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Cell swelling activates cloned Ca^{2+}-activated K^+ channels: a role for the F-actin cytoskeleton

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Abstract

Cloned Ca^{2+}-activated K^+ channels of intermediate (hIK) or small (rSK3) conductance were expressed in HEK 293 cells, and channel activity was monitored using whole-cell patch clamp. hIK and rSK3 currents already activated by intracellular calcium were further increased by 95% and 125%, respectively, upon exposure of the cells to a 33% decrease in extracellular osmolarity. hIK and rSK3 currents were inhibited by 46% and 32%, respectively, by a 50% increase in extracellular osmolarity. Cell swelling and channel activation were not associated with detectable increases in $[Ca^{2+}]_i$, evidenced by population and single-cell measurements. In addition, inhibitors of IK and SK channels significantly reduced the rate of regulatory volume decrease (RVD) in cells expressing these channels. Cell swelling induced a decrease, and cell shrinkage an increase, in net cellular F-actin content. The swelling-induced activation of hIK channels was strongly inhibited by cytochalasin D (CD), in concentrations that caused depolymerization of F-actin filaments, indicating a role for the F-actin cytoskeleton in modulation of hIK by changes in cell volume. In conclusion, hIK and rSK3 channels are activated by cell swelling and inhibited by shrinkage. A role for the F-actin cytoskeleton in the swelling-induced activation of hIK channels is suggested.

Keywords: Cell volume; rSK3 channel; hIK channel; Regulatory volume decrease; Cell shrinkage

1. Introduction

Changes in cell volume can be observed both under physiological and pathophysiological conditions. Most frequently, cell volume disturbances are due to changes in intracellular osmolyte concentration, for example during Na^+-coupled uptake of sugar and amino acids across intestinal epithelial cells [24] or as a result of stimulated secretion in, e.g., salivary or pancreatic acinar cells [30]. Although less frequent, cell volume perturbations may also reflect changes in extracellular osmolarity, e.g., during antidiuresis in inner renal medulla cells [13], diabetes, or disturbances in the secretion of antidiuretic hormone [25]. Most mammalian cell types are able to counteract such changes in cell volume, by the so-called regulatory volume increase (RVI) response after cell shrinkage, or the regulatory volume decrease (RVD) response after cell swelling. In many cell types, the early part of the RVD response is dominated by a loss of KCl and water, often through activation of K^+ and Cl^- channels [13].

The identity of the K^+ channels activated by cell swelling seems to be cell type dependent (see Refs. [13,14,44]). K^+ channels reported to mediate RVD in various cell types include voltage-dependent K^+ channels such as Kv1.3 or Kv1.5 [22], KCNQ1 channels [23], stretch-activated channels [36,39], or a Ca^{2+}- and voltage-independent K^+ channel resembling the so-called background or two-pore K^+ channels [15,31,37], which recently has been identified as TASK-2 [32].

A role for Ca^{2+}-activated K^+ channels in the RVD response has also been suggested in several cell types, including osteoblasts [45], T cells [21], cultured human epithelial cells [10,11], and tracheal epithelial cells [43]. Activation of Ca^{2+}-activated K^+ channels by cell swelling has also been described for cloned channels expressed in *Xenopus laevis* oocytes [9,42]. The type of K^+ channel(s) involved in the RVD response and the mechanism(s) linking cell swelling to channel activation are, however, not clear. A role for an increase in $[Ca^{2+}]_i$ has been suggested in some

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cell types (e.g., Refs. [11,45]). In other cell types, a rise in $[Ca^{2+}]_i$ can be observed during cell swelling, but is not required for activation of the K^+ channels (e.g., Refs. [4,35]), and in still other cell types no change in $[Ca^{2+}]_i$ can be detected (e.g., Refs. [7,38]), or a change in $[Ca^{2+}]_i$ can been seen only in a negligible number of individual cells [20].

Regulation of ion channel activity by changes in the organization of the F-actin cytoskeleton has been suggested, e.g., for the epithelial Na^+ channel [1], the cystic fibrosis transmembrane regulator, CFTR [2], IK channels during cell migration [40], voltage-gated K^+ channels [29], and cardiac ATP-sensitive channels [6] (for reviews, see Refs. [3,34]). Changes in the structure of the F-actin cytoskeleton may play an important role in cell volume regulation; generally, cell swelling is reported to cause a decrease, and cell shrinkage an increase, in cellular F-actin content (see Refs. [3,26,27,34]). Exceptions with reorganization and no quantitative change [12] or transient, swelling-induced increase in F-actin [41] have, however, also been reported. A change in the structure of the F-actin cytoskeleton is thus a possible mechanism linking cell swelling to regulation of K^+ channels.

The aim of the present study was to examine the effect of changes in cell volume on hIK channels expressed in the mammalian cell line, HEK 293, and to examine the mechanism(s) potentially linking channel activity and cell volume. The use of cloned channels expressed in a mammalian cell line has the advantage of offering a simpler and better controlled system than native cells, while the HEK 293 cell expression system still possesses the cytoskeletal structures and other signaling pathways of a mammalian cell and allows precise control of $[Ca^{2+}]_i$ in the whole-cell patch clamp experiments.

2. Materials and methods

2.1. Cells

Human embryonic kidney cells (HEK 293) expressing hIK and rSK3 channels were cultured as described previously [8,17].

2.2. Materials

Fura-2-AM, and unconjugated or rhodamin- or Alexa488-labelled phalloidin were obtained from Molecular Probes (Leiden, The Netherlands). Ionomycin, clotrimazole, and cytochalasin D (CD) were from Sigma. Apamin was obtained from Alomone Labs (Jerusalem, Israel). Concentrated stock solutions were prepared in DMSO (Fura-2-AM, clotrimazole, CD), 96% ethanol (ionomycin), or isoosmotic extracellular solution containing 0.2% BSA (apamin). Fluorophore-conjugated phallodin was kept as a stock solution in methanol. Immediately before use, methanol was evaporated and phalloidin was dissolved in saponin buffer (see below). The final concentration of DMSO was always kept below 0.1%, a concentration that did not interfere with the measurements. All other chemicals were obtained from Sigma.

2.3. Solutions

2.3.1. Patch clamp experiments

Extracellular solutions: Isoosmotic KCl–mannitol: 94.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 100.0 mM D-mannitol, 10.0 mM HEPES (pH 7.4). Isoosmotic NaCl–D-mannitol: 4.0 mM KCl, 56.4 mM NaCl, 26.1 mM Na-gluconate, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 100.0 mM mannitol, 10.0 mM HEPES (pH 7.4). Hyperosmotic and hypoosmotic solutions (1.5 and 0.67 times the normal osmolarity, respectively) were prepared as isoosmotic solution, with addition/omission of D-mannitol to adjust the osmolarity. *Intracellular solution*: 64.0 mM KCl, 30.0 mM KOH, 10.0 mM EGTA, 100.0 mM D-mannitol, 20.0 mM HEPES (pH = 7.20). $CaCl_2$ was added to a final, free Ca^{2+} concentration of 180 or 300 nM, and $MgCl_2$ was added to a final, free Mg^{2+} concentration of 1.0 mM, as calculated using Eqcal software.

2.3.2. Light scattering/fura-2 experiments

Isoosmotic NaCl solution: 140.0 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM HEPES, pH 7.40. The osmolarity was adjusted to 1.5 or 0.67 times the normal osmolarity by addition/omission of NaCl.

2.3.3. Quantitative F-actin assay

MOPS buffer: 10.0 mM MOPS, 5.0 mM EGTA, 20.0 mM K_2HPO_4, 2 mM $MgSO_4$, pH 6.9 (NaOH). *Saponin buffer*: MOPS buffer supplemented with 0.1% (w/v) saponin. *TBS*: 150 mM NaCl, 10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.3 (NaOH).

2.4. Experimental procedure

2.4.1. Electrophysiology

The EPC-9 (HEKA Electronics, Lambrect, Germany) patch clamp amplifier was controlled by computer via an ITC-16 interface. Data were acquired at three to five times the filtration rate using the Pulse software (HEKA). Pipettes (resistance 2–3 MΩ) were pulled from borosilicate glass (Modulohm, Copenhagen, Denmark) by a DMZ-Universal Puller (Zeitz Instruments, Augsburg, Germany). A custom-made perfusion chamber (volume 15 μl) with a fixed AgCl|Ag-pellet electrode (In Vivo Metric, Healdsburg, CA) was mounted on the stage of an inverted microscope (Olympus).

2.4.2. Whole-cell patch clamp

The cells were seeded at low density 2–12 h prior to experiments. For experiments with CD including parallel controls, the cells were seeded on coverslips 16–30 h before the onset of experiments. Coverslips containing the HEK 293 cells expressing hIK or rSK3 channels were mounted in the perfusion chamber, and continuously perfused with

extracellular salt solution by a gravity-driven perfusion system with a flow of approximately 1 ml/min. In the whole-cell voltage clamp experiments, the series resistance (R_S) as well as the cell capacitance were followed on-line and compensated by 70%. The initial R_S was always below 5 MΩ, and usually remained constant throughout the experiments. The voltage protocol used was a linear ramp from −100 to +100 mV, 200-ms duration, applied every 5 s. Experiments were discarded if R_S increased above 10 MΩ or if slow capacitance cancellation failed. All experiments were performed at room temperature (20–22 °C).

2.4.3. Cell volume and $[Ca^{2+}]_i$

Coverslips containing HEK 293 cells (seeded at approximately 80% confluency 2–12 h prior to experiments) were loaded with 2 μM fura-2-AM in NaCl solution for 20 min at 37 °C, washed and placed in polystyrene cuvettes in a computer-controlled fluorescence spectrophotometer (RatioMaster, PTI, Photomed, Køge, Denmark). In experiments measuring changes in cell volume, hypoosmotic exposure was obtained by reducing NaCl, not using D-mannitol, as intact cells normally shrink in mannitol solutions due to loss of KCl. In the patch clamp experiments, the use of D-mannitol had no effect on cell volume, as both bath and pipette solutions contained D-mannitol. All experiments were performed under continuous flow at 37 °C. For near-simultaneous measurements of cell volume changes (light scattering) and $[Ca^{2+}]_i$ (fura-2), the following monochromator settings were used: Light scattering, excitation 589 nm, emission 595 nm; fura-2, excitation 340 and 380 nm, emission 525 nm.

2.4.4. Determination of the relative cell volume

The magnitude of the light scattering signal varies between different coverslips and different batches of cells, and is inversely related to the change in cell volume. Thus, results are presented as the inverse of the normalized signal, i.e.,

$$\text{Relative cell volume} = 1/(I/I_0) = I_0/I$$

The background signal for the light scattering measurements was determined using a coverslip without cells. In all experiments, the signal was at least three times above the background. Potential changes in the background signal were investigated for each change in the composition of the experimental solutions and for all added drugs/inhibitors. Changes in the background signal were negligible under the experimental conditions employed in this study, some variation between different coverslips could, however, be detected (not illustrated). Thus, no correction for background was performed.

The light scattering signal was shown to be a linear function of changes in osmolarity in the range from 0.5 to 1.5 times normal osmolarity (not shown). The RVD rate was calculated from the light scattering measurements using the linear part of the curve after maximal cell swelling was obtained.

2.4.5. The fura-2 ratio

The 525-nm emission intensity after excitation at 340 and 380 nm was used to determine the fura-2 ratio (340:380) and thus to evaluate $[Ca^{2+}]_i$. The signal was at least three times above background (unloaded cells), and background subtraction was performed prior to calculation of the fura-2 ratio.

2.4.6. Quantitative F-actin assay

Net cellular F-actin content was estimated using a quantitative rhodamine–phalloidine extraction assay essentially as described previously [33], except that cells were attached to multiwell plastic dishes (70,000 cells/well), thus eliminating the need for centrifugation. Stimulation with anisoosmotic media was carried out at 37 °C. The assay was verified to be linear over a range of 20,000–120,000 cells per well. Nonspecific rhodamine labeling was evaluated by binding competition with a 100-fold excess of unlabelled phalloidin, and was found to be negligible.

2.5. Statistical analysis

The data are presented as individual experiments representative of at least three independent experiments, or as mean ± S.E. of at least three independent experiments. Statistical significance was tested using Student's *t*-test.

3. Results

3.1. Regulation of hIK and rSK3 channels by changes in cell volume

The effect of changes in cell volume on hIK channels expressed in the mammalian cell line HEK 293 was investigated using the whole-cell patch clamp technique. Fig. 1A shows IV curves for HEK 293 cells expressing hIK channels recorded after obtaining a stable current level; the channels were activated by 300 nM calcium in the pipette solution. The IV curves were measured under isoosmotic conditions, during cell swelling induced by hypoosmotic exposure under isoosmotic conditions (33% reduction in osmolarity by omission of D-mannitol) and in the presence of the IK channel inhibitor clotrimazole (1 μM).

The IV curve shows the characteristics of hIK channels; i.e., the current is weakly inward rectifying and is inhibited by clotrimazole [16–19]. Fig. 1A clearly demonstrates that hIK channels, preactivated by calcium, are activated further by cell swelling induced by exposure to a hypoosmotic solution.

The activation of hIK channels by cell swelling is further illustrated in Fig. 1B, which shows the current value at −80 mV as a function of time. The current values were sampled from IV curves as shown in Fig. 1A. Fig. 1A and B are from the same experiment. Since the clamp potential is −80 mV and K^+ concentrations are symmetrical across the membrane, activation of hIK channels will cause an increase in the inward current, seen as a downward deflection. It can be

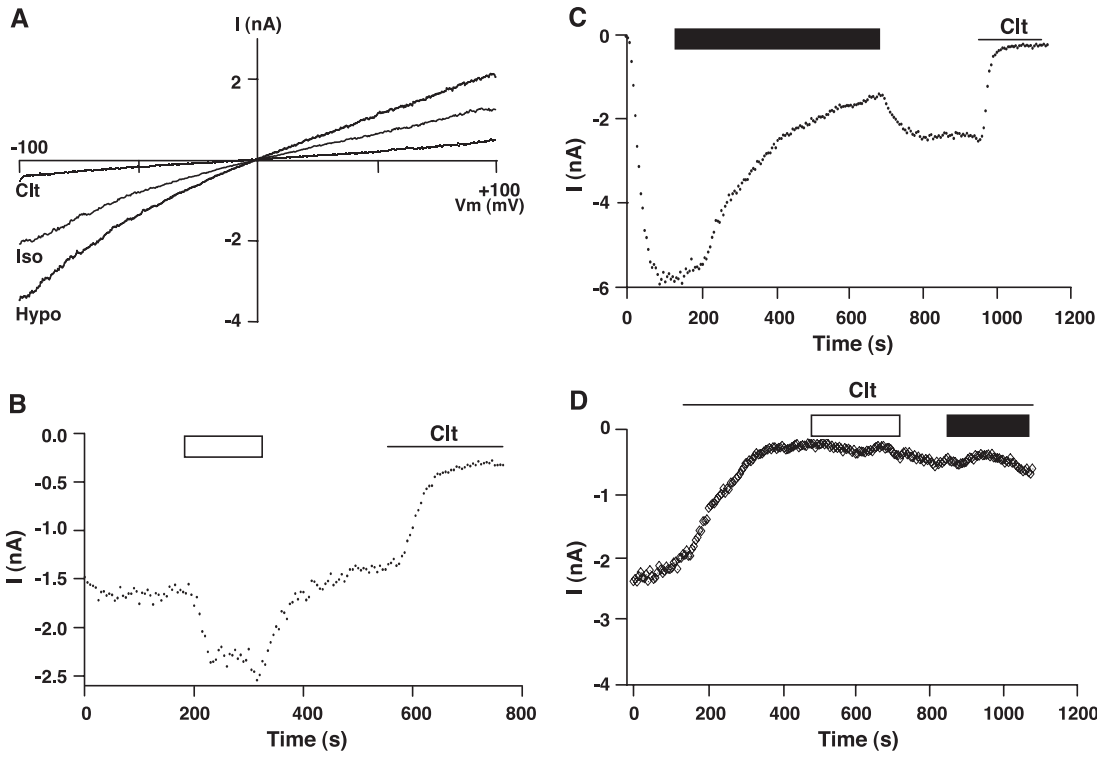

Fig. 1. hIK channels are activated by cell swelling and inhibited by cell shrinkage. Panel A shows three IV curves from a whole-cell patch clamp experiment performed on HEK 293 cells stably expressing hIK channels. The experiment was performed with a KCl–mannitol Ringer containing a $[Ca^{2+}]_i$ of 300 nM in the pipette and KCl–mannitol Ringer in the bath, thus giving symmetrical K^+ concentrations. A voltage ramp from −100 to +100 mV, 200-ms duration was applied every 5 s and the cells were exposed to hypoosmotic Ringer (33% reduction in osmolarity, by omission of D-mannitol). The IV curves illustrate the recorded hIK current during isoosmotic and hypoosmotic conditions as well as after addition of the hIK inhibitor clotrimazole (1 μM), as indicated. Panel B shows the mean current measured at −80 mV as a function of time from the experiment also presented in Panel A. The cells were exposed to hypoosmotic Ringer as indicated by the open bar or to the hIK channel inhibitor clotrimazole (1 μM) as indicated by the line. The data are from a single experiment representative of a total of five experiments giving similar results. Panel C: The data show the mean current measured at −80 mV as a function of time from a whole-cell experiment performed under the same conditions as described for Panel B, except that the cells were exposed to an increase in the extracellular osmolarity (hyperosmotic solution, 50% increase in osmolarity by addition of D-mannitol) as indicated by the closed bar. Clotrimazole (1 μM) was added as indicated by the line. The data are from a single experiment representative of a total of five experiments giving similar results. Panel D: The data show the mean current measured at −80 mV as a function of time from a whole-cell experiment performed under the same conditions as described for Panels B and C, except that the cells were treated with clotrimazole (1 μM) as indicated by the line and exposed to a hypo- or hyperosmotic solutions in the presence of clotrimazole as indicated by the open and closed bar, respectively. The data are from a single experiment representative of a total of four experiments giving similar results. The mean and S.E. values are given in Fig. 2.

seen from Fig. 1B that a stable current level due to activation of hIK channels by the pipette Ca^{2+}-concentration was obtained before exposure to the hypoosmotic cell swelling. Fig. 1B furthermore illustrates that the swelling-induced activation of the hIK channels was reversible, and addition of the inhibitor clotrimazole at the end of the experiment strongly inhibited the current, confirming that the measured current was hIK specific. Fig. 1C shows a different whole-cell patch clamp experiment, in which the cell was exposed to hyperosmotic solution (1.5 times the normal osmolarity by addition of D-mannitol, indicated by the closed bar), causing osmotic cell shrinkage. The experiment demonstrates that cell shrinkage causes a partly reversible inhibition of hIK channel activity.

When HEK 293 cells expressing hIK channels were exposed to hypo- or hyperosmotic solutions in the presence of clotrimazole (1 μM), no swelling-induced change in current could be detected (Fig. 1D). In the presence of clotrimazole, the swelling-induced increase in hIK current, calculated as the maximal K^+ current under hypoosmotic cell swelling with subtraction of the current under isoosmotic conditions, was thus -0.2 ± 0.1 nA ($n=4$). This further supports that the changes in current induced by cell swelling or shrinkage are carried exclusively by expressed hIK channels and not by endogenous channels in the HEK cells.

Similar findings were obtained in HEK 293 cells expressing rSK3 channels. The data from whole-cell patch clamp experiments with exposure of HEK 293 cells expressing hIK or rSK3 channels to hypo- or hyperosmotic KCl–mannitol solutions are summarized in Fig. 2. The pipette calcium concentration was buffered at 300 nM for hIK-expressing cells and 180 nM for rSK3-expressing cells. The figure shows the mean change in current upon anisoosmotic exposure, in percentage of the current measured under isoosmotic conditions. Fig. 2 illustrates that cell swelling significantly activated both hIK ($95 \pm 46\%$, $n=5$) and rSK

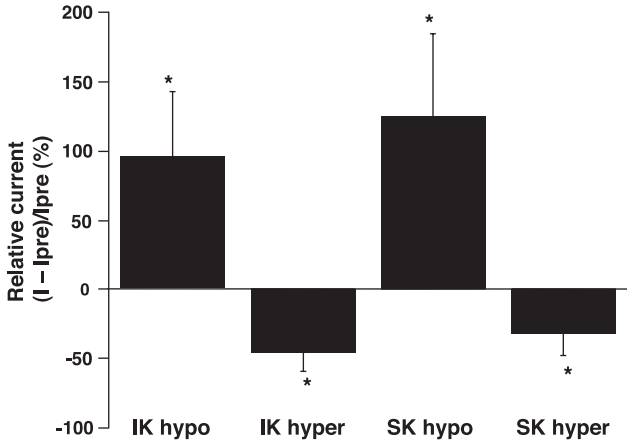

Fig. 2. Summary of the effect of cell volume changes on whole-cell currents measured with symmetrical K^+ concentrations across the membrane. The figure shows the increase and decrease in hIK or rSK3 channel activity after cell swelling and cell shrinkage, respectively. The values are obtained from whole-cell patch clamp experiments performed with 300 nM (hIK) or 180 nM (rSK3) free $[Ca^{2+}]_i$ in the pipette, and KCl–mannitol Ringer in the bath (symmetrical K^+ concentration; please see the legend to Fig. 1 and Materials and methods for details). I is the current during maximal cell swelling or shrinkage, and I_{pre} is the current under isoosmotic conditions. The data are given as the mean ± S.E. of the percentage change, with control (isoosmotic) values being 0% (hIK: hypo and hyper $n=5$; rSK3: hypo $n=4$, hyper $n=3$). *Significantly different from isoosmotic control ($P<0.05$).

3 channels (125 ± 59%, $n=4$) ($P<0.05$), and, conversely, cell shrinkage inhibited hIK (− 46 ± 13%, $n=5$) and rSK 3 channels (− 32 ± 16%, $n=3$) ($P<0.05$).

3.2. Swelling-activated Cl^- channels do not contribute to the swelling-induced current

The observation that no swelling-induced change in current activity could be measured at − 80 mV under symmetrical K^+ concentrations if the cells were treated with clotrimazole indicates that the increase in current detected under these conditions in the *absence* of clotrimazole is due to activation of hIK channels. HEK 293 cells possess several types of Cl^- channels [47], including swelling-activated Cl^- channels (e.g., Ref. [32]; Nathalie Hélix, Dorte Strøbæk and Palle Christophersen, personal communication). Activation of swelling-sensitive Cl^- channels would most likely have altered the shape of the IV curves illustrated in Fig. 1A. Contribution from Cl^- channels to the swelling-induced current thus seems unlikely under the experimental conditions used in the present study (see below). To exclude that the apparent activation of hIK channels by cell swelling was due to a confounding effect of Cl^- currents, experiments were also performed under conditions eliminating contribution from Cl^- channels: The extracellular solution was NaCl-D-mannitol and the current was measured at E_{Cl}, which is 0 mV under these experimental conditions. This procedure is similar to the method described by Riquelme et al. [37], except that no correction for the small dilution of intracellular Cl^- during cell swelling was performed.

Fig. 3 shows that under these experimental conditions, a significant, reversible increase in whole-cell current could still be detected during cell swelling (33% reduction in osmolarity by omission of D-mannitol). Please note that under these experimental conditions, the K^+ current is outwardly directed, activation of the hIK channels is thus seen as a positive current (upward deflection). Addition of clotrimazole (1 μM) at the end of the experiment strongly inhibited the current, confirming that it was hIK specific. A slower (variable time course, starting to activate after approximately 2–5 min) swelling-induced increase in current could also be detected at − 79.5 mV, i.e., at E_K, indicating activation of Cl^- channels (not illustrated). This slow activation of the swelling-induced Cl^- channels endogenously expressed in HEK 293 cells is in agreement with findings by Nathalie Hélix, Dorte Strøbæk and Palle Christophersen (personal communication), and indicates that interference from endogenous swelling-activated Cl^- channels is absent during short periods of swelling. Therefore, the apparent lack of contribution of Cl^- channels in the experiments performed under symmetrical KCl concentrations is most likely due to the relatively short (approximately 1.5–2.5 min without clotrimazole; 1.5–3 min with clotrimazole) exposure to the hypoosmotic solution.

Table 1 summarizes the mean swelling-induced change in current at 0 mV (NaCl-D-mannitol solution), with varying free calcium concentrations in the pipette. As seen, a permissive level of calcium intracellularly is required in order for the hIK channels to be further activated by cell swelling. At free intracellular calcium concentrations of 0 or 100 nM, no increase in current could be detected upon cell swelling (see Table 1) and, accordingly, no effect of clotrimazole (1 μM) on the swelling-induced current was

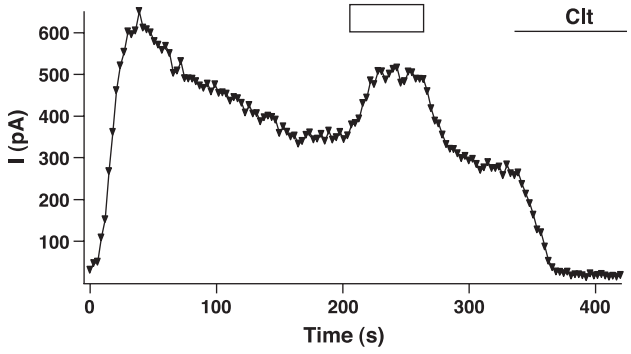

Fig. 3. Swelling-induced activation of hIK measured at E_{Cl}. Whole-cell patch clamp experiments performed on hIK expressing HEK 293 cells. The bath contained NaCl-D-mannitol Ringer, the pipette solution was KCl–mannitol intracellular solution with 180 nM $[Ca^{2+}]_i$, and K^+ current was monitored at E_{Cl} (0 mV). Cell swelling was induced by a reduction of the extracellular osmolarity by 33% (omission of D-mannitol) as indicated by the open bar. Clotrimazole (Clt, 1 μM) was added at the end of the experiment as indicated by the line. Data are from a single experiment representative of a total of seven experiments giving similar results. Mean ± S.E. are given in Table 1.

Table 1
Swelling-induced changes in hIK current activity

Calcium concentration in pipette solution (nM)	Swelling-induced increase in hIK current (nA)
0	0.0 ± 0.1 (8)
100	0.0 ± 0.1 (6)
180	0.5 ± 0.3 (7)*
300	0.7 ± 0.5 (4)*

Whole-cell patch clamp experiments performed on hIK expressing HEK 293 cells. The bath contained NaCl-D-mannitol Ringer. K^+ current was monitored at E_{Cl} (0 mV). The table shows the swelling-induced increase in K^+ current after a reduction of the extracellular osmolarity by 33% (by omission of D-mannitol). The hypoosmotic solution was applied after a stable level of K^+ current was obtained (due to activation of hIK channels by the pipette Ca^{2+} concentration). The swelling-induced K^+ current was calculated as the difference between the current level during hypoosmotic exposure and the current level under isoosmotic conditions. The cells were exposed to hypoosmotic solution after a stable current level was obtained. Data are given as mean ± S.E. with the number of experiments indicated in parenthesis.

* Significantly different from the value obtained at 0 nM calcium in the pipette ($P < 0.05$).

observed ($n = 6$, not illustrated). At 180 and 300 nM $[Ca^{2+}]_i$, a significant swelling-induced activation of hIK channels could be seen. Even though the activation of hIK channels seems greater at 300 nM compared to 180 nM, this difference was not statistically significant.

3.3. Near-simultaneous detection of cell volume and $[Ca^{2+}]_i$

The swelling-induced activation of hIK and rSK3 channels is expected to cause a loss of K^+ from the cells, which—if simultaneous opening of Cl^- channels takes place—will lead to a loss of KCl and water, and thus to a reduction in cell volume (RVD response). The whole-cell patch clamp experiments performed in NaCl-D-mannitol solution indicated that a more prolonged cell swelling can open volume-activated Cl^- channels in the HEK 293 cells. Thus, if K^+ loss is limiting for the RVD process in HEK 293 cells, an activation of hIK or rSK3 channels would be expected to increase the rate of the RVD in cells expressing these channels, although activation of a channel by cell swelling is not per se proof of its relevance to the RVD process. To test the possible contribution of hIK and rSK3 channels to the RVD response, and confirm that the channels are indeed activated by cell swelling in an intact cell, changes in cell volume after exposure to hypoosmotic NaCl solution were measured using light scattering. To investigate whether cell swelling causes an increase in $[Ca^{2+}]_i$ in the HEK 293 cells that might be responsible for channel activation, simultaneous measurements of $[Ca^{2+}]_i$ were performed using the ratiometric fluorescent probe, fura-2.

Fig. 4 shows such an experiment, where hIK expressing HEK 293 cells were exposed to hypoosmotic cell swelling as indicated by the open bar, and the relative cell volume (light scattering) as well as the change in $[Ca^{2+}]_i$ (fura-2 signal) were followed with time. In these experiments, the hypoosmotic solution (33% reduction in osmolarity) was prepared by omission of NaCl (not using D-mannitol). The figure clearly shows that the cells rapidly swell upon exposure to the hypoosmotic solution, followed by an RVD response. When the cells were returned to isoosmotic solution after 15 min in hypoosmotic solution, they showed a RVD–RVI response, i.e., they shrunk below the resting level followed by an RVI response. This is generally considered to be due to loss of intracellular osmolytes during the RVD response, resulting in lower steady state intracellular osmolarity, causing the cells to sense the isoosmotic solution as being hypertonic. Fig. 4 also shows that no detectable increase in $[Ca^{2+}]_i$ could be measured during cell swelling. Only a slight decrease in the fura-2 signal at both wavelengths was detected. This reflects the effects of a change in tonicity/viscosity on the fluorophore, and differs from the increase at 340 nm and decrease at 380 nm expected from an increase in $[Ca^{2+}]_i$. Addition of the calcium ionophore ionomycin at the end of the experiment elicited the expected increase at 340 and decrease at 380 nm, confirming that the method was valid.

3.4. Single-cell measurements confirm the absence of a detectable increase in $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ during cell swelling may not occur simultaneously in all cells in a population, as reported for example for Erhlich ascites tumour cells [20]. Thus, fluores-

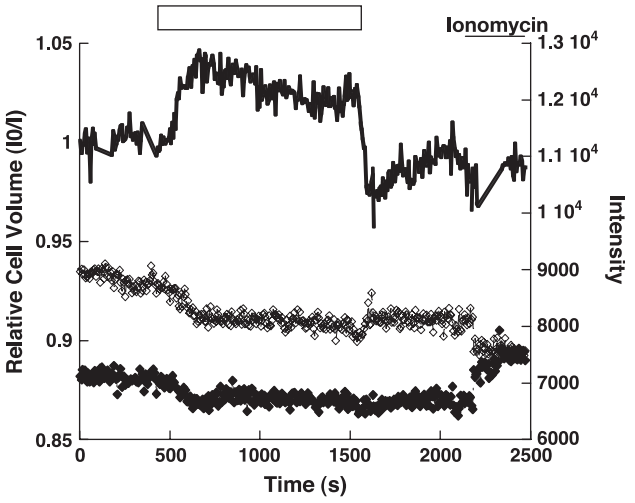

Fig. 4. Simultaneous measurements of changes in cell volume and $[Ca^{2+}]_i$. Light scattering/fura-2 experiment performed on HEK 293 cells expressing hIK channels. The closed graph represents the relative cell volume, the open and closed diamonds represent the fura-2 signal after 380 and 340 nm excitation, respectively. The cells were exposed to hypoosmotic NaCl solution (33% reduction in osmolarity by reduction in NaCl) as indicated by the open bar. Ionomycin (5 μM) was added as indicated by the line. The data are from one experiment, representative of a total of five experiments giving similar results.

cence microscopy with digital image processing was performed on fura-2-loaded HEK 293 cells expressing hIK channels in order to further investigate whether an increase in $[Ca^{2+}]_i$ could be detected during cell swelling in this cell type.

Such an experiment is shown in Fig. 5, where the change in $[Ca^{2+}]_i$ over time was followed in four cells during exposure to hypoosmotic cell swelling (33% reduction in osmolarity by reduction of NaCl). The figure confirms the findings from the light scattering/fura-2 experiments described above, i.e., no increase in the fura-2 ratio (340:380 nm), corresponding to a rise in $[Ca^{2+}]_i$, could be detected during cell swelling in any of the single cells studied. Fig. 5 also clearly shows that ionomycin caused a significant increase in the fura-2 ratio, confirming the validity of the method.

3.5. hIK and rSK3 channels contribute to the RVD response

The rate of the RVD response was measured using light scattering (see Materials and methods) and the results obtained under different experimental conditions are summarized in Fig. 6. The cells were exposed to hypoosmotic solution (33% reduction in osmolarity by reduction in NaCl) for 15 min in the absence (control) or presence of channel inhibitors.

The figure shows the rate of RVD for HEK 293 cells expressing either hIK or rSK3 channels in the presence of the channel inhibitors clotrimazole (1 μM) and apamin (100 nM), respectively, given relative to controls without addition of inhibitors (control = 1.0). The figure demonstrates that inhibition of hIK or rSK3 channels in cell expressing these channels significantly reduced the rate of RVD compared to parallel controls ($P < 0.05$). The large value of S.E. for rSK3-expressing cells is due to the fact that the inhibitor in some cases not only strongly abolished the RVD response, but caused a slight, continuous cell swelling, perhaps reflecting a larger activation of the more Ca^{2+}-sensitive SK channels at resting $[Ca^{2+}]_i$. It should be noted that expression of the hIK, but not rSK3, channels reduced the rate of RVD compared to wt HEK 293 cells ($P < 0.05$). The degree of swelling was the same in all three cell types (not illustrated). There was no significant difference in the rate of RVD between hIK- and rSK3-expressing cells, and although the rate of RVD in rSK3-expressing cells showed a tendency to be slower than in wt HEK 293 cells, there was no significant difference. The reason for the reduced rate of RVD in hIK-expressing cells is not clear, but may reflect downregulation of another membrane transport protein involved in the RVD response in the hIK-expressing cell line.

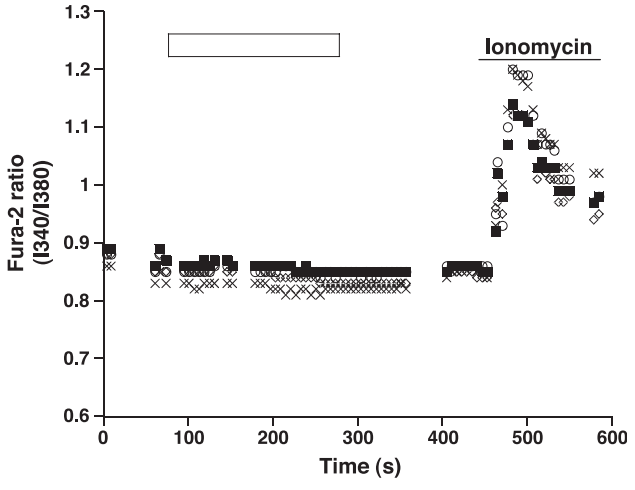

Fig. 5. Measurement of $[Ca^{2+}]_i$ in individual HEK 293 cells using fluorescence microscopy. Fluorescence microscopy with digital image processing was used to measure changes in $[Ca^{2+}]_i$ in four individual HEK 293 cells loaded with fura-2. $[Ca^{2+}]_i$ (the fura-2 ratio I340/I380) was followed as a function of time in four individual cells exposed to hypoosmotic NaCl solution (33% reduction in osmolarity by reduction in NaCl), as indicated with the open bar, or ionomycin (final concentration 5 μM), as indicated by the line. The figure is from a single experiment representative of a total of four experiments, with at least four cells per experiment, giving similar results.

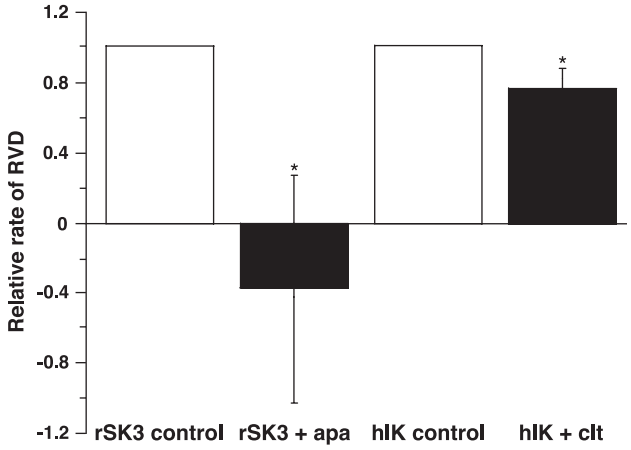

Fig. 6. The rate of RVD measured using light scatter—summary of experiments. The rate of RVD in HEK 293 cells expressing rSK3 or hIK channels was measured from light scattering data using linear regression as described in Materials and methods. The figure shows the rate of RVD in the presence of the channel inhibitors apamin (100 nM) or clotrimazole (1 μM), calculated relative to parallel controls without inhibitor. The data are given as the mean ± S.E. of five experiments with rSK3 and four with hIK channels, respectively. *Statistically different from control ($P < 0.05$).

3.6. Cell swelling changes the net cellular F-actin content

Cell swelling has been reported to change the structure of the F-actin cytoskeleton, which may in turn affect ion channel activity (see Refs. [3,27,34] and the Introduction). The effect of hypoosmotic cell swelling and hyperosmotic cell shrinkage on the net cellular F-actin content in HEK 293 cells expressing hIK channels was investigated. The F-actin content after cell shrinkage, given relative to isoosmotic control, was 1.06 ± 0.04, 1.04 ± 0.04, and 0.94 ±

0.04 at 1, 5, and 10 min after hyperosmotic exposure, respectively ($n=5$). Cell shrinkage thus caused a reversible increase in the cellular F-actin content, the values at 1 and 5 min hyperosmotic exposure were higher, and the value at 10 min lower ($P<0.05$) than isoosmotic control.

Fig. 7 shows the mean of the cellular F-actin content 1, 5, and 10 min after exposure of the cells to a hypoosmotic solution. The values are given relative to isoosmotic control. As seen, cell swelling rapidly induced a significant decrease in cellular F-actin content (1 and 10 min: $P<0.05$).

3.7. CD inhibits swelling-induced activation of hIK channels

Fig. 8A shows the effect of cytochalasin D (CD; 1 and 10 μM), a drug commonly used to depolymerize F-actin, on the net cellular F-actin content in HEK 293 cells expressing hIK channels. The figure shows the net cellular F-actin content, relative to control without CD, at 1, 3, and 10 min after addition of CD.

The data demonstrate that 1 μM CD caused a significant ($P<0.05$) decrease in the mean of the relative cellular F-actin content at 1, 3, and 10 min, while the higher concentration of 10 μM caused a significant ($P<0.05$) decrease only at 1 min exposure to CD, followed by an increase back to the level found in control conditions at 3 min exposure to CD. At 10 min exposure there is a tendency towards an increased polymerization, i.e. a value higher than control, but this is not statistically significant ($P<0.10$). Increased polymerization of F-actin in cells treated with high concentrations of CD has been reported for other cell types. The complex effects of CD on cellular F-actin found in the present study are thus in agreement with observations from other cell types [5,28,46], and underline the importance of verifying the effect of cytochalasins on the specific cell type used.

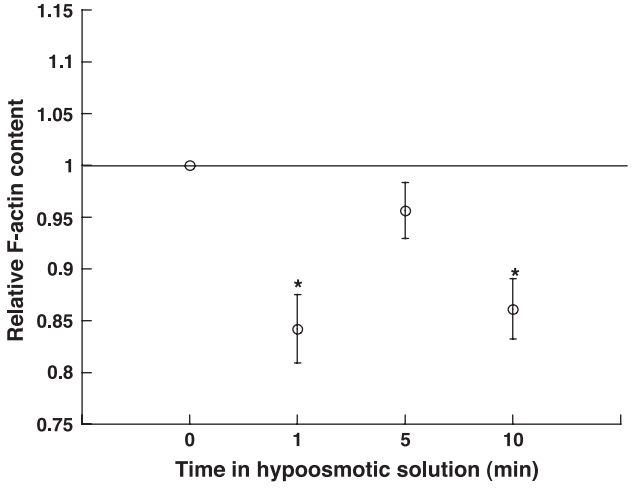

Fig. 7. Effect of changes in cell volume on net cellular F-actin content in HEK 293 cells. HEK 293 cells expressing hIK channels were exposed to hypoosmotic NaCl–Ringers at time zero (50% reduction in osmolarity by changes in NaCl concentration) and the resulting changes in net cellular F-actin content were measured at different time points. The data are given relative to isoosmotic control and are shown as the mean ± S.E. of five experiments. *Significantly different from control ($P<0.05$).

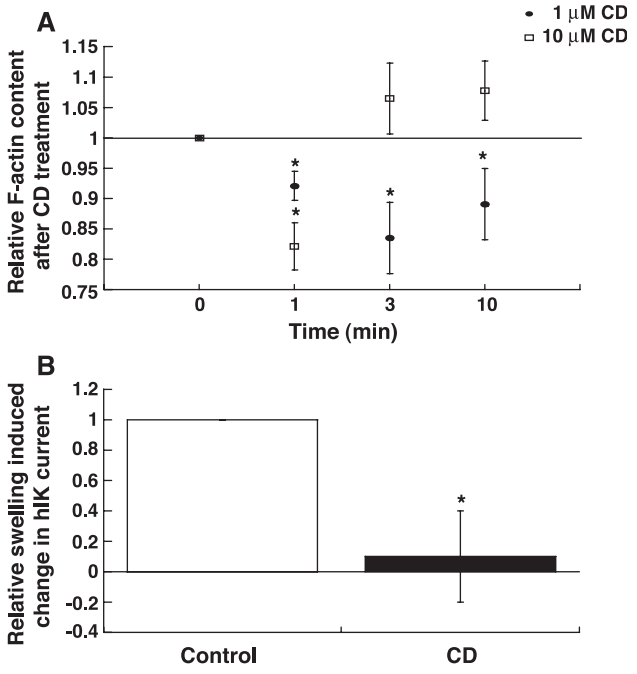

Fig. 8. The effect of cytochalasin D (CD) on net cellular F-actin content and swelling-induced activation of hIK channels. Panel A: HEK 293 cells expressing hIK channels were exposed to 1 μM (closed circles) or 10 μM (open squares) CD at time zero, and the resulting changes in net cellular F-actin content were measured at different time points. The data are given relative to control without CD and are shown as mean ± S.E. of four experiments. Panel B: The change in hIK current after cell swelling (reduction of the extracellular osmolarity by 33% by omission of D-mannitol, NaCl–mannitol Ringer extracellularly) was detected using whole-cell patch clamp. The swelling-induced change in hIK current is given relative to control without CD; the pipette solution contained a free calcium concentration of 180 nM. Control: The cells were exposed to hypoosmotic solution after a stable current level was obtained ($n=7$). CD: The cells were pretreated with CD (1 μM) for approximately 2–5 min until a stable current level was obtained, and exposed to hypoosmotic solution containing CD ($n=8$). *Significantly different from control ($P<0.05$).

To test the possible role of the F-actin cytoskeleton in the swelling-induced activation of hIK channels, whole-cell patch clamp experiments were performed on HEK 293 cells expressing these channels, and cell swelling was induced in the presence of CD (1 μM, added to the extracellular solution) after pretreatment with the drug for 2–5 min. Fig. 8B shows the swelling-induced change in hIK current relative to control, and it can be seen that CD significantly inhibited the swelling-induced activation of hIK channel activity at 180 nM intracellular calcium ($P<0.01$). It should be noted that the high concentration of CD (10 μM), which only caused a significant decrease in net cellular F-actin content at 1 min exposure, had no significant inhibitory effect on the swelling-induced activation of hIK channels ($n=3$, KCl solution in bath, not illustrated) estimated using the same experimental procedure as described for 1 μM CD.

4. Discussion

The present study demonstrates that hIK channels expressed in the mammalian cell line HEK 293 are further activated by cell swelling when $[Ca^{2+}]_i$ is elevated to levels allowing the channels to be preactivated (Figs. 1–3 and Table 1), and conversely, are inhibited by cell shrinkage (Figs. 1, 2). rSK3 channels expressed in HEK 293 cells were found to be regulated by changes in cell volume in a similar manner (Fig. 2). Activation of the channels by cell swelling could be confirmed in intact cells using light scattering to measure changes in cell volume; in HEK 293 cells expressing the channels, addition of hIK or rSK3 channel inhibitors significantly reduced the rate of RVD (Fig. 6), confirming that the channels contribute to the loss of K^+ during the RVD response when present.

Several mechanisms for coupling of cell volume changes and K^+ channel activation have been proposed. Activation of mIK channels expressed in *X. laevis* oocytes by cell swelling has been reported [42]. In that study, it was proposed that the mIK channels were activated by Ca^{2+}, although only minor increases in $[Ca^{2+}]_i$ could be detected during cell swelling. In a previous study, we found that swelling-induced activation of hIK and rSK3 channels expressed in *X. laevis* oocytes was independent of changes in $[Ca^{2+}]_i$, but dependent upon an intact F-actin cytoskeleton [9]. In human tracheal cells, swelling has also been reported to modulate IK activity. In these cells, the mechanism linking IK channel activation to cell volume changes was suggested to involve the cystic fibrosis transmembrane regulator (CFTR) in an indirect manner, and a role for changes in the F-actin cytoskeleton or ATP was tentatively suggested [43]. In human T lymphocytes, endogenous expression of IK channels increases dramatically upon activation, and these channels have been shown to play a role for the RVD response in this cell type, with channel activation presumably linked to an increase in $[Ca^{2+}]_i$ [21].

In the present study, cell swelling did not induce a detectable increase in $[Ca^{2+}]_i$ in HEK 293 cells (Figs. 4, 5). This indicates that stimulation of rSK3 and hIK channel activity by cell swelling occurred independently of changes in $[Ca^{2+}]$, in agreement with the observation from *X. laevis* oocytes [9].

A certain level of $[Ca^{2+}]_i$ was, however, found to be required in order for the hIK channels to be activated by cell swelling (Table 1). The contribution of both rSK3 and hIK channels to the RVD response indicated by the lower rate found in the presence of channel inhibitors suggests that the resting level of $[Ca^{2+}]_i$ in intact HEK 293 cells is sufficient to permit further activation of the expressed channels by cell swelling. The lack of swelling-induced activation of hIK channels at 100 nM $[Ca^{2+}]_i$ (Table 1) in the whole-cell patch clamp experiments could be due to a resting level of $[Ca^{2+}]_i$ above 100 nM in the HEK 293 cells, or more likely that the mechanism(s) linking cell swelling to channel activation may function at lower levels of $[Ca^{2+}]_i$ in the intact cell compared to a whole-cell patch clamp experiment, where dilution of cellular components and signaling molecules may take place.

The present study has demonstrated changes in the net cellular F-actin content in HEK 293 cells exposed to changes in cell volume. In cells expressing hIK channels, cell swelling caused a rapid, reversible decrease in the net cellular F-actin content (Fig. 7), while cell shrinkage had the opposite effect. This is in agreement with the findings in most cell types studied (see the Introduction). CD, in a concentration of 1 μM, caused a significant decrease in the net cellular F-actin content measured at 1, 3, and 10 min exposure to the drug. Furthermore, if cells were preincubated with 1 μM CD for 2–5 min followed by cell swelling induced in the presence of CD, the swelling-induced activation of hIK channel activity was significantly inhibited (Fig. 8). The observation that the swelling-induced activation of hIK channels is not significantly affected by pretreatment with a high concentration of CD (10 μM) for more than 1 min, i.e., a treatment that does not cause a significant decrease in net cellular F-actin content, indicates that the inhibition by the low concentration of CD is due to a disruption of the F-actin cytoskeleton and not to more unspecific effects of the drug. It also stresses the need to carefully examine the results of CD treatment in each cell type used as discussed in detail by Mills et al. [28].

Significant reductions in net cellular F-actin concentration are observed within 1 min after cell swelling. The changes thus occur within a time frame relevant for activation of the channels (see Fig. 1). Taken together with the fact that treatment with a concentration of CD, which decreases the F-actin polymerization, abolishes the swelling-induced activation of hIK channels, this indicates that an intact F-actin cytoskeleton is important for activation of the channels by cell swelling. The notion that the swelling-induced activation of hIK channels may depend on an interaction with the F-actin cytoskeleton is in agreement with our previous study on hIK and SK3 channels expressed in *X. laevis* oocytes [9], as well as with a study on migrating MDCK cells, which suggested an interaction between cell volume, F-actin filaments, and IK channels [40].

Finally, the finding that the swelling-induced activation of hIK or rSK3 expressed in HEK 293 cells contributes to the RVD response, confirms a possible physiological role for these channels in cell volume regulation, not only in cells in which swelling is associated with an increase in $[Ca^{2+}]_i$, but also in cells with no detectable swelling-induced increases in $[Ca^{2+}]_i$.

In summary, cell swelling stimulates, and cell shrinkage inhibits, hIK and rSK3 channel activity. The activation of hIK channels by cell swelling does not depend on an increase in $[Ca^{2+}]_i$, but a certain level of $[Ca^{2+}]_i$ is required. The swelling-induced activation of hIK channels is dependent upon the integrity of the F-actin cytoskeleton, indicat-

ing that direct or indirect interaction between F-actin and hIK channels may be important.

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