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Swelling-Activated K^+ Efflux and Regulatory Volume Decrease Efficiency in Human Bronchial Epithelial Cells

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Abstract. This study describes the correlation between cell swelling-induced K^+ efflux and volume regulation efficiency evaluated with agents known to modulate ion channel activity and/or intracellular signaling processes in a human bronchial epithelial cell line, 16HBE14o$^-$. Cells on permeable filter supports, differentiated into polarized monolayers, were monitored continuously at room temperature for changes in cell height (T_c), as an index of cell volume, whereas ^{86}Rb efflux was assessed for K^+ channel activity. The sudden reduction in osmolality of both the apical and basolateral perfusates (from 290 to 170 mosmol/kg H_2O) evoked a rapid increase in cell volume by 35%. Subsequently, the regulatory volume decrease (RVD) restored cell volume almost completely (to 94% of the isosmotic value). The basolateral ^{86}Rb efflux markedly increased during the hyposmotic shock, from 0.50 $\pm$ 0.03 min^{-1} to a peak value of 6.32 $\pm$ 0.07 min^{-1}, while apical ^{86}Rb efflux was negligible. Channel blockers, such as $GdCl_3$ (0.5 mM), quinine (0.5 mM) and 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB, 100 μM), abolished the RVD. The protein tyrosine kinase inhibitors tyrphostin 23 (100 μM) and genistein (150 μM) attenuated the RVD. All agents decreased variably the hyposmosis-induced elevation in ^{86}Rb efflux, whereas NPPB induced a complete block, suggesting a link between basolateral K^+ and Cl^- efflux. Forskolin-mediated activation of adenylyl cyclase stimulated the RVD with a concomitant increase in basolateral ^{86}Rb efflux. These data suggest that the basolateral extrusion of K^+ and Cl^- from 16HBE14o$^-$ cells in response to cell swelling determines RVD efficiency.

Key words: Stretch-activated channel — K^+ channel — Tyrosine kinase — cAMP

Introduction

Cells are endowed with mechanisms that regulate their volume during osmotic stress conditions. Osmolality changes in the bathing medium trigger cellular processes that restore steady-state cell volume. In the case of a hyposmotic challenge, the rapid swelling of the cells is usually counteracted by the active extrusion of osmolytes followed by the passive leaving of water, a process commonly referred to as the regulatory volume decrease (RVD). Osmotically active substances involved in this process consist mainly of inorganic ions (e.g., K^+ and Cl^-) in the short term and organic molecules (e.g., amino acids, sugars, methylamines and polyalcohols) in the long term (Lang et al., 1998; Jakab et al., 2002).

Regulatory mechanisms involved in the RVD have been studied mainly by inducing a sudden decrease in the osmolality of the bathing medium, although in physiological conditions cells never face such drastic changes in osmolality. Nonetheless, information from this type of experiment has offered insight into the underlying mechanisms.

RVD is a complex process, and its efficiency depends on the proper coordinated function of many cellular constituents. Ion channels, intracellular ion concentrations, various ion and solute membrane transporters, intracellular signaling processes, cytoskeletal changes and cytosolic ionic strength are all reported to be involved in RVD (Mongin & Orlov, 2001). Recently, it has been acknowledged that aquaporins, channels that facilitate transcellular water transport, play an important role in RVD (Liu et al.,

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2006; Kida et al., 2005; Matsuki et al., 2005). The relative contribution of these structures and processes to RVD is cell type-dependent and not clearly established. The coordinated effluxes of K^+ and Cl^- are considered by far the principal mechanism for RVD in most cell types (Sardini et al., 2003). In epithelial cells, the sequence of events leading to cell volume restoration following a hyposmotic shock seems to be triggered by an increase in cytosolic Ca^{2+} concentration, which induces K^+ efflux (Fernandez-Fernandez et al., 2002; Pasantes-Morales & Morales Mulia, 2000). Subsequently, with a delay from the onset of K^+ efflux, the pathway for Cl^- efflux is activated (Pasantes-Morales & Morales Mulia, 2000). These K^+ and Cl^- fluxes generate the osmotic gradient that drives water flow, at least partly, through aquaporins (Liu et al., 2006; Verkman, 2005; Lang et al., 1998).

In this study, polarized 16HBE14o$^-$ human airway cell monolayers, originally derived from the surface epithelium of mainstream, second-generation bronchi, were used as an *in vitro* model. 16HBE14o$^-$ is a well-established cell line that retains tight junctions and vectorial ion transport (Cozens et al., 1994). Since the efficiency of RVD as a function of K^+ efflux has not been clearly shown before, we investigated the role of RVD-activated pathways for K^+ efflux in relation to the restoration of cell volume. We measured K^+ efflux in the presence and absence of pharmacological agents known to interfere with the volume regulatory processes. We found a correlation between RVD efficiency and swelling-activated K^+ efflux, which is inhibited by 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB), a Cl^- channel inhibitor, and stimulated by an adenylyl cyclase activator, forskolin, among others.

Materials and Methods

Cell Culture

The immortalized human bronchial epithelial cell line 16HBE14o$^-$ was provided by Dr. K. Kunzelmann (University of Freiburg, Germany). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. The growth medium consisted of a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 nutrient medium (DMEM/F12), supplemented with 3.22 mM L-glutamine, 161 ml/l fetal calf serum, 80,000 U/l penicillin and 80 mg/l streptomycin. Cells were seeded at a density of $10^5/cm^2$ on permeable filter supports (pore size 0.2 μm, Anopore; Nunc Intermed, Roskilde, Denmark). The polarized monolayers were used after 10 days of growth. For this study, 16HBE14o$^-$ cells of passages 90–100 were utilized.

Solutions

The isosmotic solution contained (all in mM) 145 NaCl, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 1.3 $CaCl_2$, 1 $MgCl_2$ and 5 D-glucose (290 ± 2 mOsm/kg H_2O). The hyposmotic bathing solution (170 ± 2 mOsm/kg H_2O) was prepared by omitting 65 mM NaCl from the isosmotic solution. The K^+-free loading solution, used in the ^{86}Rb-efflux studies, contained (all in mM) 145 NaCl, 1.6 Na_2HPO_4, 0.4 NaH_2PO_4, 1.3 $CaCl_2$, 1 $MgCl_2$ and 5 D-glucose (290 ± 2 mOsm/kg H_2O). The bathing solution containing $GdCl_3$ was buffered with 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) to avoid the precipitation of Gd^{3+} with phosphates. For this purpose, we used a solution composed of (all in mM) 139 NaCl, 5 Na-HEPES, 3.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$ and 5 D-glucose (290 ± 2 mOsm/kg H_2O). The osmolality of all solutions was verified with a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany). The pH of all solutions was 7.40 ± 0.05.

Chemicals

^{86}RbCl was obtained from Amersham (Arlington Heights, IL). $GdCl_3$, quinine, forskolin, tyrphostin and genistein were from Sigma (St. Louis, MO); NPPB was from Research Biochemicals (Natick, MA).

Cell Height Measurements

Cell height (T_c) was used as an index for cell volume according to a method developed in this laboratory (Van Driessche, De Smet & Raskin, 1993). In brief, before seeding the cells, the filters were coated with gelatin containing fluorescent microspheres of 1 μm diameter (L-5081; Molecular Probes, Eugene, OR). The apical surface of the epithelium was labeled with fluorescent avidin-coated microbeads (F-8776, Molecular Probes). T_c was recorded as the vertical distance between the points of maximal light intensity of the lower and upper beads. T_c was expressed as a percentage change from control values. Control values (T_c^{ISO}) were those recorded just before the hyposmotic shock was applied and are reported in each figure legend. *N* indicates the number of experiments and *n*, the number of cells used to calculate the averaged T_c values. From the measured T_c, 1 μm was subtracted, as a correction for the diameter of the microspheres.

^{86}Rb-Efflux Measurements

Cell monolayers were loaded with 3 μCi of ^{86}Rb in a K^+-free loading solution bathing the basolateral surface. Cell monolayers were mounted in a two-compartment Ussing-type chamber. The nominal surface area of the monolayer exposed to the perfusates was 1.54 cm^2. Both compartments were continuously perfused. Rapid basolateral washout was achieved by vigorously stirring with a motor-driven magnetic stirring bar. The apical and basolateral perfusates were collected serially in test tubes every 2 min. The activity of ^{86}Rb released into perfusates was assayed with a beta counter (Tri-Carb 2100 TR; Packard, Downers Grove, IL). Prior to each experiment, the remaining tracer attached to the cell surface and filter support was washed out by perfusing both sides of the monolayer for 30 min with the isosmotic solution. At the end of each experiment, the cells were lysed with a 1N NaOH solution, to estimate the total ^{86}Rb radioactivity remaining in the cells. Radioactivity in each collected sample was expressed as a percentage of the total amount in the cells at that time. We assumed that the total amount of radioactivity accumulated in the cells equals the radioactivity in the collected perfusates (including the washes) plus the radioactivity in the cell lysates.

^{86}Rb-Efflux Measurements in the Presence of Pharmacological Agents

Cell monolayers were loaded with 3 μCi of ^{86}Rb from basolateral fluid in a K^+-free loading solution, as mentioned above. These

experiments were carried out in six-well cluster plates, using the monolayers attached to the filters and not removed from their cups. The remaining tracer attached to the cell surface and filter support was then removed by incubating both sides of the monolayers for 40 min in the isosmotic solution, containing the particular pharmacological agent to be tested. The apical and basolateral bathing solutions were collected every 30 min. At the end of the experiment, the remaining ^{86}Rb radioactivity in the monolayer was extracted by treating cells for 20 min with a 1N NaOH solution. The data are presented as ^{86}Rb release per specified time period (30 min) and expressed as radioactivity released in each collected sample as a percentage of the total amount present in the monolayer at the beginning of the 30-min time period.

Experimental Design

Measurements of T_c and ^{86}Rb efflux were performed using a similar experimental protocol, consisting of three measurement periods during which the osmolality of the bathing solutions was altered simultaneously on both sides of the monolayer: I, isosmotic (290 mOsm/kg) condition for 30 min; II, hyposmotic (170 mOsm/kg) condition for 30 min; III, isosmotic (290 mOsm/kg) condition for 20 min. For the measurements of ^{86}Rb efflux performed in the presence of pharmacological agents, the duration of period III was set at 30 min. All experiments were performed at room temperature of 22°C.

Statistics

Data are presented as means ± standard error of the mean (SEM). Unpaired Student's *t*-tests were used to compare two group means, with $P < 0.05$ being statistically significant.

Results

Effect of Hyposmotic Shock on Cell Volume and ^{86}Rb Efflux: Control Experiments

The sudden reduction in osmolality on both the apical and basolateral sides of the monolayer (Fig. 1, upper panel) evoked a rapid increase in T_c by 35% within 4 min (Fig. 1, middle panel). Cell volume increase was followed by the RVD, during which cell volume declined in two successive phases: a rather fast phase in the beginning, followed by a much slower phase. During the rapid RVD phase, T_c decreased by 15% within 5 min, whereas the slow RVD phase restored cell volume almost completely, to 6% above the isosmotic level observed before the hyposmotic challenge. The occurrence of the RVD suggests the existence of pathways activated for osmolyte release. When returning the monolayer to the isosmotic solutions, the cells experienced an additional shrinkage by 33%, indicating loss of osmolytes during the RVD. A post-RVD regulatory volume increase phase was not observed, indicating that the 16HBE14o$^-$ cells were not able to restore their volume to the control level when returning to isosmotic conditions, a feature that has been reported with some other epithelial cells.

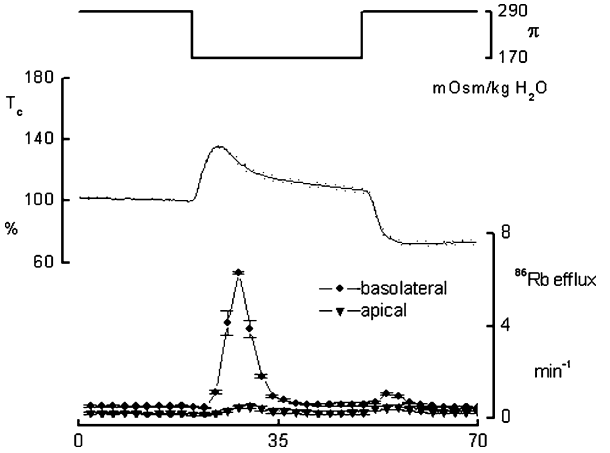

Fig. 1. T_c and ^{86}Rb efflux during hyposmotic shock in 16HBE14o$^-$ cells. The osmolality on both sides of the monolayer was simultaneously reduced to 170 mOsm/kg (*top*). The time courses of T_c ($N = 6$, $n = 94$) and ^{86}Rb efflux ($N = 6$) are shown (*middle* and *bottom*, respectively). T_c is shown as a percent of control, where 100% denotes $T_c^{ISO} = 12.62 \pm 0.31$ μm and is defined as the T_c value in isosmotic conditions prior to hyposmotic shock. Data are shown as mean ± SEM.

The major intracellular osmolyte is K^+, and RVD in most cell types is mainly achieved by net loss of K^+ and its electrical opponent Cl^-. Therefore, we investigated the contribution of the volume-activated pathway for K^+ efflux by measuring ^{86}Rb efflux. The ^{86}Rb efflux through both the apical and basolateral cell membranes increased during hyposmotic shock (Fig. 1, bottom panel). Compared to the marked increase in basolateral ^{86}Rb efflux (from 0.50 ± 0.03 min^{-1} under isosmotic conditions to a peak value of 6.32 ± 0.07 min^{-1} during hyposmotic challenge), the release of ^{86}Rb into the apical fluid was much smaller. This apical ^{86}Rb efflux is also observed in isosmotic solutions and not appreciably affected by hypotonicity (*data not shown*). As the amount of the ^{86}Rb release into the apical fluid appears to be constitutive and, thus, presumably not related to volume regulation, we restricted our investigation to the basolateral ^{86}Rb efflux, which will be shown from this point onward. It is worth noting that the peak in T_c was achieved 4 min after inducing the hyposmotic challenge, whereas basolateral ^{86}Rb efflux reached a maximum 8 min after initiation of the osmotic perturbation. This delay suggests that cell volume expansion does not directly activate the cation release pathway. When the epithelium was returned to the isosmotic solution, ^{86}Rb efflux slightly increased from 0.62 ± 0.07 min^{-1} (i.e., the value reached at the end of hyposmotic challenge) to a peak of 1.08 ± 0.05 min^{-1}. Next, the ^{86}Rb efflux returned toward the level attained during the prehyposmotic challenge.

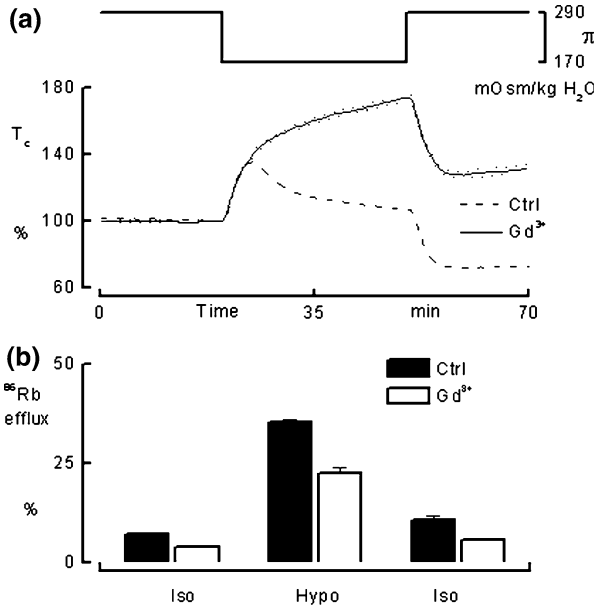

Fig. 2. Effect of gadolinium on (*A*) RVD, where $T_c^{ISO} = 13.09 \pm 0.41$ μm ($N = 5, n = 83$) compared to the control (*dotted line*). (*B*) ^{86}Rb efflux (*unfilled bars, N = 6*) compared to the ^{86}Rb efflux in control conditions (*filled bars, N = 6*). $GdCl_3$ (0.5 mM) was added to both sides of the monolayer throughout the experiment. Data are shown as mean ± SEM.

Effects of Pharmacological Agents on Cell Volume and Osmolyte Efflux

Next, we investigated the contribution of a number of channels known to be activated for K^+ efflux during RVD.

Gadolinium

To explore the involvement of stretch-sensitive channels, likely to be activated during cell swelling, we evaluated the effect of gadolinium, a commonly used blocker of stretch-activated ion channels. In the presence of $GdCl_3$ (0.5 mM), T_c increased steadily during the hyposmotic challenge to 175% above control after 30 min (Fig. 2A). When restoring the isosmotic perfusion solutions, cell volume remained 30% above the prehyposmotic level, indicating a net gain of osmolytes during hyposmotic shock. The ^{86}Rb efflux measured during the 30-min period of hyposmotic challenge increased about fivefold, from 4.06 ± 0.12% to 22.49 ± 1.61% ($P < 0.05$) (Fig. 2B, unfilled bars). The reduction in ^{86}Rb efflux during each period was almost constant, amounting to about 58% ($P < 0.05$) of the corresponding control cells. Thus, gadolinium abolished the RVD response and significantly inhibited ^{86}Rb efflux.

Quinine

In the following series of experiments, we focused on the involvement of K^+ channels by introducing quinine, an inhibitor of K^+ channels, in the solutions at both sides of the epithelium. In the presence of quinine, approximately 10 min after inducing hyposmotic shock, T_c reached a plateau (about 56% above the prehyposmotic level) (Fig. 3A). When 16HBE14o$^-$ monolayers were returned to the isosmotic bathing solutions in the presence of quinine, cell volume was completely restored, suggesting that osmolyte efflux was counteracted by an influx of osmolytes at a similar rate. The alkaloid did not influence ^{86}Rb efflux under isosmotic conditions (i.e., periods I and III), when ^{86}Rb release was similar to the respective control values. By contrast, during hyposmotic shock, ^{86}Rb efflux increased from 7.1 ± 0.20% to 22.51 ± 0.86 %, reaching about 70% of the corresponding control value ($P < 0.05$). Thus, like gadolinium, quinine abolished RVD and partially (by about 30%) inhibited ^{86}Rb efflux.

NPPB

Because the volume modification is too large to be dependent on a single charged species (K^+), which would induce a charge separation that cannot be supported by mammalian cell membranes, half of the osmolyte extrusion is established by an ion with opposite charge to keep net efflux electroneutral. Therefore, we applied NPPB to block Cl^- channels and observed an abolishment of RVD (Fig. 4A). In the presence of NPPB, T_c increased by about 50% during hyposmotic shock. When the osmolality of the bathing fluids was restored, the cells shrunk back close to the cell volume prior to the hyposmotic challenge, indicating no net loss of osmolytes during hyposmotic shock in the presence of NPPB. This hypothesis was confirmed by measurements of ^{86}Rb efflux. In the presence of NPPB (period II), ^{86}Rb efflux was not significantly different from the other periods (I and III) (Fig. 4B).

Tyrphostin 23 and Genistein

Tyrosine kinases are described to play a role in cell volume regulation (Tilly et al., 1993). Hence, we evaluated the effects of two tyrosine kinase inhibitors, tyrphostin 23 and genistein, on cell volume regulation in 16HBE14o$^-$ epithelia. In the presence of tyrphostin 23, RVD was abolished (Fig. 5A), while genistein only slowed down the RVD (Fig. 6A). Compared to the control, ^{86}Rb efflux during the hyposmotic challenge period was diminished by 45% ($P < 0.05$) in the presence of tyrphostin 23 (Fig. 5B) and by 55% ($P < 0.05$) when genistein was included in the solutions (Fig. 6B).

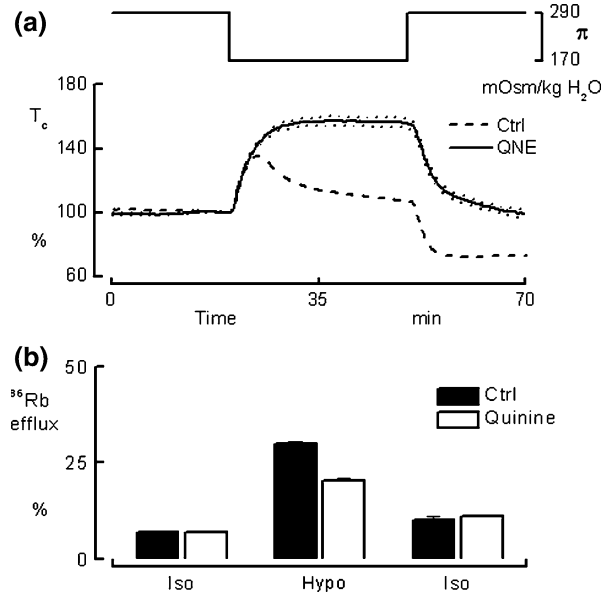

Fig. 3. Effect of quinine on (*A*) RVD, where T_c^{ISO} = 14.52 ± 0.37 μm (N = 4, n = 57) compared to the control (*dotted line*). (*B*) ^{86}Rb efflux (*unfilled bars, N* = 6) compared to the ^{86}Rb efflux in control conditions (*filled bars, N* = 6). Quinine (0.5 mM) was added to both sides of the monolayer throughout the experiment. Data are shown as mean ± SEM.

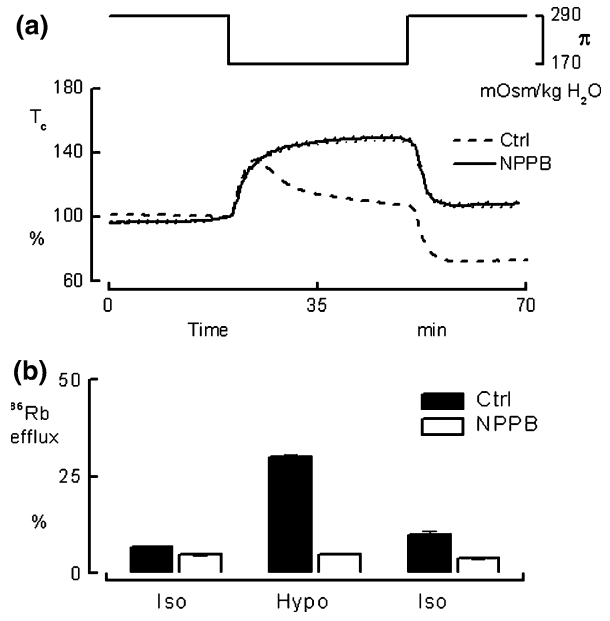

Fig. 4. Effect of NPPB on (*A*) RVD, where T_c^{ISO} = 13.33 ± 0.38 μm (N = 4, n = 71) compared to the control (*dotted line*). (*B*) ^{86}Rb efflux (*unfilled bars, N* = 6) compared to the ^{86}Rb efflux in control conditions (*filled bars, N* = 6). NPPB (100 μM) was added to both sides of the monolayer throughout the experiment. Data are shown as mean ± SEM.

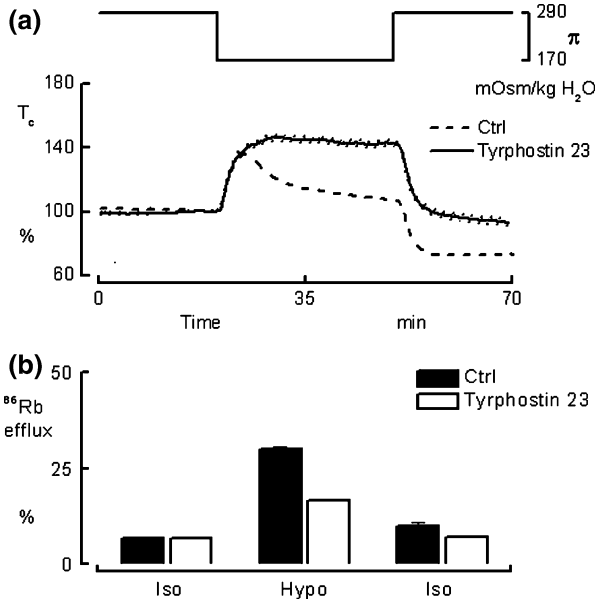

Fig. 5. Effect of tyrphostin 23 on (*A*) RVD, where T_c^{ISO} = 12.72 ± 0.60 μm (N = 5, n = 61) compared to the control (*dotted line*). (*B*) ^{86}Rb efflux (*unfilled bars, N* = 6) compared to the ^{86}Rb efflux in control conditions (*filled bars, N* = 6). Tyrphostin (100 μM) was added on both sides of the monolayer throughout the experiment. Data are shown as mean ± SEM.

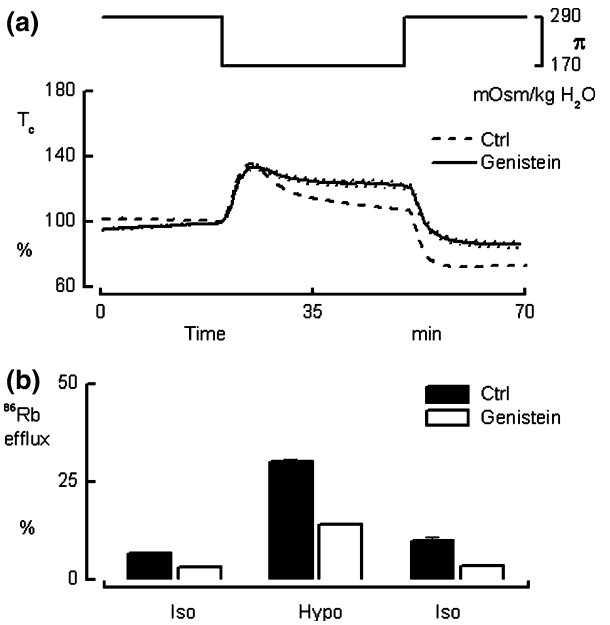

Fig. 6. Effect of genistein on (*A*) RVD, where T_c^{ISO} = 14.26 ± 0.43 μm (N = 6, n = 68) compared to the control (*dotted line*). (*B*) ^{86}Rb efflux (*unfilled bars, N* = 6) compared to the ^{86}Rb efflux in control conditions (*filled bars, N* = 6). Genistein (150 μM) was added on both sides of the monolayer throughout the experiment. Data are shown as mean ± SEM.

Forskolin

Cell volume regulation has been reported to be influenced by the intracellular cyclic adenosine monophosphate (cAMP) level (Lang et al., 1998). By activating adenylyl cyclase using forskolin, the RVD in 16HBE14o$^-$ cells was slightly accelerated, without

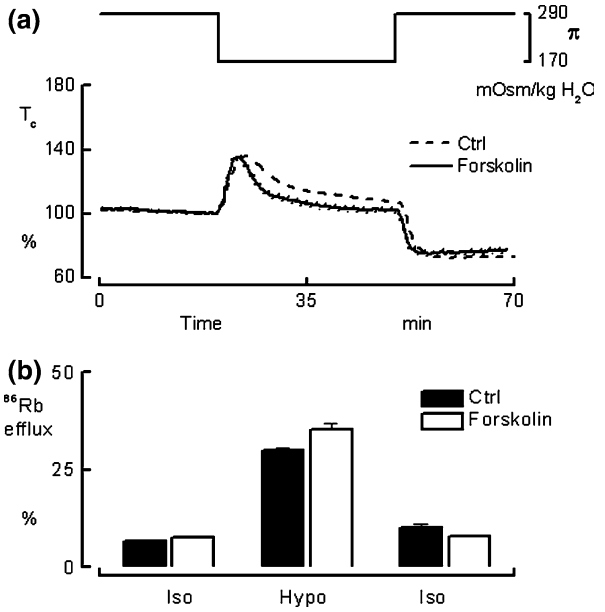

Fig. 7. Effect of forskolin on (*A*) RVD, where $T_c^{ISO} = 11.37 \pm 0.40$ μm ($N = 4$, $n = 57$) compared to the control (*dotted line*). (*B*) ^{86}Rb efflux (*unfilled bars, N* = 6) compared to the ^{86}Rb efflux in control conditions (*filled bars, N* = 6). Forskolin (5 μM) was added to both sides of the monolayer throughout the experiment. Data are shown as mean ± SEM.

altering significantly the peak level of cell swelling induced by hyposmotic challenge (Fig. 7A). Compared to the control, ^{86}Rb efflux during hyposmotic shock was increased by 21% ($P < 0.05$) (Fig. 7B), whereas forskolin had no effect on ^{86}Rb efflux under isosmotic conditions (i.e., periods I and III).

Discussion

In most cell types, including 16HBE14o$^-$ cells (Boudreault & Grygorczyk, 2004; Fernandez-Fernandez et al., 2002), a hypotonic shock elicits RVD. The efficiency of RVD depends on many factors. In epithelial cells, the involvement of K^+ and Cl^- channels (Lock & Valverde, 2000; Wang, Morishima & Okada, 2003) as well as stretch-activated channels (SACs) (Boudreault & Grygorczyk, 2002) in RVD are well documented. In addition, previous reports suggest that signaling processes, mainly Ca^{2+}-dependent (Hoffmann & Dunham, 1995; Lang et al., 1998), are associated with RVD. In a recent report (Fernandez-Fernandez et al., 2002), a K^+ current flowing through maxi K^+ channels was substantially increased during RVD in 16HBE14o$^-$ cells, which led to the conclusion that these channels mediate RVD.

In our experiments, Gd^{3+} abolished the RVD in 16HBE14o$^-$ cells. Similar observations were made in another study (Fernandez-Fernandez et al., 2002). We noticed a continuous increase in cell volume during hyposmotic shock in the presence of Gd^{3+} without reaching a clear-cut plateau level. It is feasible that the SACs allow passage of K^+ and some anions, thus contributing to cell volume regulation during hypotonic stress, or that the SACs are nonselective for cations (Lang et al., 1998). At present, relatively little information is available about their activity in relation to RVD. Ca^{2+} entering the cells through the SACs is thought to activate Ca^{2+}-sensitive K^+ and Cl^- channels (Jakab et al., 2002). Gd^{3+}, a nonspecific inhibitor of SACs, is thus able to block the calcium increase (taking place via SACs) during hyposmotic stress in epithelial (Urbach et al., 1999) and nonepithelial (Miyauchi et al., 2000; Viana et al., 2001) cells. Hence, the absence of RVD in 16HBE14o$^-$ cells in the presence of Gd^{3+} (Fig. 2A) is likely due to the inhibition of Ca^{2+}-sensitive K^+ and Cl^- channels. Interestingly, the corresponding K^+ efflux during the hyposmotic period was only partially reduced (Fig. 2B), suggesting that a large fraction of the K^+ efflux occurs via Gd^{3+}-insensitive pathways. A hypothesis can be proposed to explain the failure of the remainder of the K^+ efflux to restore cell volume. Taking into account that SACs behave as a Ca^{2+} entry pathway, thus increasing the intracellular Ca^{2+} concentration during RVD, it is possible that the failure to establish the RVD in the presence of Gd^{3+} may be related to the impairment of Ca^{2+} signaling pathways involved in RVD. This view is supported by recent findings related to the Gd^{3+} blockade of RVD. In addition to inhibiting SACs, Gd^{3+} blocks transient receptor potential vanilloid 4 (TRPV4), a nonselective cation channel reported to be involved in cell volume regulation in a Ca^{2+}-dependent manner (Fernandez-Fernandez et al., 2002; Becker et al., 2005; Liu et al., 2006). Liu and coworkers (2006) reported that TRPV4 and aquaporin 5 (AQP5) concertedly control the RVD in salivary epithelial cells. Thus, it is feasible to establish a functional link between the Gd^{3+}-sensitive TRPV4, thought to support the hypotonicity-induced Ca^{2+} entry in 16HBE14o$^-$ cells (Fernandez-Fernandez et al., 2002), and AQP5, which are most likely expressed in these cells (Kreda et al., 2001; King, Kozono & Agre, 2004) and involved in RVD.

The involvement of K^+ channels, distinct from the Gd^{3+}-sensitive K^+-efflux pathways, in RVD was confirmed using quinine, a widely used epithelial K^+ channel blocker (MacLeod, Lembessis & Hamilton, 1992; Adorante & Cala, 1995; Nilius et al., 1995). Quinine most likely blocked maxi K^+ channels in our experiments as these were reported to mediate the RVD in 16HBE14o$^-$ cells (Fernandez-Fernandez et al., 2002). Although the inhibition level of K^+ efflux was similar to that seen with Gd^{3+} (Fig. 3B), the cell volume recovered toward the level before the prehyposmotic challenge, when osmolality was restored in the presence of quinine. The K^+ efflux

during the hyposmotic shock, in the presence of either the respective blockers for SACs or K^+ channels, decreased by about 30%, which was sufficient to impair the completion of RVD. Since no other major pathways for K^+ efflux are yet described, we suggest that SACs, TRPV4 channels and K^+ channels may be required for the efficiency of RVD in 16HBE14o$^-$ cells. The greater degree of cell volume changes seen with SACs and TRPV4 channel blockade strongly suggests that signaling processes (e.g., Ca^{2+}-dependent RVD) are involved in the RVD, in agreement with previous reports (Fernandez-Fernandez et al., 2002; Liu et al., 2006).

It appears that the level of RVD and the magnitude of K^+ efflux are strongly related to each other. In contrast with the blockade of SACs, TRPV4 channels and K^+ channels, the blockade of Cl^- channels inhibited completely the activation of K^+ efflux during the hyposmotic challenge. Thus, the RVD-associated Cl^- efflux appears to take place primarily via Cl^- channels, and RVD-related K^+ efflux is linked with the efficiency of the Cl^-efflux mechanism.

Many studies have reported the involvement of intracellular signaling processes in the epithelial cell RVD (Lang et al., 1998; Mongin & Orlov, 2001; Jakab et al., 2002; Wehner et al., 2003). Various kinases are considered to be involved in cell volume regulation (Lang et al., 1998). For example, volume regulatory $^{86}Rb^+$ efflux in intestinal epithelial cells was inhibited by herbimycin A and genistein, suggesting a role of protein tyrosine kinases (PTKs) in RVD (Tilly et al., 1993). Both PTK inhibitors tested in this study, genistein and tyrphostin 23, albeit not very specific, impaired RVD and decreased the corresponding K^+ effluxes in 16HBE14o$^-$ cells. The inhibition level of K^+ efflux exerted by PTK inhibitors may imply a role of PTK in controlling the swelling-sensitive efflux of Cl^- and organic osmolytes (Tilly et al., 1993; Sorota, 1995; Voets et al., 1998).

Under hyposmotic or isosmotic conditions, the volume of a variety of cells is diminished by elevated cellular cAMP levels, an effect mainly due to the activation of both Cl^- and K^+ channels (Lang et al., 1998). In addition, cAMP has been reported to upregulate AQP5 expression in lung epithelial cells, hence increasing water permeability (Yang, Kawedia & Menon, 2003; Sidhaye, Hoffert & King, 2005). In hyposmotically challenged 16HBE14o$^-$ cells, forskolin, an activator of adenylyl cyclase, increased both RVD efficiency and K^+ efflux (Fig. 7). In contrast with other cell types (Nakahari & Marunaka, 1996), forskolin had no effect on cell volume under isosmotic conditions. Moreover, since forskolin did not alter significantly the peak level of cell swelling induced by the hyposmotic challenge, the increase in cAMP concentration may be potentiated during the RVD phase. Thus, there may be a threshold for the cAMP level that led to K^+ efflux during RVD in 16HBE14o$^-$ cells. In accordance with this notion, a cAMP-activated K^+ conductance was reported in other cell types (Van Driessche & Erlij, 1988; Wang, 1995; Izu et al., 2002; Srinivas et al., 2004). As is commonly accepted, cAMP also activates Cl^- channels. For example, forskolin has been reported to enhance the amplitude of the swelling-induced Cl^- current in human cardiac myocytes (Oz & Sorota, 1995). A cAMP-dependent Cl^- conductance was observed in rat hepatocytes, where cAMP appears to alter the volume set point of a swelling-activated channel (Meng & Weinman, 1996). In addition, many epithelial cells, including 16HBE14o$^-$ cells (Cozens et al., 1994), express the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl^- channel which may promote Cl^- efflux. Our data cannot exclude the involvement of a cAMP-activated Cl^- efflux in the enhancement of the RVD induced by forskolin. It is reasonable to speculate that the increase in RVD efficiency seen in the presence of forskolin is partly determined by AQP5 upregulation as well. Previous reports showed that long-term exposure (hours) to forskolin or cAMP analogues induced an increase in AQP5 expression in lung epithelial MLE-12 cells (Yang et al., 2003; Sidhaye et al., 2005). However, short-term exposure (30 min) induced endocytosis of AQP5 (Sidhaye et al., 2005). Moreover, in the MLE-12 cell line, it has been reported that hypotonic stress reduced the AQP5 abundance between 10 and 30 min of hypotonicity (Sidhaye et al., 2006). From the data that are presented in our study, in which forskolin did not modify the peak level of cell swelling induced by the hyposmotic challenge, it can be suggested that the abundance of AQP5 was not modified by 30-min forskolin preincubation and by the onset of RVD. Whether the increased expression of AQP5 contributes to the second phase of RVD in 16HBE14o$^-$ cells has still to be assessed.

Revealing the underlying mechanisms involved in cell volume regulation in respiratory epithelial cells is beneficial for understanding the pathophysiology of cystic fibrosis because it has been shown that RVD is impaired in cystic fibrosis human airways (Vazquez, Nobles & Valverde, 2001). Restricted water efflux of transporting lung epithelia that retain water during transepithelial transport of electrolytes may lower the volume of the airway surface liquid and, in this way, hamper effective mucociliary clearance.

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