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Hypertonic activation of a non-selective cation conductance in HeLa cells and its contribution to cell volume regulation

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Abstract In whole-cell recordings on single HeLa cells, the hypertonic activation of a cation conductance with a selectivity ratio $P_{Na}:P_{Li}:P_K:P_{Cs}:P_{NMDG}:P_{Ca}:P_{Cl}$ of 1.00:0.86:0.84:0.56:0.10:0.07:0.15 was observed. This (non-selective) cation conductance was reduced to 59 and 30% of maximal stimulation by Gd^{3+} and flufenamate, respectively, but it was insensitive to amiloride (with each compound applied at 100 μmol/l). As was determined by the Coulter counter technique, the cation conductance was the main mechanism of regulatory volume increase (RVI) in HeLa cells. Whereas a significant contribution of Na^+/H^+ antiport was also detectable, Na^+-K^+-$2Cl^-$ symport most likely did not contribute to RVI.

Key words: HeLa cell; Volume regulation; Cation channel; Amiloride; Gadolinium; Flufenamate

1. Introduction

In most systems studied so far, hypertonic cell shrinkage induces a regulatory volume increase (RVI) that is achieved by the uptake of NaCl and osmotically obliged water. Ion pathways employed in this process are Na^+-K^+-$2Cl^-$ symport, Na^+/H^+ antiport, as well as (non-selective) cation channels [1–3].

Activation of cation channels upon cell shrinkage has been proven to be one of the most efficient mechanisms of RVI [3,4]. In most instances, these channels may either be inhibited by the diuretic amiloride (but are insensitive to Gd^{3+} and the anti-inflammatory drug flufenamate) or they are insensitive to amiloride (but efficiently blocked by Gd^{3+} and flufenamate; see [3] for review). From two preparations so far, however, a mixed type of pharmacological profile has also been reported [5,6].

Whereas the ion selectivity of hypertonicity-induced and *amiloride-sensitive* channels has not been studied in much detail yet, *amiloride-insensitive* channels may either be equally permeable to Na^+, K^+, Cs^+, and Li^+ but impermeable to $NMDG^+$ or exhibit a permeability to Li^+ and $NMDG^+$ that amounts to some 50% when compared to that of Na^+ [3].

It was the aim of the present study (i) to characterize the shrinkage-activated cation conductance in HeLa cells from a comparative point of view, (ii) to define the actual contribution of this conductance to the RVI process with reference to Na^+-K^+-$2Cl^-$ symport and Na^+/H^+ antiport, and (iii) to hereby provide the basis for a future study on the interplay between cell volume regulation and proliferation vs. apoptosis in this model system [7].

2. Materials and methods

2.1. Cell culture

Human cervix HeLa cells were grown as monolayers in minimum essential medium supplemented with 10% fetal bovine serum, 40 IU/ml penicillin G, and 100 μg/ml streptomycin at 37°C under a 95% air–5% CO_2 atmosphere. For sub-culturing, phosphate-buffered saline containing 0.25% trypsin and 0.02% EDTA was used to detach cells from the plastic substratum of the culture flasks. For the experiments, cells were resuspended by mechanical detachment and then stored in gently stirred culture medium for 15–300 min at 37°C in an incubator.

2.2. Electrophysiology

Membrane currents were recorded from single detached HeLa cells in the whole-cell mode of the patch-clamp technique. Patch pipettes were pulled from 1.4 mm (OD) and 1.0 mm (ID) borosilicate glass capillaries (Asahi Rika-Glass Industry, Kobe, Japan) on a programmable multi-stage laser pipette puller (P-2000, Sutter Instruments, Novato, CA, USA) and had resistances of 2–4 MΩ. A motorized micromanipulator (model 5171, Eppendorf, Hamburg, Germany) was used for the positioning of pipettes. An Ag–AgCl pellet (Warner Instruments, Hamden, CT, USA) served as the reference electrode (except in the Cl^- substitution experiments where a custom-made salt bridge containing 3 M KCl in 2% agar was used). Pipette offsets, series resistance, and capacitive transients were compensated on the patch-clamp amplifier (Axopatch 200A, Axon Instruments, Union City, CA, USA). Currents were digitized with an AD converter (Digi-Data 1200A, Axon Instruments) at 4 kHz and filtered with the built-in four-pole Bessel filter at 1 kHz. Data acquisition and analysis were done with the pCLAMP 6.0 software package (Axon Instruments). Liquid junction potentials of pipettes were calculated on the basis of the pCLAMP 8.0 software (Axon Instruments) and compensated for. Holding voltage was −30 mV and voltage ramps from −80 to +20 mV and 1 s duration were applied every 10 s, unless otherwise noted. In most instances, currents were normalized to cell capacitance that was obtained from the readings on the patch-clamp amplifier.

The pipette solution (pH 7.3) contained (in mmol/l): NaCl, 26; Na-gluconate, 69; $MgCl_2$, 1; TEA-Cl, 2; Na_2-ATP, 2; Na_2-GTP, 0.5; HEPES, 10; EGTA, 1. Osmolality was adjusted to 300 mosmol/kg-H_2O by addition of mannitol under osmometric control (OM802, Vogel, Germany). The bath solution (pH 7.5) contained (in mmol/l): NaCl, 94; Na-gluconate, 6; $MgCl_2$, 1; $CaCl_2$, 1; TEA-Cl, 2; HEPES, 10. Osmolality was adjusted to 450 mosmol/kg-H_2O (hypertonic) or 340 mosmol/kg-H_2O ('isotonic'). The latter value was found to be essential to avoid the spontaneous activation of *hypo*tonicity-induced Cl^- currents (not shown). From the above ionic gradients, the equilibrium potentials $E_{Cl} = -30$ mV and $E_{Na} = 0$ mV can be computed.

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2.3. Determination of cell volumes

Changes in cell volumes were quantified by electronic cell sizing on a Coulter-type analyzer (CDA-500, Sysmex, Japan). The set-up is equipped with a computer-based routine and the mean volume of a population of cells is calculated with reference to the volume distribution of latex beads of a defined size. Isotonic and hypertonic solutions (of pH 7.3) contained (in mmol/l): NaCl, 95; KCl, 4.5; $MgCl_2$, 1; $CaCl_2$, 1; $NaHCO_3$, 10; HEPES, 5. Osmolalities were adjusted to 300 and 450 mosmol/kg-H_2O by addition of mannitol. Solutions were always prepared immediately before experimental use to achieve reproducible HCO_3^- concentrations. Because in HeLa cells the presence of HCO_3^- was found to be essential for a sizeable RVI to occur (see Section 3.4) and because of the known strong chemical interference of HCO_3^- with Gd^{3+} [8] it was not possible to test for the effect of the latter on RVI.

2.4. Statistical analysis

Data are presented as means ± S.E.M. with n denoting the number of cells tested. For comparison of data sets, Student's t-tests for paired and unpaired data were employed as appropriate.

3. Results and discussion

3.1. Hypertonic activation of currents

In single detached HeLa cells, increasing bath osmolality from 340 to 450 mosmol/kg-H_2O led to a sizeable increase of whole-cell currents (Fig. 1A). At $V_h = E_{Cl} = -30$ mV (i.e. with no contribution of Cl^- conductance), currents equalled -1.49 ± 0.28, -4.24 ± 0.63, and -1.39 ± 0.27 pA/pF, for the isotonic control, after 5 min of hypertonic stress (when current activation was maximal), and at 6–8 min after change back to isotonicity, respectively (Fig. 1B). Under each experimental condition, currents were linear within the tested range of −80 to +20 mV (Fig. 1C) with a membrane conductance of 41.8 ± 8.3, 152.7 ± 26.8, and 40.4 ± 8.4 pS/pF for the above time protocol. Whereas zero current voltage, under isotonic conditions, was variable and in the range of +5 to −10 mV (not shown), that of the fully activated current was highly reproducible and equalled -1.7 ± 0.4 mV on average.

In an additional set of experiments, membrane currents in HeLa cells were monitored by use of a voltage-step (rather than a ramp) protocol. This was done to test for a possible voltage and/or time dependence of the volume-activated currents in this system. As is depicted in Fig. 2A, however, no evidence for such a behavior could be observed and the results obtained were virtually identical to those determined by means of the voltage ramps (see Fig. 2B).

3.2. Ion selectivity

In the next series of measurements, the ion selectivity of the hypertonicity-induced membrane conductance was determined. To this end, following maximal osmotic activation, bath Na^+ was changed to various substitutes and the resultant changes in zero current voltage were determined. An experiment in which Na^+ was changed to $NMDG^+$ is exemplified in Fig. 3. In additional experiments, Cl^- was replaced by gluconate. Taken together, these recordings revealed shifts in reversal potential upon change to Li^+, K^+, Cs^+, $NMDG^+$, and Ca^{2+} that amounted to -3.9 ± 0.6 ($n = 4$), -4.5 ± 0.7 ($n = 4$), -14.6 ± 1.0 ($n = 5$), -58.2 ± 2.4 ($n = 4$), and $-55{,}8 \pm 5.7$ mV ($n = 4$), respectively. Substitution of Cl^- shifted zero current voltage by -2.6 ± 0.9 mV ($n = 6$). From this (and by use of the Goldmann–Hodgkin–Katz equation) a permeability ratio of $P_{Na}:P_{Li}:P_K:P_{Cs}:P_{NMDG}:P_{Ca}:P_{Cl}$ of 1:0.86:0.84:0.56:0.10:0.07:0.15 can be computed for the hypertonicity-induced cat-

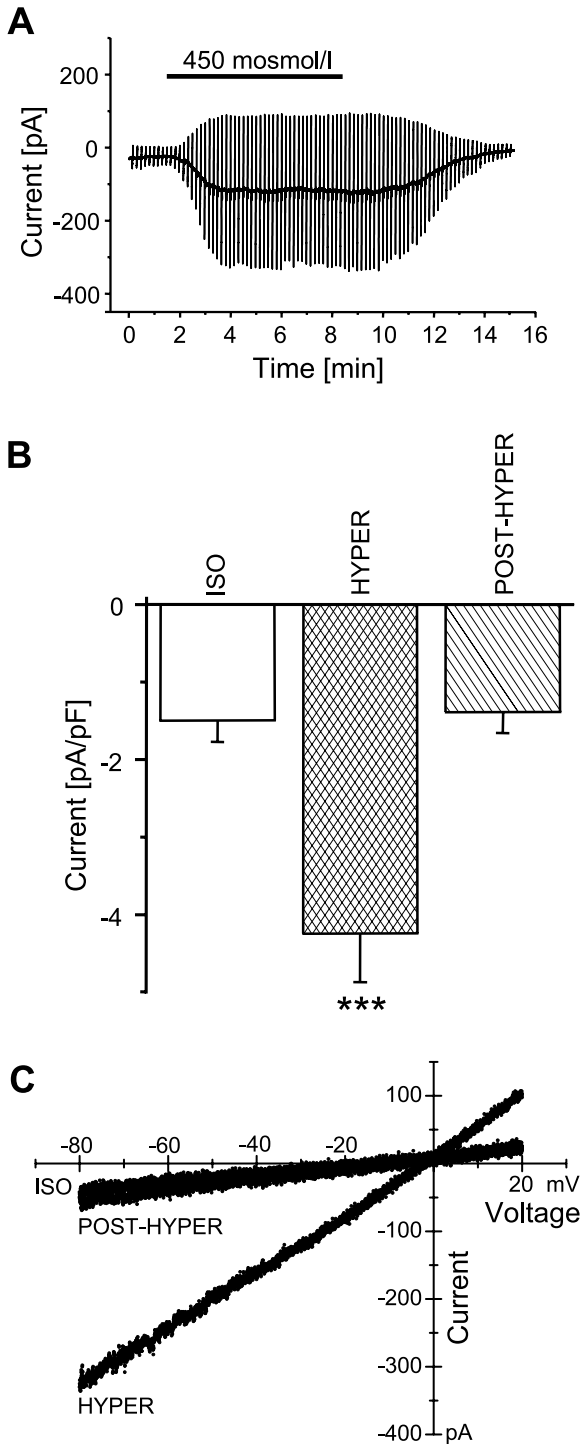

Fig. 1. Hypertonic activation of membrane currents in HeLa cells. A: Typical whole-cell recording at $V_h = E_{Cl} = -30$ mV. Current deflections result from voltage ramps (from −80 to +20 mV, see C) of 1 s duration that were applied every 10 s. For the time indicated, bath osmolarity was increased from 340 to 450 mosmol/kg-H_2O. Note the resultant increase in current and membrane conductance. B: Summary of the effects of hypertonic stress on membrane currents (obtained at −30 mV; $n = 7$). See text for details. $^{**}P < 0.001$, significantly different from control. C: Current–voltage relations at 340 mosmol/kg-H_2O (ISO), at 5 min in 450 mosmol/kg-H_2O (HYPER), and at 8 min after change back to isotonic conditions (POST-HYPER). Representative experiment.

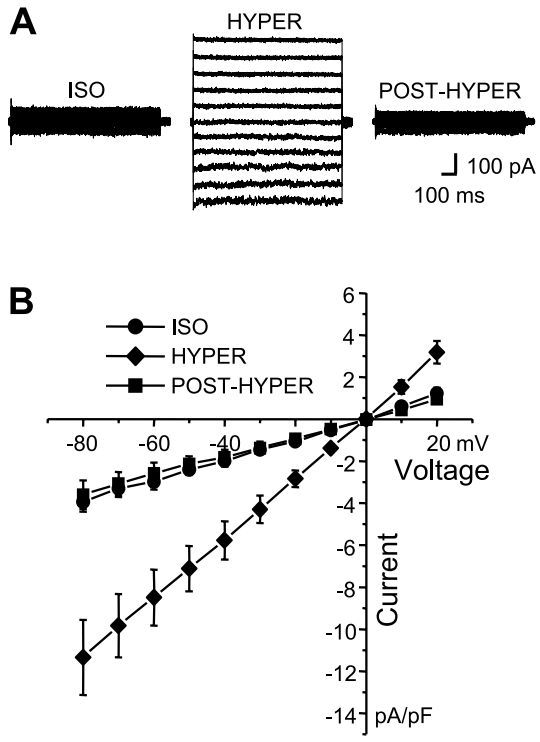

Fig. 2. Monitoring the hypertonic activation of membrane currents by use of voltage steps (from −80 to +20 mV, with 10 mV increment). Holding voltage was −30 mV and pulses of 1 s duration were applied every 10 s. See text for details. A: Typical experiment. B: Summary of results ($n = 5$).

ion conductance of HeLa cells (see Table 1). Thus, with respect to its selectivity, this conductance would be comparable to the (volume-activated and) *amiloride-sensitive* cation channel found in rat hepatocytes [9], Ehrlich–Lettre ascites tumor cells [6], and human red blood cell ghosts [10] that does not discriminate much between Na^+, K^+ (and Li^+). Alternatively, it may be related to the *second* of the two groups of *amiloride-insensitive* cation channels. Whereas in both of these groups channels were found to be equally permeable to Na^+ and K^+, the *first* type of channel exhibits a significantly lower permeability to Li^+ when compared to Na^+ in combination with a sizeable permeability to $NMDG^+$ (with a P_{NMDG}/P_{Na} of 0.56). These channels were described for human nasal epithelium [11] and the CaCo-2 cell line [12]. The *second* type of *amiloride-insensitive* channels is equally permeable to Na^+ and Li^+ but virtually impermeable to $NMDG^+$. These channels were found in the human colon cell line HT29, the mouse cortical collecting duct cell line M1, BSC-1 renal epithelial cells, A10 vascular smooth muscle cells, and Neuro-2a cells [13–15]. Of note, however, from both groups of these *amiloride-insensitive* (and hypertonicity-induced) channels comparable permeabilities for Na^+ and Cs^+ were reported so that the P_{Cs}/P_{Na} ratio of 0.56 observed in the present study would clearly be a peculiarity.

Table 1
Ion selectivity of the hypertonicity-induced conductance in HeLa cells

	P_x/P_{Na}	P	
Li^+	0.86 ± 0.01**	0.0052	(from 1)
K^+	0.84 ± 0.02**	0.0064	(from 1)
Cs^+	0.56 ± 0.02***	0.000035	(from 1)
$NMDG^+$	0.10 ± 0.01***	0.0000032	(from 1)
Ca^{2+}	0.07 ± 0.02***	0.000018	(from 1)
Cl^-	0.15 ± 0.02***	0.000019	(from 1)
	0.15 ± 0.02**	0.00314	(from 0)

Data are given as permeabilities relative to Na^+ (P_x/P_{Na}) and P values were computed with reference to 1 or 0 as indicated ($n = 4$–5 for each experimental condition).

3.3. Pharmacology of cation conductance

We then determined the pharmacological profile of the hypertonicity-induced cation conductance in HeLa cells. A typical recording exemplifying the experimental protocol used is depicted in Fig. 4A and results are summarized in Fig. 4B. As is obvious from the figure, (100 μmol/l) amiloride did not significantly change currents which equalled 91.4 ± 5.2% of maximal hypertonic stimulation ($n = 4$). In contrast, Gd^{3+} and flufenamate (applied at the same concentration) reduced currents to 58.8 ± 14.5% ($n = 4$, $P < 0.05$) and 30.3 ± 11.6% ($n = 5$, $P < 0.01$), respectively. Together with its ion selectivity, these results strongly imply a close correlation of the volume-activated channel in HeLa cells to the *second* group of *amiloride-insensitive* channels, as was outlined above (see Section 3.2).

We then tested for a possible molecular correlation of the HeLa conductance to TRP channels. These non-selective cation channels belong to a family of gene products that are structurally related to the light-activated transient receptor potential (Trp) channel from *Drosophila melanogaster* and that appear to be ubiquitously expressed [3,16–18]. As was reported so far, channels of this type were activated under *hypo-* rather than *hyper*tonic conditions and they exhibited a distinct permeability to Ca^{2+}. Of note, however, TRP channels were also found to be effectively blocked by Gd^{3+}. Interestingly, there is a selective inhibitor of these channels available (SKF-96365) and, in fact, this compound (at 100 μmol/l) led to a slight but significant reduction of hypertonicity-induced currents in HeLa cells that amounted to 26.1 ± 5.3% ($n = 5$, $P < 0.01$, Fig. 4B).

3.4. Volume regulation

In the next series of experiments, the contribution of the hypertonicity-induced cation channel to the RVI process was defined. In particular, this was done with reference to the other mediators of Na^+ import under hypertonic conditions,

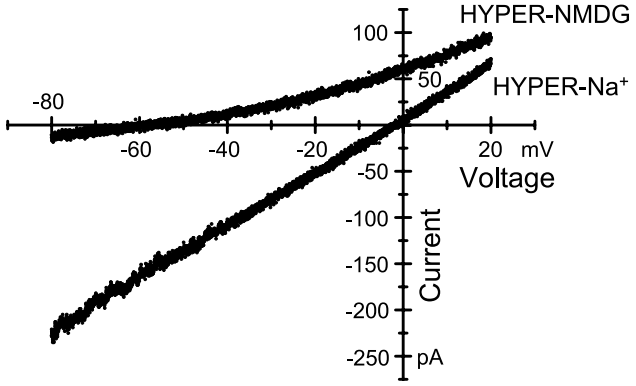

Fig. 3. Membrane currents after maximal hypertonic activation in symmetrical Na^+-containing solutions (HYPER-Na^+) and after complete substitution of Na^+ in the bath by $NMDG^+$. Typical experiment.

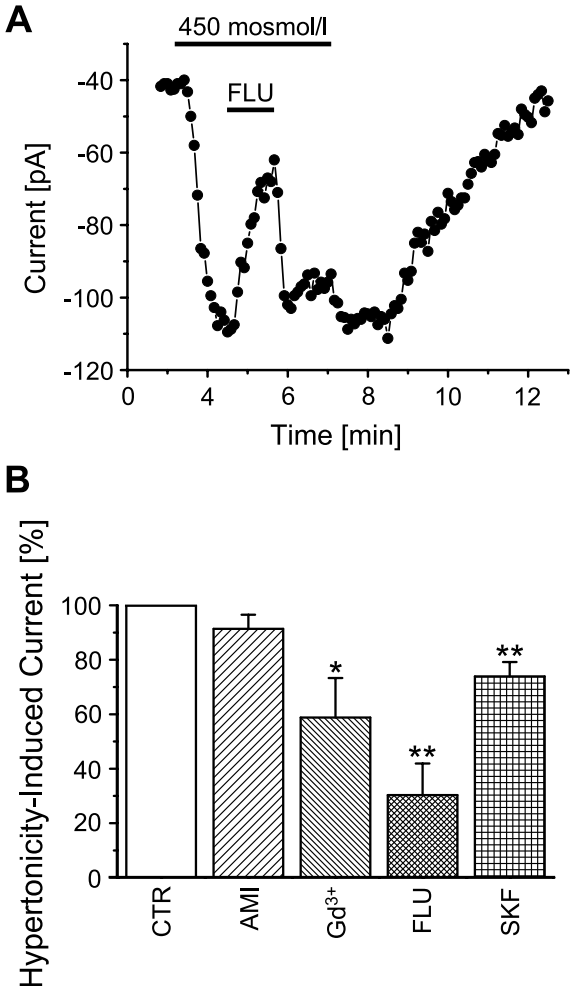

Fig. 4. Pharmacology of hypertonicity-induced currents. A: Typical experiment with flufenamate (FLU) added to the superfusate at 100 μmol/l for the time indicated. Note the reversible inhibition of current after maximal osmotic activation. B: Summary of results. Amiloride (AMI), Gd^{3+}, flufenamate (FLU), and SKF-96365 (SKF) were used at 100 μmol/l each. $n = 4$–5 for each compound. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control.

namely Na^+-K^+-$2Cl^-$ symport and Na^+/H^+ antiport. Because the latter is known to operate in parallel to Cl^-/HCO_3^- antiport (and in order to allow for a quasi-physiological RVI to occur) these measurements were performed in the presence of HCO_3^- (see Section 2.3). Also of note in this respect is the finding that for a sizeable RVI in HeLa cells to occur HCO_3^- appears to be essential (Okada, unpublished observation).

As was determined by means of Coulter counter techniques, increasing osmolality from 300 to 450 mosmol/kg-H_2O led to an initial decrease of cell volume that, at 5 min of hypertonic stress, equalled 77.6 ± 0.6% of control. Thereafter, cell volume slowly increased again so that, after 90 min, a new steady-state value of 86.1 ± 1.2% was achieved ($n = 10$, Fig. 5A). This is equivalent to an RVI of 38.1 ± 4.6% when referred to the initial amount of cell shrinkage ($P < 0.001$, Fig. 5B).

In the presence of 100 μmol/l furosemide, a selective blocker of Na^+-K^+-$2Cl^-$ symport, RVI equalled 49.7 ± 6.4% ($n = 10$) which is not significantly different from control conditions. This strongly suggests that this transport is not employed in the RVI of HeLa cells. With 100 μmol/l amiloride, RVI equalled 19.8 ± 2.3% ($n = 9$, $P < 0.01$). Since there was no significant effect of the compound on cation conductance (see Section 3.3) and because, at 100 μmol/l, amiloride will almost completely block Na^+/H^+ antiport (NHE1) [19,20] its effect here is very likely to reflect the actual contribution of this antiport to RVI. In the presence of 100 μmol/l flufenamate, RVI was −10.4 ± 6.7% ($n = 9$). This is different from control (with $P < 0.001$) but it does not significantly differ from zero. In other words, the RVI process was completely blocked under these conditions which, first, supports our patch-clamp data. Second, these results strongly suggest that the cation conductance is the main mechanism of RVI in HeLa cells.

In the presence of 100 μmol/l SKF-96365, a numerical RVI value of −38.2 ± 8.3% was obtained ($n = 9$, $P < 0.001$). This was due to a second slow phase at which cell volume was continuously further decreasing, under these conditions (Fig. 5A). The effect is much stronger than one would expect from the relatively weak inhibition of hypertonicity-induced currents by SKF-96365 (see Section 3.3). Accordingly, the compound appears to exert pronounced additional modes of acting on cell volume that await further investigation.

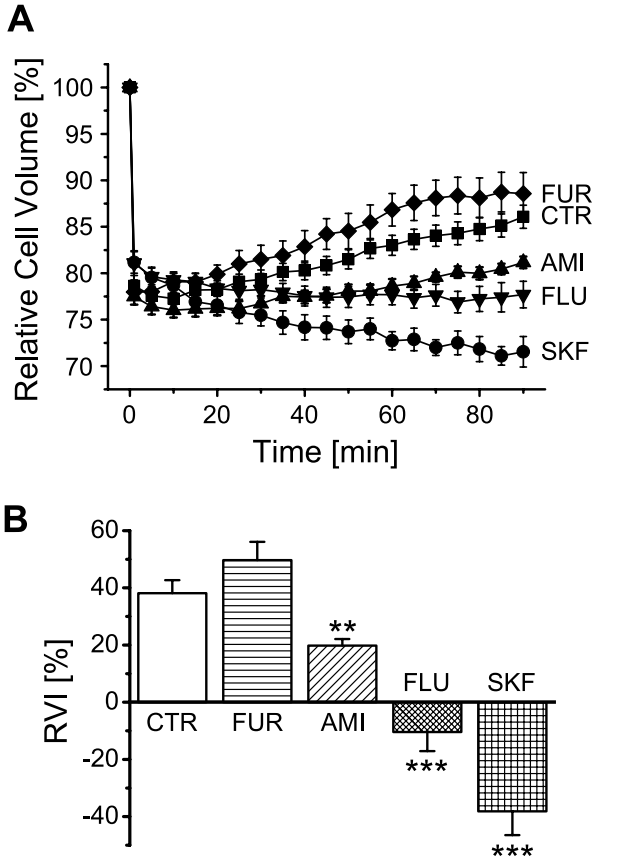

Fig. 5. A: Changes of cell volume upon hypertonic stress (300→450 mosmol/kg-H_2O) that were determined by Coulter counter technique (see text for details). Experiments were performed under control conditions (CTR) and in the presence of furosemide (FUR), amiloride (AMI), flufenamate (FLU), or SKF-96365 (SKF). $n = 9$–10 for each experimental condition. B: RVI computed from the cell shrinkage at 5 min and from the volumes at 90 min of hypertonicity (see A).

3.5. Conclusions

In conclusion, we observe the hypertonic activation of a cation conductance in HeLa cells which does not discriminate much between Na^+, Li^+, and K^+ but which exhibits a significantly lower permeability to Cs^+. Permeabilities to $NMDG^+$, Ca^{2+}, and Cl^- are minor. Most probably, the cation conductance is the main mechanism of RVI in HeLa cells but a significant contribution of Na^+/H^+ antiport is also very likely. Na^+-K^+-$2Cl^-$ symport does not appear to be employed in the RVI of this system.

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