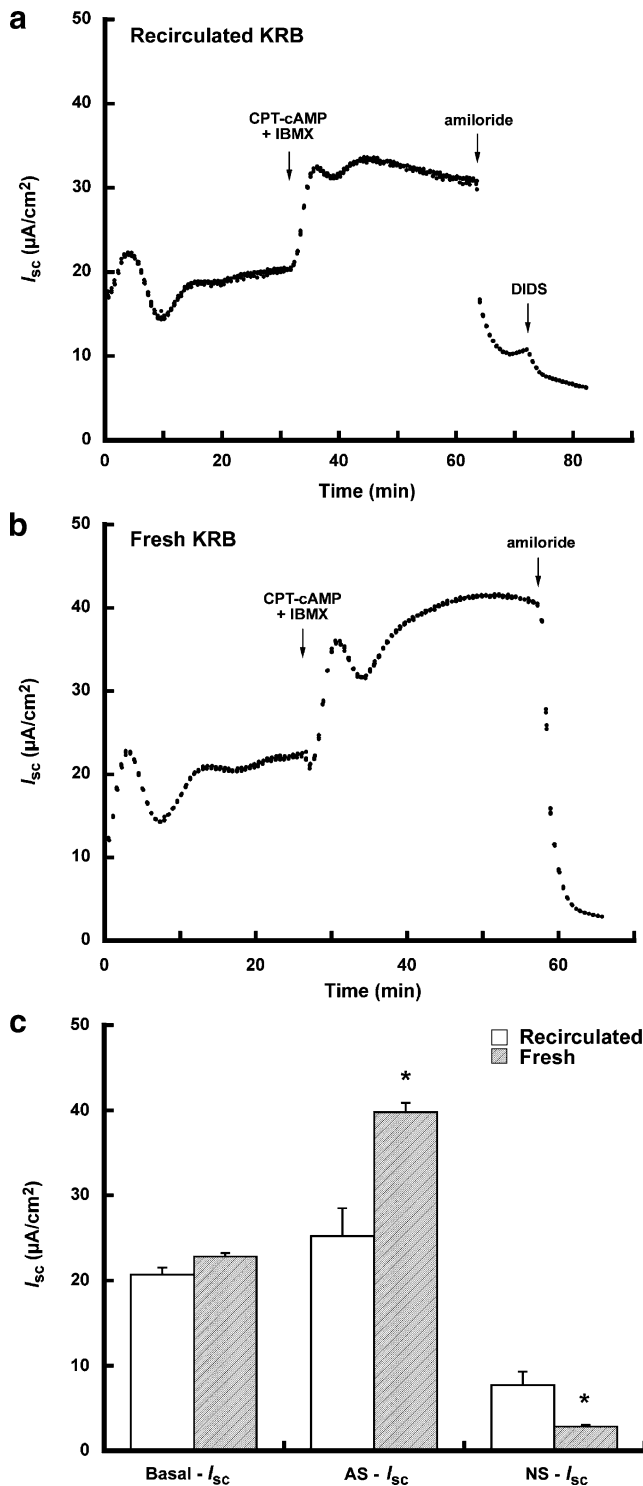


Fig. 6 Effect of thapsigargin on the nonamiloride-sensitive component ($NS-I_{sc}$) and prevention of that effect by 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS). All experiments were performed in the presence of 10 μM luminal amiloride. **a** Mean $NS-I_{sc}$ values for seven experiments at four time points: just before adding thapsigargin to the apical and basolateral solutions, 3–5 min after addition of 1 μM thapsigargin to the apical and basolateral solutions (*TG-initial*), 15–20 min after thapsigargin addition (*TG-late*), and 3–5 min after the addition of 300 μM DIDS to the apical solution. * Significantly different compared with $NS-I_{sc}$ before thapsigargin treatment, $P < .001$; † significantly different compared with both the initial and late $NS-I_{sc}$ after thapsigargin, $P < .001$. **b** Mean values of the change in $NS-I_{sc}$ produced by thapsigargin: the initial peak (*gray bars*) and the late response (*open bars*) for control experiments (as in **a**) and in three experiments in which 300 μM DIDS was added before thapsigargin. * Significantly different compared with the corresponding control values, $P < .001$

to cAMP treatment was exaggerated, and the broader peak was more sustained (Fig. 9b). As shown by the summary of four such sets of paired experiments (Fig. 9c), hexokinase had no effect on the basal I_{sc} measured just before cAMP addition; however, after cAMP, $AS-I_{sc}$ was significantly higher ($40.4 \pm 2.9 \mu A/cm^2$ vs. $29.7 \pm 2.8 \mu A/cm^2$, $P < 0.05$) with hexokinase, whereas the DIDS-sensitive $NS-I_{sc}$ was not significantly different from zero and significantly less

than in the control group ($-0.3 \pm 0.2 \mu\text{A}/\text{cm}^2$ vs. $3.6 \pm 0.7 \mu\text{A}/\text{cm}^2$, $P < 0.01$). Thus, all of the above data were consistent with the hypothesis that the accumulation of extracellular ATP causes the time-dependent increase of NS- I_{sc} and decrease of AS- I_{sc} that were observed in those experiments in which the bathing solutions were recirculated.



Measurement of ATP released from MDCK cells

ATP released from polarized MDCK cells was determined by a bioluminescence assay, in which a luminometer was used to measure light production from ATP consumption in the luciferin-luciferase reaction [20]. MDCK cells were grown to confluence in 12-mm permeable supports (Milli-cell), and the ATP was measured in the apical or basolateral solution by adding the reagents only to that chamber. As shown in Fig. 10, under basal conditions, apically directed ATP release produced a light emission of 16.4 ± 2.1 ALU (artificial light units), whereas basolaterally directed ATP release was nearly zero (0.7 ± 0.2 ALU). In ten paired experiments, addition of $100 \mu\text{M}$ cAMP and $100 \mu\text{M}$ IBMX to the apical solution significantly increased the apically directed ATP release from 16.0 ± 2.1 ALU (basal) to 51.1 ± 9.4 ALU ($n=10$, $P < 0.005$), whereas addition of the same volume of solution without cAMP and IBMX (sham control for the possible effects of manipulating the monolayers) had no significant effect (17.4 ± 3.5 ALU basal vs. 15.1 ± 2.3 ALU sham; $n=4$, $P=0.29$). Treatment with cAMP had no significant effect on basolaterally directed ATP release (0.8 ± 0.3 , $n=8$, $P > 0.5$).

Discussion

Previous work [10] has shown that the triply transfected FL-MDCK cells used in these experiments (a subclone of $\alpha_{\text{F}}\beta_{\text{F}}\gamma_{\text{F}}$ MDCK cells used in the experiments of Morris et al. [2]) expressed all three rat-ENaC subunits and CFTR, and that it was a very useful model epithelium for the study of ENaC regulation. In the current study, we examined in greater detail the relationship between Cl^- secretion and Na^+ absorption in this epithelium.

Time-dependent changes in NS- I_{sc} and AS- I_{sc}

FL-MDCK cells uniformly exhibited a high initial I_{sc} , which was almost completely eliminated by $10 \mu\text{M}$

Fig. 7 Time course of the short-circuit current (I_{sc}) in experiments with and without recirculation of the bathing solutions. The sequence of treatments was the same in these paired experiments: $100 \mu\text{M}$ cyclic adenosine monophosphate (cAMP) plus $100 \mu\text{M}$ isobutylmethylxanthine (IBMX) were added to both apical and basolateral solutions, followed by $10 \mu\text{M}$ amiloride to the apical solution. **a** Apical and basolateral Krebs-Ringer bicarbonate (KRB) solutions were recirculated through the two sides of the Ussing chamber from 10-ml reservoirs. 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS) ($300 \mu\text{M}$) was added to the apical solution near the end of the experiment. **b** In this paired experiment, fresh KRB solution was perfused through both sides of the Ussing chamber at the same rate ($1 \text{ ml}/\text{min}$) as for the recirculated solutions in **a**. **c** Summary results for four sets of paired experiments such as those in **a** and **b**. *Significantly different from control (with recirculation as in **a**), $P < .001$

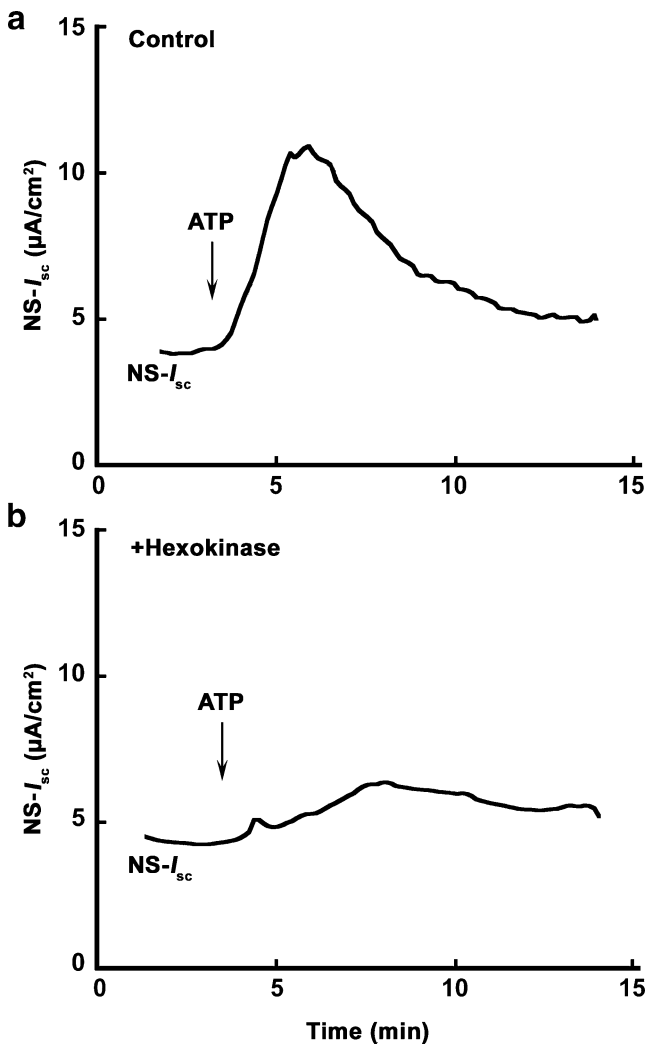


Fig. 8 Effect of adenosine triphosphate (ATP) on the nonamiloride-sensitive component ($NS-I_{sc}$) and prevention of that effect by hexokinase. All experiments were performed in the presence of 10 μ M apical amiloride. **a** Effect of 100 μ M ATP added to the apical solution on $NS-I_{sc}$. Mean values of five such experiments are given in the text. **b** Effect of 100 μ M ATP on $NS-I_{sc}$. In these experiments, 6 U/ml hexokinase was continuously present in the bathing solution. The same results were obtained in three such experiments

amiloride added to the apical solution. The initial I_{sc} fell to a nadir within 15 min and then increased slowly (Figs. 2b and 3b) due to an increase in $NS-I_{sc}$. This progressive rise of $NS-I_{sc}$ occurred consistently regardless of whether amiloride was present or not and whether the epithelium was continuously short-circuited or was left in the open-circuit condition with only intermittent short-circuiting to measure I_{sc} . Moreover, the time-dependent increase of $NS-I_{sc}$ could not be inhibited by the further addition of amiloride but was partially blocked by apical DIDS, which is consistent with anion secretion (see below).

The time course of the I_{sc} response to cAMP using these FL-MDCK cells was like that observed in the previous studies of Morris and Schafer [2] and in other studies of

similar epithelia [6, 9, 27]. There was an initial transient increase lasting less than 6 min, followed by a broader peak, which decayed over the next 30 min (Fig. 3). Morris and Schafer [2] have shown that when these experiments are conducted in a Cl^- -free solution, the initial transient spike was absent and I_{sc} was stable for at least 30 min. The initial transient and the broader peak of I_{sc} were attributed to, respectively, stimulation of Cl^- secretion and Na^+ absorption by cAMP [2]. Interestingly, the effect of cAMP on I_{sc} was also time dependent. With late-cAMP treatment (Fig. 3b), the decay of I_{sc} was faster and the magnitude of $NS-I_{sc}$ was larger. As shown in Fig. 4, there was a significant inverse correlation between $AS-I_{sc}$ and $NS-I_{sc}$ both in the absence and in the presence of cAMP. This correlation does not indicate a causal relationship between the rise in $NS-I_{sc}$ and the fall in $AS-I_{sc}$, but suggests that whatever process is responsible for the time dependence produces reciprocal changes in $NS-I_{sc}$ and $AS-I_{sc}$.

Cl^- channels associated with $NS-I_{sc}$

As discussed previously [2], we attributed $NS-I_{sc}$ to Cl^- secretion because in the presence of apical amiloride, Cl^- was the only ion present in sufficient concentration to account for it. Furthermore, glibenclamide blocked the initial rise in $NS-I_{sc}$ in response to subsequent cAMP treatment, whereas DIDS blocked the broader late transport response but not the early peak (Fig. 5). Glibenclamide is a well-established inhibitor of CFTR at the concentration (200 μ M) used in these experiments. Although glibenclamide also inhibits ATP-activated K^+ channels [28, 29], previous studies have shown that the apical membrane of MDCK cells has no measurable K^+ conductance [10, 30]. Although DIDS effectively inhibits Cl^- channels such as CaCC as well as the outwardly rectifying Cl^- channel (ORCC), and the CLC family of Cl^- channels, it does not inhibit CFTR [31, 32]. These results indicate that at least two types of Cl^- channels contribute to $NS-I_{sc}$ and that they respond differently to cAMP.

Based on these data, we have attributed the early, glibenclamide-sensitive, increase in $NS-I_{sc}$ after cAMP treatment to CFTR, which we have demonstrated is expressed in the apical membrane of these FL-MDCK cells [10]. CaCC appears to be responsible for the later increase in $NS-I_{sc}$ after cAMP treatment. As shown in Fig. 6, we found that thapsigargin, which moderately increases $[Ca^{2+}]_i$ [22], produced a transient spike and a sustained increase in $NS-I_{sc}$ that could be completely blocked by apical DIDS as expected for a CaCC [33]. Furthermore, cAMP increases $[Ca^{2+}]_i$ in MDCK cells [21], and thus a CaCC may contribute to the DIDS-sensitive component of the $NS-I_{sc}$ response to cAMP. A CaCC has also been described in the M-1 and mMCD-K2 cell lines [34, 35] and in primary

cultures of rabbit proximal and distal tubule cells [36]. It should also be noted, however, that the molecular composition of CaCC is not as yet known. Thus, we were unable to provide unequivocal evidence for its existence in the FL-MDCK cell line.

Endogenous ATP release inhibits amiloride-sensitive Na^+ absorption

When the MDCK monolayers were continuously perfused with fresh apical and basolateral KRB solutions rather than recirculated solutions, the time-dependent increase of NS- I_{sc} and decrease of total I_{sc} was prevented (Fig. 7). AS- I_{sc} was also significantly increased during the continuous perfusion, suggesting that inhibition of ENaC was prevented by washing out accumulated metabolites (Fig. 7c). Furthermore, our experiments showed that hexokinase, a scavenger of extracellular ATP, produced the same effects on AS- I_{sc} and NS- I_{sc} as the perfusion of fresh KRB solutions (Fig. 9). In the presence of hexokinase, AS- I_{sc} was significantly higher but the DIDS-sensitive NS- I_{sc} was significantly lower than in control experiments, indicating that extracellular ATP caused the inhibition of ENaC and activation of NS- I_{sc} , the latter possibly via a CaCC.

Extracellular ATP is a well-established agonist for purinergic receptors, leading to an increase of intracellular $[\text{Ca}^{2+}]$ and activation Ca^{2+} -activated Cl^- channels [24, 37, 38]. In parallel, ATP attenuates amiloride-sensitive Na^+ absorption in a variety of tissues including airway [39–41] and renal epithelia [42–44]. Similarly, activation of CFTR also increases Cl^- secretion and inhibits ENaC in native epithelial tissues [14, 45]. Because CFTR has been suggested to potentiate ATP release [46, 47] and it has been shown to be expressed in this MDCK cell line [10], we examined a possible contribution of ATP release to the time-dependent transport in FL-MDCK cells. As shown in Fig. 8a, addition of 100 μM ATP stimulated anion secretion, possibly via a Ca^{2+} -activated Cl^- channel, which was inhibited by DIDS, and this effect was prevented in the presence of hexokinase (Fig. 8b).

Endogenous release of ATP by the FL-MDCK monolayers was demonstrated by the bioluminescence assay experiments shown in Fig. 10. Consistent with measurements in cortical CD (see [48]), ATP was shown to be released into the apical solution under basal conditions, and this release was augmented more than three-fold by cAMP

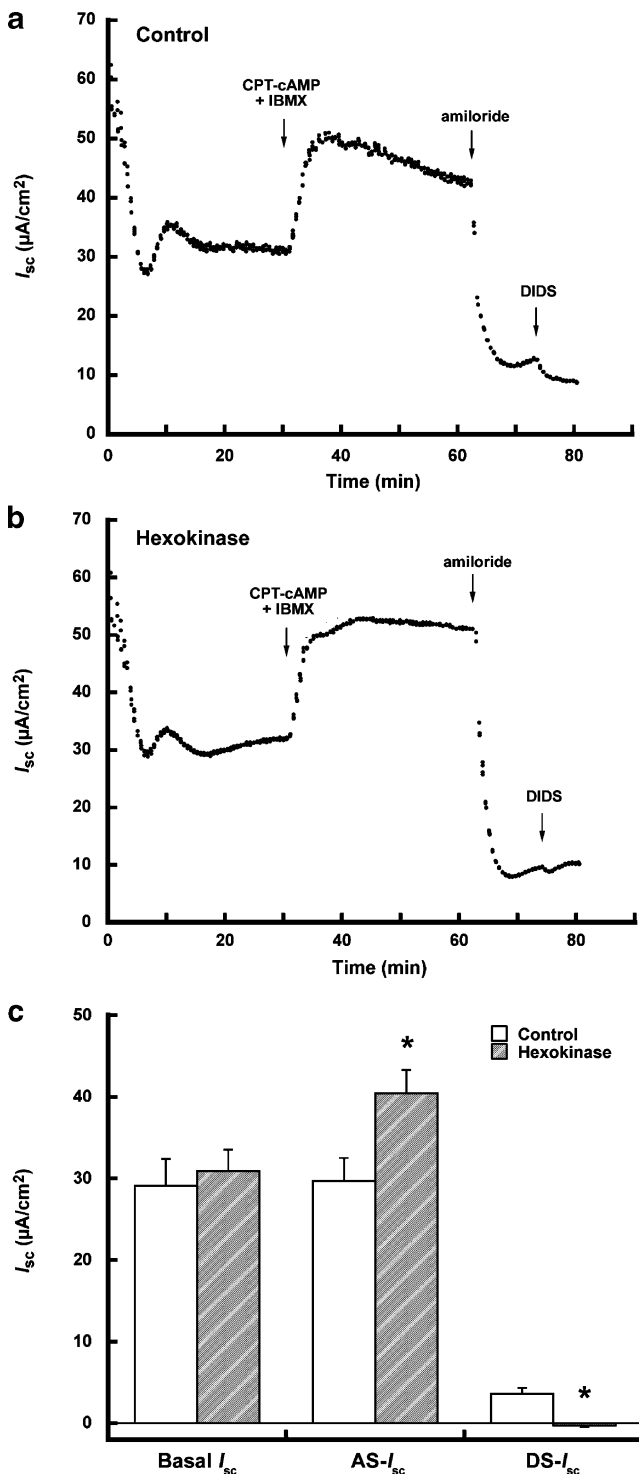


Fig. 9 Time course of the short-circuit current (I_{sc}) response to cyclic adenosine monophosphate (cAMP) treatment in the absence and presence of hexokinase. Both types of experiments followed the same general protocol: A mixture of 100 μM (4-chlorophenylthio) (CPT)-cAMP plus 100 μM isobutylmethylxanthine (IBMX) was added as indicated, followed by 10 μM apical amiloride and 300 μM apical 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS). **a** In this experiment, the Krebs-Ringer bicarbonate (KRB) solution had no hexokinase added. **b** A paired experiment in which 6 U/ml hexokinase was continuously present in apical and basolateral bathing solution. **c** Average data for four paired experiments: the mean values of I_{sc} immediately before cAMP was added (*Basal- I_{sc}*), amiloride-sensitive (*AS- I_{sc}*) at 30 min after cAMP addition (*AS- I_{sc}*), and DIDS-sensitive I_{sc} (*DS- I_{sc}*). * Significantly different compared with the corresponding control values

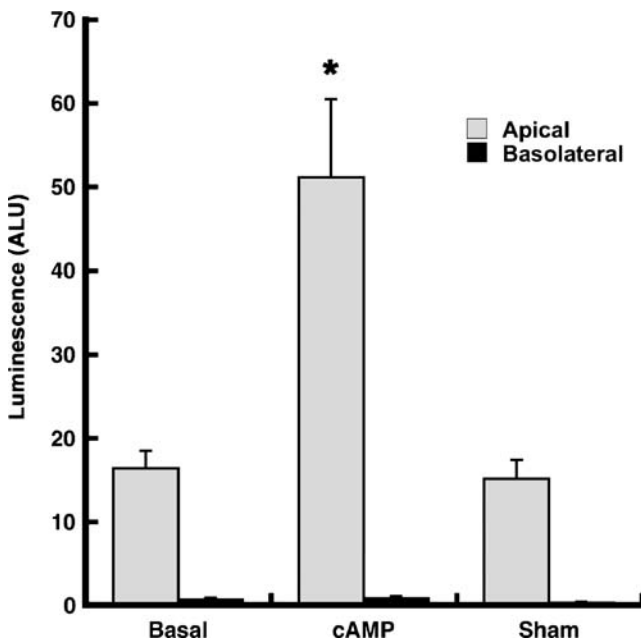


Fig. 10 Adenosine triphosphate (ATP) release across the apical and basolateral membranes of FL-Madin-Darby canine kidney (MDCK) monolayers. ATP release into the apical and basolateral solutions was measured for FL-MDCK monolayers in 12-mm culture inserts using the luciferin-luciferase assay. To measure apical ATP release, these reagents were present only in the apical solution ($n=14$), and they were present only in the basolateral solution to measure basolateral release ($n=11$). The monolayers were first incubated with 200 μ l of standard Opti-minimal essential medium (MEM) solution on each side to measure *basal* ATP production. The monolayers were then removed from the instrument and either 1 μ l of a stock solution of 8-(4-chlorophenylthio) (CPT)-cAMP + isobutylmethylxanthine (IBMX) stock solution (*cAMP*, $n=10$; final concentrations: 100 μ M cAMP and 100 μ M IBMX) or 1 μ l of plain Opti-MEM medium (*Sham*, $n=4$) were added. Average values of the light emission in arbitrary light units (ALU) \pm standard error of the mean (SEM) are given. * Significantly different from basal and sham, $P < 0.005$

treatment. In contrast, there was no significant release of ATP into the basolateral solution either without or with cAMP treatment.

ATP has been shown to inhibit $AS-I_{sc}$ and stimulate $NS-I_{sc}$ in other epithelia of distal nephron origin, including M-1 [43], mIMCK-K2 [42], and A6 cells [49]. Release of endogenous ATP into the extracellular medium has been proposed as a general response to hypotonicity and mechanical stimulation in many types of cells, including MDCK and A6 cells [49, 50]. Ostrom et al. [51] demonstrated that mechanical stimulation increases ATP release from MDCK cells as well as COS-7 and HEK-293 cells, and they proposed that ATP acts to alter the set point for a variety of signal transduction pathways. Praetorius et al. [50] also observed that mechanical stimulation caused ATP release in MDCK cells and that this release was associated with a transient increase in $[Ca^{2+}]_i$.

Because of the opposite actions of ATP in the extracellular apical membrane, it can serve to shift the balance

between active NaCl absorption and active NaCl secretion in the MDCK cell, as shown by the hypothetical model in Fig. 11. It is also quite important to note that the evidence we have presented for ATP accumulation in the extracellular fluid with longer incubation periods in Ussing chambers raises a caution for all such experiments, and it suggests that recirculation of the bathing solution, which has been in common use, should be avoided.

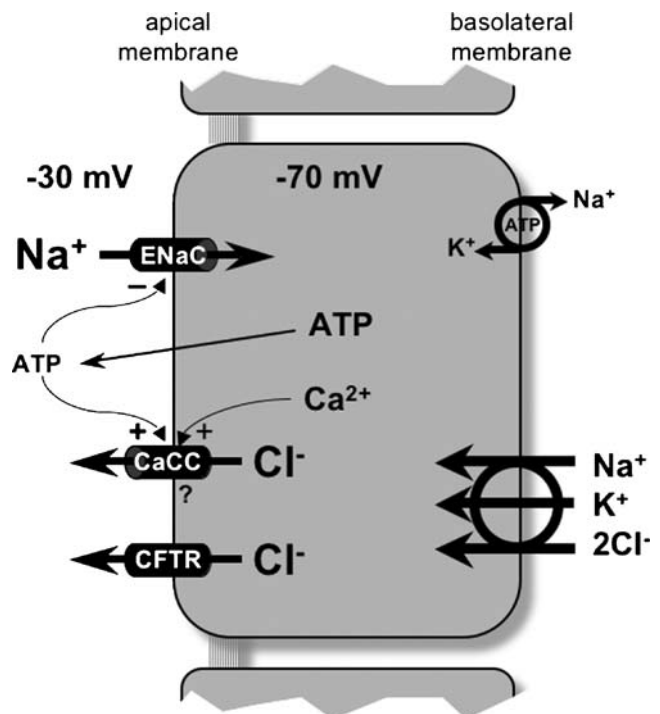


Fig. 11 Model for the role of adenosine triphosphate (ATP) in regulating Na^+ absorption and Cl^- secretion in Madin-Darby canine kidney (MDCK) epithelia. The epithelial Na^+ channel (ENaC) mediates active Na^+ absorption and produces the amiloride-sensitive component of the short-circuit current ($AS-I_{sc}$). Because of the increased Na^+ conductance produced by ENaC, it also depolarizes the apical membrane, producing a negative transepithelial voltage (V_{te}) of -30 mV. The basolateral membrane contains the ubiquitous Na, K-ATPase and, putatively, an Na-K-2Cl cotransporter, which actively accumulates Cl^- in the cell. Cl^- can then move across the apical membrane down its electrochemical potential gradient through Cl^- channels, which include the glibenclamide-sensitive cystic fibrosis transmembrane conductance regulator (CFTR) channel, and a 4, 4'-diisothiocyano-2, 2'-disulfonic acid stilbene (DIDS)-sensitive, calcium-activated Cl^- channel (CaCC). The net secretory movement of Cl^- gives rise to the amiloride-insensitive component of the short-circuit current ($NS-I_{sc}$). Our results show that ATP is secreted across the apical membrane. The pathway for ATP secretion is not known, but it has been proposed that it may also occur via CFTR [46]. When ATP accumulates in the apical extracellular space (e.g., when the solution bathing this space is restricted in volume or is recirculated), ATP inhibits ENaC and stimulates CaCC (and possibly CFTR or other Cl^- channels), resulting in a fall in $AS-I_{sc}$ and an increase in $NS-I_{sc}$

Implications for renal pathophysiology

It must be recognized that the FL-MDCK cell line suffers from the limitations of any cell culture system when attempting to apply these results to an understanding of normal renal physiology or pathophysiology. Furthermore, even the type-1 MDCK cells used in these studies are heterologous, and their origin cannot be ascribed definitively to any nephron segment. Nevertheless, the ability of ATP to inhibit ENaC-mediated Na^+ absorption and stimulate Cl^- secretion, as shown in these experiments with FL-MDCK cells and in similar epithelia [42, 43, 49], suggests a role for such signaling in the nephron. Significant amounts of ATP are released from epithelial cells originating from all regions of the nephron, and multiple P2Y and P2X receptors have been identified in all segments of the nephron [48]. Thus, changes in the luminal ATP concentration in the cortical CD and other aldosterone-sensitive segments of the distal nephron, in which ENaC mediates Na^+ transport, may be a physiologic signal for diminished Na^+ reabsorption. Also, Wilson et al. [19] demonstrated significant accumulation of ATP in cyst fluid obtained from patients with autosomal dominant polycystic kidney disease. Thus, based on our observations in MDCK cells, it seems reasonable to speculate that ATP might favor cyst enlargement and consequent exacerbation of polycystic disease by inhibiting Na^+ reabsorption and stimulating Cl^- secretion [19, 52].

Acknowledgments This work was part of a dissertation submitted in partial fulfillment of the requirements for the Ph.D. degree awarded to Y. Xie by the School of Graduate Studies at the University of Alabama at Birmingham in December 2004. We are particularly grateful to Dr. Ryan G. Morris, who developed the retrovirally transfected cell line used in these studies. We thank Dr. Erik Schwiebert in this department for allowing us to use his luminometer and to Ms. E. Welty for teaching us the methodology of ATP measurement with this instrument. This study was supported in part by NIH grant DK-25519-21 and a predoctoral fellowship (R464-CR02) from the Cystic Fibrosis Foundation. Some of the data in this study have been presented previously in abstract form (FASEB J 2003; 17: A1225).

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