Swelling-activated cation-selective channels in A6 epithelia are permeable to large cations

JINQING LI, PATRICK DE SMET, DANNY JANS, JEANNINE SIMAELS, AND WILLY VAN DRIESSCHE

Laboratory of Physiology, K. U. Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium

Li, J. inqing, Patrick De Smet, Danny J ans, Jeannine Simaels, and Willy Van Driessche. Swelling-activated cation-selective channels in A6 epithelia are permeable to large cations. Am. J. Physiol. 275 (Cell Physiol. 44): C358–C366, 1998.—Effects of basolateral monovalent cation replacements (NaCl by LiCl, KCl, CsCl, methylammonium, or guanidinium) on permeability to 86Rb of volume-sensitive cation channels (VSCC) in the basolateral membrane and on regulatory volume decrease (RVD), elicited by a hypotonic shock, were studied in A6 epithelia in the absence of apical NaCl uptake. A complete and quick RVD occurred only when the cells were perfused with NaCl or LiCl saline. With both cations, hypotonicity increased basolateral 86Rb release (Rb1), which reached a maximum after 15 min and declined back to control level. When the major cation was KCl, CsCl, methylammonium, or guanidinium, the RVD was abolished. Methylammonium induced a biphasic time course of cell thickness (Tc), with an initial decline of Tc followed by a gradual increase. With KCl, CsCl, or guanidinium, Tc increased monotonously after the rapid initial rise evoked by the hypotonic challenge. In the presence of KCl, CsCl, or methylammonium, Rb1 remained high during most of the hypotonic period, whereas with guanidinium blockage of Rb1 was initiated after 6 min of hypotonicity, suggesting an intracellular location of the site of action. With all cations, 0.5 mM basolateral GdCl3 completely blocked RVD and fully abolished the Rb1 increase induced by the hypotonic shock. The lanthanide also blocked the additional volume increase induced by CsCl, KCl, guanidinium, or methylammonium. When pH was lowered from 7.4 to 6.0, RVD and Rb1 were markedly inhibited. This study demonstrates that the VSCCs in the basolateral membrane of A6 cells are permeable to KCl, RbCl, CsCl, methylammonium, and guanidinium, whereas a marked inhibitory effect is exerted by GdCl3, protons, and possibly intracellular guanidinium. LiCl, 86Rb efflux; renal epithelia; volume-sensitive cation channel; gadolinium; protons; regulatory volume decrease

When exposed to a hypotonic environment, most cells initially swell and then, within several minutes, shrink back toward their original volume by a complex process known as regulatory volume decrease (RVD) (3, 12, 21). RVD is mediated by the loss of intracellular solutes, including KCl and Cl−, accompanied by an obligatory decrease of cell water content. Several ion transport mechanisms, such as KCl channels, Cl− channels, poorly selective cation channels, anion channels, and cotransporters, have been reported to be involved in RVD (11, 13). Like most cell types, A6 epithelial cells show an RVD after swelling caused by dilution of the basolateral solution (6, 7). In this preparation, RVD is also mediated by KCl and Cl− efflux and to a smaller extent by amino acid excretion. This study focuses on the investigation of the cation pathway used for the release of KCl. In a previous study (5), we demonstrated that KCl release occurs through a volume-sensitive cation channel (VSCC) in the basolateral membrane of A6 cells that is blockable by lanthanides, suggesting that it belongs to the class of poorly selective channels. Nonselective cation channels have been widely reported in various epithelial cells (19, 25). Some of them are mechanosensitive or stretch sensitive (4) and might be involved in RVD. In this paper, we extend our study of the VSCC, aiming at the investigation of the permeability properties of this pathway. Because previous studies demonstrated that, in A6 cells, KCl is the cation predominantly excreted during RVD, we focused our experiments on the influence of different monovalent cations on KCl release. We chose to use 86Rb as a substitute for KCl (22) because of the striking similarities of the physical and chemical properties of both cations and the fact that both have a comparable permeability in many transport systems. Except for NaCl and LiCl, all the other investigated monovalent cations appeared to enter the cells through the relatively poorly selective cation pathway, activated by cell swelling. In A6 epithelia, this VSCC seems to provide the pathway for cationic osmolyte release during RVD, rather than the highly selective KCl pathway.

MATERIALS AND METHODS

Preparation. A6 cells were obtained from Dr. J. P. Johnson (Univ. Pittsburgh, Pittsburgh, PA) and cultured on permeable Anopore filter supports (pore size 0.2 µm) (Nunc Intermed, Roskilde, Denmark) as described previously (25). The cells were seeded at a density of 104/cm2. For our experiments, we used confluent monolayers of cells of passages 104–112 that were cultured between 8 and 22 days. The epithelial monolayers were mounted in the respective chambers for monitoring cell thickness (Tc) and for measuring 86Rb efflux. Tc measurement. The method used for recording Tc has been previously described in detail (26). The filter supports were coated with fluorescent microspheres of 1 µm diameter (LS081, Molecular Probes, Eugene, OR) embedded in a thin gelatin layer. The apical membrane of the epithelium was labeled with fluorescent avidin-coated microbeads (F8776, Molecular Probes) by exposing the apical surface for 30 min to 1 ml of an isotonic NaCl Ringer solution that contained 2 µl/ml of the beads. The microbeads that did not attach to the membranes were washed off by rinsing the apical compartment with NaCl Ringer solution. The preparation was mounted on the microscope stage in a modified Ussing chamber and short-circuited. The distance between a basolateral reference bead and an apical bead was recorded as Tc. The size of the microsphere was taken into account, and Tc values were corrected by subtracting 1 µm from the measured heights and expressed in percentage of the control. Control values were recorded just before the hypotonic shock was applied and are reported in Figs. 1–7. N represents...
the number of tissues, and n represents the number of cells used to calculate the average.

$^{86}$Rb efflux. The epithelium was loaded with $^{86}$Rb by exposing the basolateral membrane to isotonic K$^+$-free loading solution containing 50 µCi of $^{86}$Rb for 1 h. The loading solution (262 mosmol/kgH$_2$O) contained (in mM) 135 NaCl, 1 CaCl$_2$, and 2.5 NaHCO$_3$. The epithelial monolayers loaded with $^{86}$Rb were gently rinsed with isotonic NaCl Ringer solution and then mounted in Ussing-type chambers with two compartments. Before the collection of samples was started, the remaining tracer attached to the cell surface and filter support was washed off by perfusing the chamber halves for 15 min. During this preefflux period, the basolateral perfusate was isotonic NaCl solution, whereas the apical side was exposed to hyposmotic N-methyl-D-glucamine-HCl (NMDG-Cl) solution. Rapid washout of the tracer at the basolateral side was guaranteed by vigorously stirring this compartment with a magnetic stirring bar. During the experiment, both compartments were continuously and separately perfused with the desired solutions according to the different protocols. The samples (perfused solutions) were continuously collected in the counting tubes by a suction pump. The tubes were changed at 3-min intervals. At the end of the experiment, radioactivity ($^{86}$Rb) remaining in the epithelium was extracted by treating the cells with a 5% TCA solution. Five additional samples for apical and basolateral compartments of this solution were collected. Radioactivities in all efflux samples and in the TCA extracts were quantified by gamma counting. Data are expressed as a rate constant: the ratio of the radioactivity released from the cells per minute to the counts remaining in the cells at that moment (20).

$^{134}$Cs uptake. $^{134}$Cs uptake experiments were performed on polarized epithelia that remained in the filter holders. The apical surface was exposed to hyposmotic NMDG-Cl solution. Isoosmotic basolateral solution contained 70 mM Cs$^+$ and 112 mM sucrose (see Solutions). Hyposmotic conditions were obtained by removing sucrose. As described below (Experimental protocols), the experiments consisted of three periods (isosmotic, experimental, isosmotic). During the second period, the basolateral surface was exposed to 20 µCi $^{134}$Cs. The tracer was removed from the filter during the third period by carefully washing at 10°C with isotonic solution. $^{134}$Cs accumulated in the cell was obtained by treating the cells with 1 M NaOH solution. $^{134}$Cs in the cell extract and loading solution was determined by Cerenkov counting. The concentration of cold Cs$^+$ in the loading solution was calculated from data provided by the manufacturer. For the calculation of the intracellular concentration of Cs$^+$, we assumed a $T_c$ of 7 µm (6). Cs$^+$ uptake was determined under the following (experimental) conditions: 1) isosmotic, 2) hyposmotic, 3) isosmotic with 0.5 mM Gd$_3^+$ in the basolateral solution, and 4) hyposmotic with 0.5 mM Gd$_3^+$ in the basolateral solution.

Experimental protocols. The experimental protocol consisted of three periods. During the first period (30 min), we exposed the basolateral side of the epithelium to the isosmotic solution (260 mosmol/kgH$_2$O). The osmolality of the basolateral bath was shockwise reduced from 260 to 140 mosmol/kgH$_2$O during the second period (60 min) by withdrawing sucrose or NaCl. Finally, the osmolality of the basolateral bath was restored to isosmotic during the third period (30 min) of the experiment. Throughout the experiments, the apical side of the tissues was exposed to a Na$^+$-free hyposmotic solution (140 mosmol/kgH$_2$O). Protocols used for $T_c$ measurements were exactly identical to those for the $^{86}$Rb efflux experiments. In the experiments with Gd$_3^+$, GdCl$_3$ (0.5 mM) was added in the basolateral compartment at least 15 min before the tracer was sampled.

Solutions. The apical bathing solution was hyposmotic (140 mosmol/kgH$_2$O) and contained (in mM) 70 NMDG$^+$, 2.5 K$^+$, 2.5 HCO$_3^-$, 1 Ca$^{2+}$, and 72 Cl$^-$ (pH 8.0). Basolateral solutions contained (in mM) 70 X, 5 K-HEPES, 1 Ca$^{2+}$, and 72 Cl$^-$ (pH 7.4), where X represents one of the cations tested (Na$^+$, Li$^+$, Cs$^+$, K$^+$, guanidinium [(NH$_2$)$_2$CNH$_2$]), or methylammonium (CH$_3$$_2$NH$_3^+$). The osmolality of these solutions was 140 mosmol/kgH$_2$O (hyposmotic). Isosmotic solutions (260 mosmol/kgH$_2$O) were prepared by adding sucrose (112 mM) or 65 mM NaCl. To study the effect of pH, bathing solutions were buffered with 5 mM HEPES for the solutions with pH 7.4 and with 5 mM MES for the solutions with lower pH values. To study the effect of Gd$_3^+$, 0.5 mM GdCl$_3$ was present in the respective basolateral bathing solutions during all three experimental periods. All chemicals were obtained from Sigma, Fluka, or Merck. $^{86}$Rb and $^{134}$Cs were purchased as chloride salt from Amersham.

**RESULTS**

Control experiments. Previously, we demonstrated that A6 epithelia were able to recover their volume after they had been swollen by hypotonic basolateral solutions (6, 7). In these studies, we reduced the osmolality by removing NaCl from the bath. Such a procedure gives rise to drastic changes in ionic strength that could interfere with the mechanisms involved in volume recovery. When volume regulation is studied in the presence of different cations, the salt withdrawal procedure could result in responses that depend on the type of cation removed. Therefore, in this study, we wanted to lower the osmolality by removing the same type of osmolyte. For this purpose, we chose to use sucrose, thereby keeping the ionic strength and ion concentrations constant during the entire experiment. The effects of NaCl and sucrose removal on volume recovery and $^{86}$Rb efflux are compared in Fig. 1. Figure 1A shows that both procedures resulted in a similar time course of cell swelling and volume recovery. The time constants of the RVD were comparable, that is, 4.1 and 3.4 min for NaCl and sucrose withdrawal, respectively. The peak values of $T_r$ were 163 ± 2% (n = 59) and 154 ± 2% (n = 73) with NaCl and sucrose removal, respectively. The slightly lower $T_r$ peak reached with sucrose could be related to differences in intracellular composition (cellular osmotic activity) as a consequence of the lower ionic strength during the isotonic period.

The results of $^{86}$Rb release showed that the basolateral $^{86}$Rb efflux ($R_{B}^{86}$Rb) was markedly lower in experiments in which the osmolality was reduced by sucrose withdrawal. $R_{B}^{86}$Rb reached a maximum (4.9 ± 0.3 min$^{-1}$, N = 6) after 9 min when NaCl was removed, whereas the peak value (2.7 ± 0.4 min$^{-1}$, N = 6) for sucrose withdrawal experiments was reached after 15 min. With both procedures, apical $^{86}$Rb efflux ($R_{A}^{86}$Rb) was negligible in isosmotic as well as in hypotonic conditions. For clarity, only the $R_{A}^{86}$Rb data recorded with sucrose removal are shown. Furthermore, we will refer to the $T_r$ trace and $R_{B}^{86}$Rb data recorded with sucrose as control data.
during the initial phase of the hypotonic shock. This reduction of \( R_{\text{Rb}}^{\text{bl}} \) however, does not give rise to a noticeable inhibition of the volume recovery, suggesting an effect of sucrose on the diffusion delay of \( ^{86}\text{Rb} \) into the basolateral bath.

Effect of basolateral \( \text{Li}^+ \). \( \text{Li}^+ \) and \( \text{Na}^+ \) have similar chemical properties. As with \( \text{Na}^+ \), a perfect RVD was recorded with \( \text{Li}^+ \), which brought back the cells to their original thickness (Fig. 2A). With \( \text{Li}^+ \) as the principal cation, \( T_c \) reached a peak (180 ± 3%) that was noticeably larger than with \( \text{Na}^+ \) (164 ± 2%). Also, the time constant of the \( T_c \) decline was clearly longer with \( \text{Li}^+ \) (6.5 min) than with \( \text{Na}^+ \) (3.4 min). The larger peak value and time constant recorded with \( \text{Li}^+ \) show that this cation exerts an inhibitory effect on volume recovery. On the other hand, in the \( ^{86}\text{Rb} \) efflux measurement, an almost identical activation of the \( R_{\text{Rb}}^{\text{bl}} \) to the hypotonic shock was obtained as with \( \text{Na}^+ \) (Fig. 2B). In the presence of \( \text{Li}^+ \), \( R_{\text{Rb}}^{\text{bl}} \) rose from 0.20 ± 0.03 to 2.9 ± 0.1 min\(^{-1}\) (with \( \text{Na}^+ \) control, 2.7 ± 0.4 min\(^{-1}\)) within 15 min. The results show that with both \( \text{Li}^+ \) and \( \text{Na}^+ \) the same pattern is obtained for the RVD and the associated \( ^{86}\text{Rb} \) efflux. The effect of \( \text{Li}^+ \) on \( T_c \) peak and increased time constant of \( T_c \) decline cannot be therefore attributed to an effect on the \( \text{K}^+ \) efflux.

Effect of basolateral \( \text{Cs}^+ \). Because the size of \( \text{Cs}^+ \) compares well with that of \( \text{K}^+ \) and \( \text{Rb}^+ \), we verified whether this cation can pass through the VSCC. When we exposed the basolateral side of the A6 cells to \( \text{Cs}^+ \)-containing (70 mM) hypotonic saline, RVD was completely abolished (Fig. 3A). Moreover, after an

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The 86Rb efflux measurements reflect the elevated level of cellular water content. Rb accumulation of osmolytes during the hypotonic shock were completely blocked. Tc remained constant at the initial “peak” level (Fig. 3A). After isosmotic conditions were restored, Tc returned to the control level recorded before the hypotonic shock, which confirms that osmolyte accumulation did not take place. The inhibitory effect of Gd3+ on the influx of osmolytes (Cs+) suggests that it occurs through the lanthanide-sensitive pathway activated by cell swelling and responsible for the RVD. Gd3+ (0.5 mM) blocks the outward Rb+ flux. Also, an inhibition of K+ entry can be expected. This was confirmed in our 134Cs uptake studies that demonstrated that, in the presence of Gd3+, the uptake was in iso- and hyposmotic conditions 16.0 ± 3.8 and 40.9 ± 4.1 mM (N = 6), respectively. This reveals a net hyposmotically induced uptake of 24.9 ± 5.1 mM. Part of the Gd3+-insensitive Cs+ uptake could be explained as an exchange of Cs+ for K+.

The effect of basolateral K+. Similar and even more pronounced effects on the response to a hypotonic challenge as described above for Cs+ were found when basolateral Na+ was replaced by K+. Figure 4A shows initial fast increase of 65%, Tc further increased up to 224 ± 7% within 60 min after the initiation of the hypotonic shock. This secondary increase of Tc is consistent with this idea, that is, that the cells gain additional amounts of solute, followed by water. It should be noted that the accumulation of osmolytes during the hypotonic treatment is also reflected in the elevated level of cell volume after the osmolality is returned to normal (260 mosmol/kgH2O). The 86Rb efflux measurements (Fig. 3B) showed that the Rb+ increase was markedly delayed, reaching a maximum of 2.0 ± 0.1 min⁻¹ after 30 min. It is clear that, during the initial phase of the hypotonic shock, Rb+ increased noticeably slower than in the control experiment, indicating an inhibitory effect of Cs+ on the Rb+ efflux. This inhibitory effect of Cs+ is therefore most likely caused by a competition of Rb+ and Cs+ for the binding sites in the channel. The steady increase in Tc over the entire hypotonic period suggests that Cs+ enters the cell, thus giving rise to an accumulation of osmolytes and the subsequent increase of cellular water content. Rb+ remained on a higher plateau level during the entire hypotonic phase (1.7 ± 0.1 min⁻¹), which seems to be evoked by the additional swelling caused by the influx of Cs+. We verified this hypothesis by measuring 134Cs uptake in a set of paired experiments. In isosmotic conditions, we obtained an apparent uptake of Cs+ of 15.8 ± 2.4 mM (N = 6), an amount which might be at least partly attributed to accumulation of isotope in the filter support of the cells and should therefore be considered as background. During hypotonicity, we measured an uptake of 79.4 ± 10.3 mM Cs+ (N = 6). Consequently, hypotonicity gives rise to an increase of Cs+ uptake by 63.6 ± 7.2 mM.

These results suggest that the volume-sensitive pathway is permeable to Cs+. This was confirmed by the experiment in the presence of Gd3+ in the basolateral bathing solution. Previously, we demonstrated that 0.5 mM of the lanthanide completely blocked RVD as well as the associated increase of Rb+ (5). We therefore used this potent inhibitor of volume regulatory mechanisms to probe the permeability of the cation pathway for Cs+. In the presence of Gd3+, both the additional swelling of the cells as well as the elevated level of the Rb+ during the hypotonic shock were completely blocked. Tc remained constant at the initial “peak” level (Fig. 3A). After isosmotic conditions were restored, Tc returned to the control level recorded before the hypotonic shock, which confirms that osmolyte accumulation did not take place. The inhibitory effect of Gd3+ on the influx of osmolytes (Cs+) suggests that it occurs through the lanthanide-sensitive pathway activated by cell swelling and responsible for the RVD. Gd3+ (0.5 mM) blocks the outward Rb+ flux. Also, an inhibition of K+ entry can be expected. This was confirmed in our 134Cs uptake studies that demonstrated that, in the presence of Gd3+, the uptake was in iso- and hyposmotic conditions 16.0 ± 3.8 and 40.9 ± 4.1 mM (N = 6), respectively. This reveals a net hyposmotically induced uptake of 24.9 ± 5.1 mM. Part of the Gd3+-insensitive Cs+ uptake could be explained as an exchange of Cs+ for K+.

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that with basolateral K+, the hypotonic shock provoked an extremely large swelling of the cells. At the end of the hypotonic period, Tc reached 373 ± 35%. The additional increase of Tc was correlated with a marked elevation of Rbbl, reached a maximum of Tc 4.7 ± 0.4 min⁻¹ after 21 min of hypotonic treatment (Fig. 4B), but, unlike with Cs⁺-containing solution, Rbbl decreased quickly and reached the control level toward the end of the hypotonic shock. The marked overshoot in Rbbl could be due to an exchange of RB⁺ for K⁺. Electro neutrality requires that cellular uptake of K⁺ is accompanied by an equal amount of anions or by the excretion of another cation type (RB⁺). Such a mechanism could account for the overshoot in Rbbl. In the presence of Gd³⁺, the additional cell swelling was markedly reduced, although not completely blunted as in the experiments with Cs⁺. In the presence of Gd³⁺, Tc increased to 248% 30 min after the hypotonic shock was applied and in addition remained approximately constant. Gd³⁺ also blocked the increase of Rbbl evoked by the hypotonic shock, although a slight increase (from 0.24 ± 0.02 to 0.60 ± 0.03 min⁻¹) remained. The fact that cell volume increase is not completely blocked by Gd³⁺, whereas the ⁸⁶Rb efflux is totally abolished, indicates that K⁺ entry proceeds partly through a pathway that is not taken by RB⁺. Indeed, K⁺ can pass the cell through the native K⁺ channels, which have a markedly lower permeability for RB⁺ (unpublished observations). After isotonic conditions were returned, this pathway also enabled the osmolyte release, which returns cells rapidly back to their original volume. This is in marked contrast with the time course of Tc in the experiments with Cs⁺ (Fig. 3). Here, cells remain swollen over a more extended time period, maintaining also that the cation pathway and thus Rbbl were activated. The delayed closure of the cation pathway is a requirement for Cs⁺ release from the cell, which should return cell volume to its original value.

Effect of basolateral methylammonium. Methylammonium, an organic monovalent cation, whose size amounts to 3.8 Å (23), was used to probe the permeability of the volume-activated cationic pathway. With this organic cation, Tc showed a biphasic response during the hypotonic challenge (Fig. 5A). After Tc reached a maximum of 167 ± 3%, it slightly declined to 155 ± 4%. Then Tc increased again and reached 171 ± 6% by the end of the hypotonic shock. This biphasic behavior suggests that, in the initial phase, volume recovery is taking place (mediated by a net efflux of osmolytes), whereas, during the last part of the hypotonic period, solute moves into the cell. The latter phenomenon resembles the volume increase observed in the experiments with Cs⁺. In the presence of Gd³⁺, the biphasic response of Tc was abolished, which gave rise to a plateau after the initial increase during the hypotonic challenge (Fig. 5A). This result suggests that both inward and outward solute movements were blocked by Gd³⁺.

⁸⁶Rb efflux experiments shown in Fig. 5B demonstrate that Rbbl increased up to 3.3 ± 0.1 min⁻¹ within 21 min after the initiation of the hypotonic challenge. Then Rbbl gradually declined, although it remained noticeably above the level reached by the end of the shock in control experiments. This result suggests that the RB⁺ pathway remained active during the hypotonic shock. The time courses of Rbbl during the initial phase of the hypotonic shock in control conditions and with methylammonium are quite comparable. Also, Rbbl reached approximately the same level. Contrary to the experiments with Cs⁺ (Fig. 3), in the beginning of the hyposmotic shock Rbbl was not inhibited by methylammonium. When Gd³⁺ was present in the methylammonium-containing solution, the Rbbl was completely abolished, confirming that the pathway activated by cell swelling was blockable by Gd³⁺. This agrees with the Tc data showing the sensitivity of both components of the biphasic behavior of Tc to Gd³⁺.

The effect of basolateral guanidinium. Guanidinium is a large organic monovalent cation (size 5.8 Å) (23). With guanidinium as principal cation, hypotonicity elicited rapid cell swelling followed by a gradual increase of cell volume (Fig. 6A). The initial Tc increase amounted to 153 ± 2% and was reached within 4 min. During the subsequent phase, Tc increased slowly, and, toward the end of the hypotonic treatment, Tc attained 220 ± 6%. This secondary increase in Tc again suggests an accumulation of intracellular solute, attributed to the entrance of guanidinium and Cl⁻. In the ⁸⁶Rb efflux experiments (Fig. 6B), Rbbl quickly increased from 0.19 ± 0.01 to 1.75 ± 0.03 min⁻¹ within 6 min after the hypotonic treatment, thereafter decreasing, and at the end of the hypotonic period reached 0.33 ± 0.02 min⁻¹. This peak is noticeably lower than that in the
control experiment. The gradual increase of $T_c$ on the one hand and the reduced $^{86}$Rb release on the other hand seem paradoxical. The former suggests an influx of guanidinium, whereas the latter appears like a blocking effect of guanidinium on the Rb$^+$ efflux. These observations may be explained by assuming a finite permeability for guanidinium that leads to the additional swelling, whereas the inhibition of $R^{\text{bl}}_{Rb}$ suggests an intracellular site for the blockage of the Rb$^+$ pathway by guanidinium.

Protons block the RVD and the $R^{\text{bl}}_{Rb}$ induced by hypotonic shock. Protons are potent blockers of many cation channels and also of some nonselective cation channels (18). Effects of symmetrical pH changes to 8.0, 7.4, 7.0, 6.0, and 5.0 on the VSCCs were examined with Na$^+$-containing basolateral bathing solution. Between pH 7.0 and 8.0, no differences were found in the RVD as well as the $R^{\text{bl}}_{Rb}$. However, RVD was considerably inhibited and delayed once the pH was lowered to 6.0 (Fig. 7A). At pH 6.0, $T_c$ initially increased to 158 ± 3% within 4 min, then decreased to 125 ± 3% within 27 min, and afterward stayed at a plateau. These observations were confirmed in the $^{86}$Rb efflux experiments (Fig. 7B). Hypotonicity increased $R^{\text{bl}}_{Rb}$ only from 0.25 ± 0.01 to 0.68 ± 0.04 min$^{-1}$. These results indicate that the pathway activated by cell swelling is sensitive to protons.

**DISCUSSION**

In this study, we examined the effects of some monovalent cations on the $^{86}$Rb efflux pathway that is involved in the RVD in polarized A6 cells. Because the basolateral membrane is highly permeable to water, a sudden decrease of the osmolality at this side of the epithelium results in a rapid volume increase, whereas osmotic perturbations at the apical surface are without effect (7). Under all conditions, cells swelled rapidly after the basolateral osmolality was reduced, indicating that the water permeability of this membrane was preserved. However, only with Na$^+$ and Li$^+$ as major cations were cells able to develop volume recovery, whereas the RVD was abolished with K$^+$, Cs$^+$, methylammonium, or guanidinium. Because we used a high concentration of the cations in these experiments, these compounds cannot be considered as simple inhibitors of the pathways for releasing osmolytes. Therefore, we have to consider two fundamental questions. 1) Is volume recovery impeded by either blockage of the release pathway for osmolytes and/or opening of a pathway that enables the influx of extracellular osmotic active substances? 2) Does the osmolyte release and uptake occur through the same or separate pathways? In relation to these questions, it should be noted that simple blockage of osmolyte release should keep volume constant at the level that was reached after the initial swelling phase, whereas osmolyte entry could give rise to additional cell swelling. This additional swelling will challenge volume regulation more and lead to further activation of the mechanisms that should take care of the RVD and result in the opening of pathways that provide osmolyte excretion under normal physiological conditions (basolateral NaCl). Because of the unusual conditions, in which cells are exposed to extracellular cations that permeate through the regulatory pathway, the opposite effect is obtained: cells will take up solute instead of releasing osmolytes, which results in additional swelling. The increased cell

![Image](https://example.com/fig6.png) Fig. 6. Effect of guanidinium on RVD and $R^{\text{bl}}_{Rb}$. Tissues were exposed to basolateral guanidinium (70 mM) during the entire experiment. Gd$^{3+}$ (0.5 mM) was added to the basolateral side at least 10 min before the beginning of the experiment. A: recording of $T_c$. Control values of $T_c$ in isosmotic conditions were as follows: with guanidinium = 7.8 ± 0.3 µm (N = 5, n = 59); with guanidinium + Gd$^{3+}$ = 7.0 ± 0.2 µm (N = 6, n = 64). B: averaged $R^{\text{bl}}_{Rb}$ data from 6 experiments. For clarity, error bars were omitted.

![Image](https://example.com/fig7.png) Fig. 7. Inhibition of RVD and $R^{\text{bl}}_{Rb}$ by protons. When pH of the bathing solutions for both sides of the cells was lowered to 6.0, both RVD and higher $R^{\text{bl}}_{Rb}$ induced by the hypotonic challenge were clearly blocked. A: recording of $T_c$. Control value of $T_c$ in isosmotic conditions at pH 6.0 was 7.7 ± 0.4 µm (N = 4, n = 42). B: averaged $R^{\text{bl}}_{Rb}$ data from 6 experiments. For clarity, error bars were omitted.
volume will lead to further activation of the pathway for osmolyte release, which is reflected in the increase of \(^{86}\text{Rb}\) efflux. Thus, under conditions in which high concentrations of permeant cations are present in the basolateral bath, a positive instead of negative feedback is taking place, giving rise to a steady increase of cell volume, as illustrated in Figs. 3, 4, and 6. As far as the experiments with high K\(^+\) are concerned, it is reasonable to assume that the RVD is inhibited by abolishing the driving force for K\(^+\) release and that the additional swelling is due to the influx of K\(^+\). Similar results were obtained with Cs\(^+\) and guanidinium, indicating that these cations enter the cells during the hypotonic phase of the experiment. On the other hand, in isosmotic conditions, no noticeable volume increase was observed after Na\(^+\) was replaced by either Cs\(^+\) or guanidinium (not shown), which indicates that, in isosmotic conditions, the permeability of the basolateral border for these cations is negligible. However, with methyammonium, cell volume increased by 7 ± 1%, demonstrating that the basolateral membrane has a finite permeability for this cation. A conspicuous increase in cell volume was recorded after Na\(^+\) was replaced by K\(^+\), most likely caused by K\(^+\) entry through the native K\(^+\) channels in the basolateral membrane. These results show that cell swelling is required to activate the permeability of the basolateral membrane for Cs\(^+\) and guanidinium.

In this study, we lowered the osmolarity by removing sucrose and found that the nonelectrolyte did not affect volume recovery, whereas \(R_{\text{Rb}}\) was markedly depressed. In this context, it should be noted that volume regulation is mediated by K\(^+\) release, whereas \(^{86}\text{Rb}\) is utilized as substitute for K\(^+\) to monitor cation efflux. A critical analysis of the use of \(^{86}\text{Rb}\) as tracer for K\(^+\) in skeletal muscle (8) demonstrated that the isotope cannot replace K\(^+\) in several transport pathways. Dorup and Clausen (8) showed that in this preparation K\(^+\) efflux was 2.3 times larger than that of \(^{86}\text{Rb}\). In a preliminary set of experiments with A6 epithelia (unpublished observations), we compared \(^{86}\text{Rb}\) and \(^{42}\text{K}\) efflux. In isosmotic conditions, \(^{42}\text{K}\) efflux was three times larger than that of \(^{86}\text{Rb}\). On the other hand, during hypotonicity evoked by NaCl removal, the efflux of both tracers reached exactly the same peak and differences in the time course were not discernible. This test indicates that \(^{86}\text{Rb}\) can be utilized to probe the cation pathway activated by cell swelling, whereas the isotope underestimates the permeability of the native K\(^+\) channels. Furthermore, it should be noted that sucrose, as well as other nonelectrolytes, affects cation permeability of ion channels (1, 17) as well as ion mobility in aqueous solutions (17). The latter effect could especially affect tracer washout through porous membranes as those used to support the monolayers. Moreover, studies on the gramicidin A channel (1) demonstrated that the reduction of the cation permeability by sucrose depends on the permeating cation species, and for this channel the effect on Rb\(^+\) was markedly larger than on Na\(^+\). When the differences in permeability for K\(^+\) and Rb\(^+\) of the native K\(^+\) channels, the dependence of the inhibitory effect on cation species, and the possible reduction of the mobility of ions by sucrose are considered, it is quite likely that residual traces of sucrose adjacent to the basolateral border can exert major effects on the tracer washout, without inhibiting the osmolyte (K\(^+\)) release from the cell.

Evidence for the fact that Cs\(^+\) passes through the K\(^+\) release pathway comes from experiments using Gd\(^{3+}\) to block the additional volume increase. Under all conditions, flux experiments showed that the lanthanide blocks the volume-activated \(^{86}\text{Rb}\) release completely, and this, as well as a previous study (5), revealed that the trivalent cation is a very potent inhibitor of the cation pathway involved in volume recovery. We also found that the blockage occurred from the intracellular side, although we could not decide whether the inhibition was due to direct binding to a receptor site at the channel or by interference with the mechanism that activates the volume regulatory processes. In Fig. 3, volume measurements demonstrate that osmolyte uptake, most likely CsCl, is inhibited by Gd\(^{3+}\), an observation that supports the hypothesis of a common pathway. Similar results obtained with guanidinium are depicted in Fig. 6. Further evidence for an identical pathway for \(^{86}\text{Rb}\) release and Cs\(^+\) uptake comes from the depression of \(R_{\text{Rb}}\) during the initial phase of the hypotonic treatment. Immediately after the hypotonic challenge is imposed, \(R_{\text{Rb}}\) is partly inhibited by Cs\(^+\), which suggests an inhibition by Cs\(^+\) from the extracellular side. This observation does not exclude the possibility that Cs\(^+\) acts at two different sites: 1) an inhibition of the \(^{86}\text{Rb}\) efflux pathway and 2) entry through separate cation channels. However, because both phenomena are blocked by Gd\(^{3+}\), this hypothesis seems unlikely. The \(^{86}\text{Rb}\) release recorded in the presence of guanidinium reveals a different mode of action of this cation. With this cation, \(R_{\text{Rb}}\) initially increased as in control conditions, which demonstrates the absence of an inhibitory effect. However, 10 min after the initiation of the hypotonic challenge, a marked inhibition of the \(^{86}\text{Rb}\) release occurred. This finding suggests that the organic cation has first to enter the cell before it can exert its inhibitory effect on the \(^{86}\text{Rb}\) release. Here also, cation entry is completely abolished by Gd\(^{3+}\).

With methyammonium as principal cation in the basolateral solution, cells showed a biphasic change in volume after the hypotonic shock was imposed. During the first phase, \(T_e\) declines, indicating the initiation of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery. Subsequently, a marked upstroke of volume recovery.
molecular weight of the permeant cations was found. From these studies, it was concluded that the size of the cation is the major determinant of permeability. In a recent study of variants of the voltage-gated Na\(^+\) channel in skeletal muscle (23), the sieving theory was challenged. The authors demonstrated that for the alanine substitution mutations the relative permeability of a subset of ammonium derivatives did not decrease in a monotonic fashion with molecular size or volume, as expected for a pure sieving model. For the mutants, this study reported a relative permeability \(P_{\text{Na}}/P_{\text{cation}}\) for guanidinium and methylammonium, which was 0.91 and 0.41, respectively. It is noteworthy that the respective diameters of these cations are 5.8 and 3.8 Å and their van der Waals volumes are 49.1 and 35.1 Å\(^3\). Although the size of guanidinium is larger than that of methylammonium, we also found that the former cation could enter the cell more easily.

Comparison of the \(R_{\text{Na}}^{\text{Rb}}\) during the hypotonic challenge and isosmotic condition shows that the activation of the \(^{86}\text{Rb}\) release pathway obviously depends on the cell swelling (10, 20). Nevertheless, it remains an open question whether the pathway is activated by direct stretch of the membrane or by a volume-sensitive intracellular messenger. The activity of the pathway is determined by cell swelling, whereas \(R_{\text{Na}}^{\text{Rb}}\) might be influenced by changes in driving force for \(\text{Rb}^+\). In the experiments with Cs\(^+\), methylammonium, and guanidinium, the entrance of the cations contributes to cell swelling, maintaining in this way the activity of the pathway. A further stimulation of the \(^{86}\text{Rb}\) release is probably caused by the depolarization of the intracellular potential by the opening of the cation pathway. The permeating cations (Cs\(^+\), methylammonium, guanidinium) can diminish the \(\text{Rb}^+\) efflux, when passing through the channel, by competing for the same binding sites in the channel. The occupancy of these sites by the permeating cations will modulate the release of \(\text{Rb}^+\).

Complete volume recovery and comparable time courses of \(R_{\text{Na}}^{\text{Rb}}\) were obtained with basolateral Na\(^+\) and Li\(^+\) solutions, although the time constant of the RVD was noticeably larger with Li\(^+\). In many transport systems, both cations have a similar permeability, e.g., for the amiloride-sensitive Na\(^+\) channel (14). However, Na\(^+\) as well as Li\(^+\) do not seem to enter the cell through the volume-activated pathway. If these cations would permeate, cells should gain extra solute and the RVD would be inhibited as with Cs\(^+\) and guanidinium. On the other hand, it is conceivable that Na\(^+\) might leave the cell through apical amiloride-sensitive Na\(^+\) channels or the basolateral Na\(^+\)-K\(^+\)-ATPase. We verified these possibilities in experiments in which the Na\(^+\)-K\(^+\)-ATPase was inhibited with ouabain (0.1 mM) as well as by blocking apical Na\(^+\) permeability with amiloride (20 μM). In both types of experiments, the volume recovery and \(R_{\text{Na}}^{\text{Rb}}\) were not different from control (data not shown), indicating that Na\(^+\) does indeed not permeate through the cation pathway.

In a previous report (5), we demonstrated that Gd\(^3+\) is a very efficient inhibitor of volume regulation. Many studies demonstrated that the lanthanide is a potent blocker of nonselective and stretch-activated channels (2, 15, 27). We found that Gd\(^3+\) acts from the intracellular side and that the lanthanide enters the cell in isosmotic conditions. Nevertheless, it remains possible that the trivalent cation can also enter the cell through the volume-activated cation channels. Our data do not exclude this possibility. With all cations tested, Gd\(^3+\) effectively occluded the volume-activated pathway, preventing additional cell swelling in the presence of Cs\(^+\), K\(^+\), guanidinium, or methylammonium. With nonpermeant cations (Na\(^+\), Li\(^+\)), volume recovery was completely blocked by Gd\(^3+\) (5). This study also revealed that protons are able to block volume recovery at pH 6.0 and below. It is conceivable that this cation exerts a direct blocking action on the VSCC, although an inhibition of the signaling pathway by cell acidification cannot be excluded.

We thank Dr. W. Zeiske for critical comments and helpful suggestions on this paper and E. Larihivire for performing the volume measurements.

This project was supported by research grants from the Fonds voor Wetenschappelijk Onderzoek (G.0235.95) and the Interuniversity Pole of Attraction Programme-Ghent University, Prime Minister’s Office-Federal Office for Scientific, Technical and Cultural Affairs (IUAP P4/23). P. De Smet is a postdoctoral fellow of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. J. Li was supported by a fellowship from the Research Council of K.U. Leuven.

Address reprint requests to W. Van Driessche.

Received 30 December 1997; accepted in final form 27 April 1998.

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