A novel hypertonicity-induced cation channel in primary cultures of human hepatocytes

Tongju Li[a], Frank ter Veld[a], Hartwig R. Nürnberger[b], Frank Wehner[a],*

[a] *Max-Planck-Institut für molekulare Physiologie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany*
[b] *Chirurgische Klinik, Klinikum Dortmund, Beurhausstrasse 40, 44137 Dortmund, Germany*

Abstract In whole-cell recordings on primary cultures of human hepatocytes, we observe the hypertonic activation of a novel type of cation channel with a permeability ratio for Na^+: Li^+:K^+:Cs^+:$NMDG^+$ of 1:1.2:1.3:1.2:0.6. With a P_{Ca}/P_{Na} of 0.7 the channel is also clearly permeable to Ca^{++}. Most likely, the channel is Cl^- impermeable but its activity critically depends on the extracellular Cl^- concentration (with the half maximal effect at 88 mmol/l). With a 64% inhibition by amiloride and a complete block by flufenamate and Gd^{3+} (at 100 µmol/l each), the channel may represent a molecular link between the amiloride-sensitive and insensitive channels reported so far.
© 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

In addition to their role in homoeostasis, the mechanisms of cell volume regulation have proven to participate in a variety of important physiological functions such as the synchronisation of transport across epithelia, the triggering of hepatic metabolism, as well as the coordination of proliferation and apoptosis [1–3].

The transporters of regulatory volume increase (RVI, in response to cell shrinkage) most commonly employed are the Na^+/H^+-antiporter NHE1 (in many instances working in parallel with Cl^-/HCO_3^- exchange) and the Na^+–K^+–$2Cl^-$ symporter NKCC1, both mediating the net-uptake of Na^+Cl^- and osmotically obliged water. In recent years, however, it became increasingly evident that a Na^+ uptake that is mediated by ion channels may function as an alternative mechanism of RVI (see [1] for review). Moreover, whenever the RVI of a given cell was studied quantitatively these non-selective hypertonicity-induced cation channels (HICCs) were found to be the main mechanism of volume regulation [4–6].

The actual contribution of HICCs to the RVI process was first studied in detail in rat hepatocytes [4,7,8]. The hepatocyte channel is effectively blocked by the diuretic amiloride [7] with an IC_{50} of 5 µmol/l [9]. In addition, rat hepatocytes express all three subunits of the ENaC (the amiloride-sensitive epithelial Na^+ channel) [9] and injection of anti-α-rENaC oligo-DNA

nucleotides into single rat hepatocytes inhibits HICC activation by 70% [10]. Additional features of the channel are the insensitivity to Gd^{3+} and to the anti-inflammatory drug flufenamate (Wehner, unpublished) and its selectivity ratio P_{Na}/P_K of 1.4 [10]. A contribution of amiloride-sensitive HICCs to the RVI process and/or a possible correlation between these channels and the ENaC have since then been found in a variety of preparations (see [1] for review).

Just based on pharmacological characteristics, one may distinguish the above type of HICC from the one that is insensitive to amiloride but effectively blocked by flufenamate and Gd^{3+}. The latter is expressed in many systems including human nasal epithelial cells [11], the human colon cell-lines CaCo-2 and HT29 [12,13], and the mouse cortical collecting duct cell-line M1 [14]. It is also found in BSC-1 monkey renal epithelial cells, A10 rat aortic smooth muscle cells, Neuro-2a cells derived from mouse neuroblastoma [13], as well as in the human cervix carcinoma cell-line HeLa [6].

Here, we describe a novel type of HICC for primary cultures of human hepatocytes that is non-selective for small monovalent cations. The activity of the channel critically depends on the presence of extracellular Cl^- and it is the first HICC exhibiting a sizeable permeability to Ca^{++}. Moreover, the channel is inhibited by Gd^{3+} and flufenamate as well as by amiloride. Hence, it may represent a molecular link between the amiloride-sensitive and insensitive HICCs reported so far.

2. Materials and methods

Primary human hepatocytes were harvested from ≈10 g pieces of healthy liver tissue as they were obtained during partial hepatectomies for tumour removal. The procedure was approved by the local ethics committee. Patients were informed that no additional tissue will be removed during surgery for study purposes. Samples were only taken if a written consent was obtained from the patient.

Hepatocytes were isolated by a 15 min perfusion with 0.05% collagenase IV (Sigma, Deisenhofen, Germany) in Williams Medium E (WME), supplemented with 5 mmol/l $CaCl_2$. Thereafter, cells were separated mechanically and washed with WME by centrifugation at $30 \times g$ for 5 min. Cells were counted in a Thomae-chamber and the amount of viable cells was determined on the basis of trypan blue exclusion. 0.5×10^6 Cells were plated on collagen-coated, gas-permeable Petriperm™ dishes (Vivascience, Hanover, Germany) and allowed to attach at 37 °C for 2 h. Non-attached cells were then washed off and hepatocytes were cultured in WME supplemented with 5 mmol/l L-glutamine, 0.06% glucose, 50 µg/ml gentamycin, 50 U/ml penicillin, 50 µg/ml streptomycin, 37 µmol/l inosine, 4.8 µg/ml hydrocortisone, 1 µg/ml insulin, 10% FCS, and 23 mmol/l HEPES (pH 7.4). Hepatocytes were maintained at 37 °C and 5% CO_2 and were used at day 1 after preparation.

*Corresponding author. Fax: +49 231 133 2669.
E-mail address:* frank.wehner@mpi-dortmund.mpg.de (F. Wehner).

Patch-clamp set-up and techniques were the same as reported previously. Briefly, patch pipettes were pulled from 1.50 mm OD and 1.17 mm ID borosilicate glass capillaries (Harvard Apparatus Ltd., Edenbridge, UK) on a programmable multi-stage pipette puller (DMZ-Universal Puller; Zeitz-Instrumente, München, Germany) and had resistances in the range of 2.0–4.0 MΩ. Pipettes were positioned by use of a motorised micro-manipulator (mini 25; Luigs und Neumann, Ratingen, Germany) and an Ag–AgCl wire served as the reference electrode, except in the Cl^- substitution experiments where a custom-made flowing junction 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 digitised with an AD converter (Digidata 1200 A; Axon Instruments) at 2 kHz and filtered with the built-in four-pole Bessel filter at 1 kHz. Data acquisition and analysis were done with the pCLAMP 8.2 software package (Axon Instruments). The same software was used for the computation of liquid junction potentials of pipettes that were compensated for (a voltage equal in size but opposite in sign to the liquid junction potential was added to the recording circuit and, following seal formation, this voltage was then cancelled out again). Holding voltage was −20 mV and voltage ramps from −80 to +40 mV and 1 s duration were applied every 10 s. In most instances, currents were normalised to the cell capacitance determined from the readings on the patch-clamp amplifier.

The isotonic bath solution (pH 7.4) contained (in mmol/l): NaCl 147.0, KCl 3.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, Na–HEPES 5.0, HEPES 5.0. Osmolality was adjusted to 300 mosmol/kg-H_2O (by addition of mannitol) under osmometric control (Knauer, Berlin, Germany). In the hypertonic test solution, the extracellular osmolality was increased to 350 mosmol/kg-H_2O.

The pipette solution (pH 7.2) contained (in mmol/l): NaCl 19.0, KCl 43.5, K-gluconate 28.5, $CaCl_2$ 0.5, $MgCl_2$ 3.0, EGTA 1.0, Na–HEPES 10, Na_2ATP 1.0. The osmolality was set to 280 mosmol/kg-H_2O. With the above transmembrane gradients, E_{Na}, E_{Cl}, and E_K equal +40, −20, and −80 mV, respectively.

In the ion-substitution experiments, extracellular Na^+ was exchanged for the mono-valent cation under consideration; Ca^{++} was isotonically increased to either 10 or 25 mmol/l in exchange for Na^+. Cl^- was partially exchanged for gluconate so that the final concentrations equalled 154.0 (control), 129.0, 104.0, 79.0, and 4.0 mmol/l. We did not compensate for the Ca^{++} buffering effect of gluconate that, in the 4.0 mmol/l Cl^- solution for instance, will reduce the actual free Ca^{++} concentration to 0.30 mmol/l. This is because, in additional control experiments with compensated Ca^{++} (3.3 mmol/l, *nominally*; 1.0 mmol/l, *free*; $n = 3$), we obtained an effect of Cl^- reduction to 4.0 mmol/l that was virtually identical to the one depicted in Fig. 4B, namely a decrease of membrane conductance to 30.1 ± 5.7% of the value at maximal hypertonic stimulation.

Relative permeabilities with respect to Na^+ were calculated from shifts in zero-current voltage according to the Goldmann–Hodgkin–Katz equation. All experiments were conducted at room temperature (18–20 °C). An exchange of solutions in the experimental chamber was completed after some 2 min but, in any instance, additional 2 min were given to insure a proper ion substitution also on the trans-side of the cells (i.e., on the part of the membrane facing the substratum).

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.

For the Cl^- dependence of cation conductance, data were fitted to the standard concentration–response form

$$I = I_1 + (I_2 - I_1)/(1 + 10^{(\log X_0 - X) \cdot P}),$$

where I is the cation current at a Cl^- concentration X. I_1 and I_2 are the baseline and maximal values obtained and X_0 is the Cl^- concentration giving a response half-way between I_1 and I_2. The Hill-slope P defines the steepness of the curve, i.e., the Cl^- sensitivity of currents close to X_0.

3. Results and discussion

In whole-cell recordings on single human hepatocytes, hypertonic stress (+50 mosmol/l mannitol) led to a significant increase of membrane currents. Fig. 1A depicts the currents obtained at E_{Na}, E_{Cl}, and E_K (where current is the sum of I_{Cl} plus I_K, I_{Na} plus I_K, and I_{Na} plus I_{Cl}, respectively). The increase of currents coincided with a significant shift of zero-current voltage from −38.7 ± 2.7 to −18.3 ± 1.6 mV ($n = 10$, $p < 0.01$; Fig. 1B) already indicative of the activation of a Na^+ permeable conductance. Both effects were almost completely reversible. From the voltage range of −40 to 0 mV, slope conductances could be computed that equalled 42.6 ± 3.8, 232.7 ± 49.3 ($p < 0.01$), and 81.7 ± 20.1 pS/pF (n.s. from control) at times 1, 6, and 12 min (with reference to Fig. 1A), respectively.

Figs. 2A and B exemplify an experiment in which the effect of (100 μmol/l) amiloride on hypertonicity-induced currents was examined. The complete pharmacological characterisation of the channel is summarised in Fig. 2C. As is obvious from the figure, the HICC was completely blocked by 100 μmol/l Gd^{3+} and flufenamate (with $p < 0.001$ each) while the inhibition by amiloride amounted to 64 ± 10% ($p < 0.01$). Accordingly, (together with the channel reported for Ehrlich–Lettre ascites tumour cells [15]) the HICC in human hepatocytes appears to be a member of a new class combining the "classical" pharmacological features of amiloride-sensitive and insensitive channels [1]. This novel type of channel will be of particular interest with respect to the future molecular characterisation of HICCs because it may represent an actual link between the two groups reported so far.

In the next series of experiments, we tested for the ion selectivity of the HICC in human hepatocytes. A typical experiment is shown in Fig. 3A and data are summarised in Fig. 3B. It was found that the channel did not discriminate much between
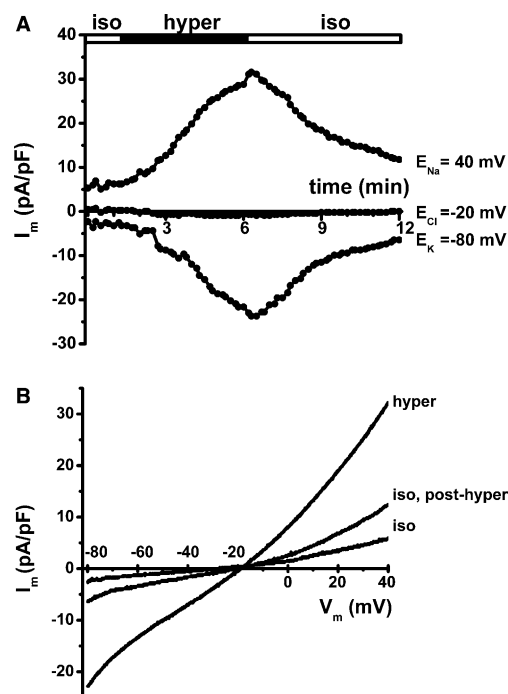

Fig. 1. Effects of hypertonic stress on the membrane currents of a single human hepatocyte in primary culture: (A) whole-cell currents at E_{Na}, E_{Cl}, and E_K, as indicated; (B) complete current-to-voltage relations determined at min 1, 6, and 12, in (A). Representative experiment.
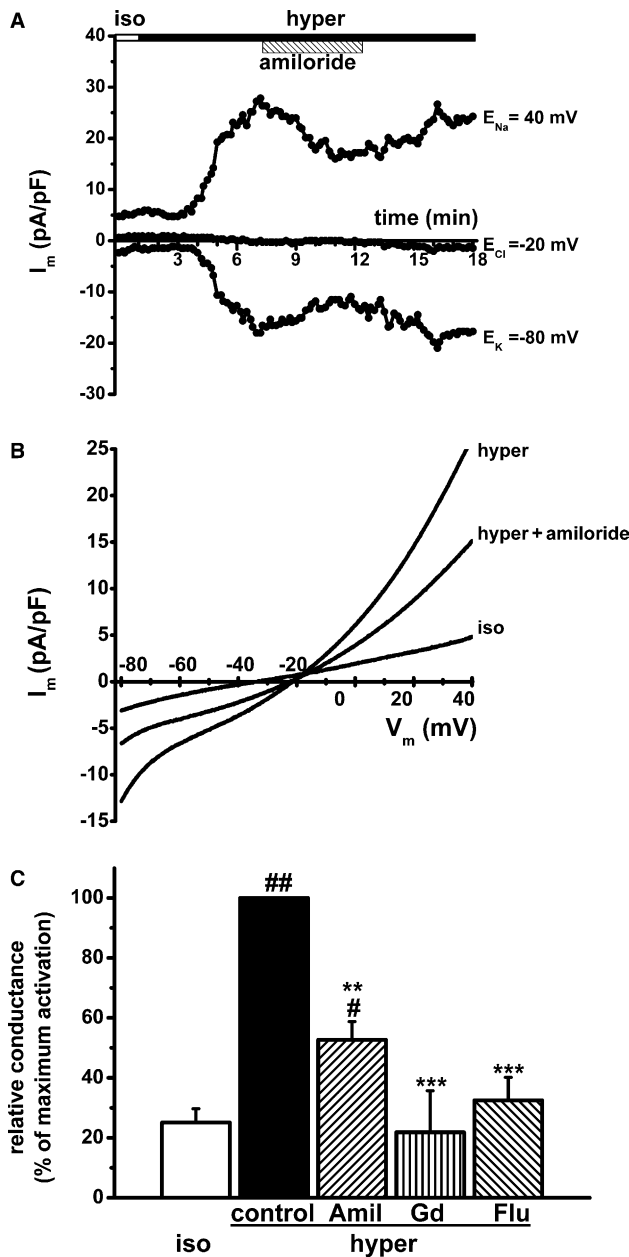
Fig. 2. Parmacology of hypertonicity-induced membrane currents: (A, B) typical experiment with 100 μmol/l amiloride; (C) summary of the experiments with amiloride, Gd^{3+}, and flufenamate (at 100 μmol/l each; $n = 5$–7). Conductances were computed from the slope of currents between −40 and 0 mV. Data were normalised to the maximal hypertonicity-induced conductance obtained, as 100% (control). (#, ##) Significantly different from the *isotonic* control with $p < 0.05$ and 0.01; (**, ***) significantly different from the *hypertonic* control, i.e., from the value at maximal stimulation, with $p < 0.01$ and 0.001.
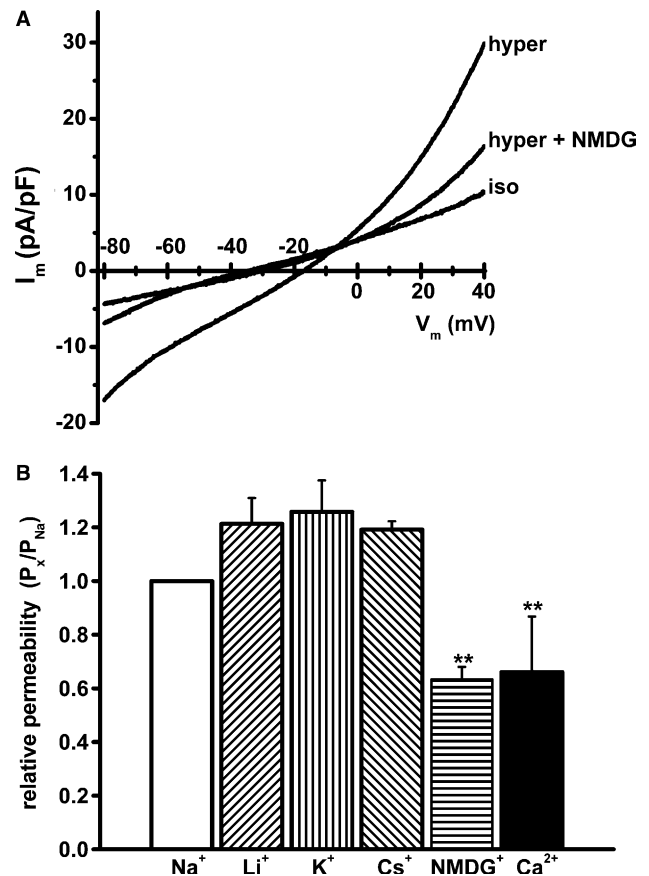

Fig. 3. (A) Following maximal hypertonic activation of currents, extracellular Na^+ was completely exchanged for $NMDG^+$. Representative experiment. (B) Summary of the ion-substitution experiments ($n = 4$–6). Permeabilities were computed with reference to P_{Na} as 1. See text for details. (**) Significantly different from Na^+ with $p < 0.01$.

small mono-valent cations so that the relative permeabilities for Li^+, K^+, and Cs^+ were 1.2 ± 0.1, 1.3 ± 0.1, 1.2 ± 0.1 with reference to Na^+ ($n = 4$ and n.s. for each of these ions). The relative permeability for $NMDG^+$, in contrast, amounted to 0.6 ± 0.1 which is significantly smaller than that for Na^+ ($n = 6$; $p < 0.01$). This is a novel selectivity pattern because the HICCs reported so far were either equally permeable to Na^+, K^+, Cs^+, and Li^+ but impermeable to $NMDG^+$ or they exhibited a permeability to Li^+ and $NMDG^+$ that amounts

to some 50% when compared to that of Na^+ [1]. If the membrane of human hepatocytes would, actually, contain a mixture of these two types of channels (rather than the novel single type we propose) one would expect relative permeabilities for Li^+ and $NMDG^+$ (with reference to Na^+) that would be somewhere between 1.0 and 0.5, and 0.5 and 0, respectively. This, however, is clearly not the case.

Most interestingly, the HICC in human hepatocytes was clearly permeable to Ca^{++} as well, with a P_{Ca}/P_{Na} of 0.7 ± 0.1 ($n = 7$; $p < 0.01$). This is a feature with no correlate to any other hypertonicity-induced channel reported so far [1]. It is of particular interest with respect to the actual molecular architecture of the HICC in human hepatocytes, because it may reflect a relationship to TRP channels. This super-family of non-selective cation channels is structurally related to the light-activated channel in *Drosophila* mediating the "transient receptor potential" [1]. These channels are ubiquitously expressed, they are employed in the sensation of a variety of chemical and physical stimuli, and some TRP channels were found to be osmo-sensitive; in most instances, TRP channels are involved in the receptor-mediated increase of cell Ca^{++} but, actually, there is a considerable variability with respect to the actual P_{Ca}/P_{Na} value [16–19].

In a series of Cl^- substitution experiments, extracellular Cl^- was reduced from 154.0 (control) to 129.0, 104.0, 79.0,
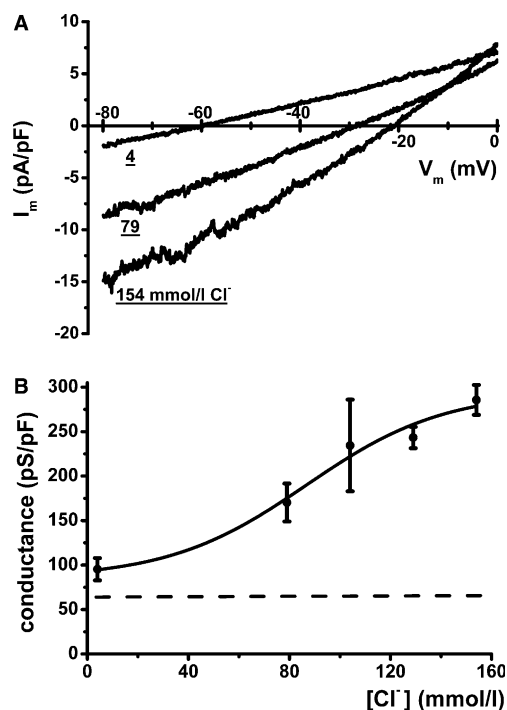
Fig. 4. (A) Effects of various Cl^- substitutions, as indicated, on hypertonicity-induced membrane currents. Typical experiment. (B) Concentration dependence of the effect of Cl^- on membrane conductance (computed from the slope of currents between -40 and 0 mV; $n = 3–6$). The dashed line indicates the average pre-stimulus (isotonic) conductance. See text for details.

and 4.0 mmol/l; with an intracellular Cl^- concentration of 69.5 mmol/l (see Section 2), this is equivalent to changes in E_{Cl} by $+4.6$, $+10.2$, $+17.4$, and $+95.1$ mV, respectively. These Cl^- substitutions led to changes in the zero-current voltage of hypertonicity-induced currents that equalled 0.1 ± 0.1 mV (n.s.), -4.7 ± 1.1 mV (n.s.), -8.7 ± 0.9 mV ($p < 0.01$), and -40.2 ± 2.6 mV ($p < 0.001$, $n = 3–6$; Fig. 4A). For the smallest change in Cl^- tested (from 154.0 to 129.0 mmol/l), this gives a P_{Cl}/P_{Na} of 0.01 or, in other words, a Cl^- permeability of the HICC that is non-significant (for the other Cl^- concentrations, computation of P_{Cl}/P_{Na} would even lead to *negative* values, namely to relative permeabilities of -0.38, -0.45, and -0.79, respectively). First of all, these results clearly show that the Cl^- permeability of the HICC in human hepatocytes is very low (in addition, they finally prove that we are, in fact, dealing with a cation channel and not with a special type of Cl^- channel that may be blocked by amiloride, flufenamate, Gd^{3+}, as well as by $NMDG^+$). Second, the pronounced shift of reversal potentials in low Cl^- solutions is very likely to reflect an actual Cl^- dependence of the HICC, as it was already reported from some other systems [11,20]. Under low Cl^- conditions, the channel would be partially inactivating again and this decrease in conductance is supposed to occur in parallel to a shift of zero-current voltage back to more negative (isotonic) values. A detailed analysis of the Cl^- effect revealed a decrease of cation conductance to 86% of control when extracellular Cl^- was reduced by just 25 mmol/l (Fig. 4B). The effect was half maximal at a Cl^- concentration of 88.6 ± 4.6 mmol/l and, very close to this point, the fitted curve also exhibited its maximal steepness

(with a Hill slope of 0.016). The sensitivity of the HICC to extracellular Cl^- may represent a safety mechanism, in terms that, under conditions of an outwardly directed Cl^- gradient, the activation of a non-selective cation channel may be too dangerous because it may lead to an actual overall release of osmolytes rather than to their net cellular uptake.

4. Conclusions

In summary, we observe the hypertonic activation of a non-selective cation channel in primary human hepatocytes. The channel is novel with respect to its cation selectivity including a sizeable permeability to Ca^{++}. The activity of the channel critically depends on the presence of extracellular Cl^- which may serve as a safety device to avoid triggering under inappropriate physiological conditions. The pharmacology of the channel combines the features of the amiloride-sensitive and insensitive channels reported so far. It may represent a molecular link between these channels and a relation to Deg/ENaC and TRP channels is possible.

References

[1] Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E. and Kinne, R.K.H. (2003) Cell volume regulation: Osmolytes, osmolyte transport, and signal transduction. Rev. Physiol. Biochem. Pharmacol. 148, 1–80.

[2] Okada, Y., Maeno, E., Shimizu, T., Dezaki, K., Wang, J. and Morishima, S. (2001) Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J. Physiol. (Lond.) 532, 3–16.

[3] Shimizu, T., Numata, T. and Okada, Y. (2004) A role of reactive oxygen species in apoptotic activation of volume-sensitive Cl-channel. Proc. Natl. Acad. Sci. USA 101, 6770–6773.

[4] Wehner, F. and Tinel, H. (1998) Role of Na^+ conductance, Na^+–H^+ exchange, and Na^+–K^+–$2Cl^-$ symport in the regulatory volume increase of rat hepatocytes. J. Physiol. (Lond.) 506, 127–142.

[5] Wehner, F., Lawonn, P. and Tinel, H. (2002) Ionic mechanisms of regulatory volume increase (RVI) in the human hepatoma cell-line HepG2. Pflugers Arch. 443, 779–790.

[6] Wehner, F., Shimizu, T., Sabirov, R. and Okada, Y. (2003) Hypertonic activation of a non-selective cation conductance in HeLa cells and its contribution to cell volume regulation. FEBS Lett. 551, 20–24.

[7] Wehner, F., Sauer, H. and Kinne, R.K.H. (1995) Hypertonic stress increases the Na^+ conductance of rat hepatocytes in primary culture. J. Gen. Physiol. 105, 507–535.

[8] Wehner, F. and Tinel, H. (2000) Osmolyte and Na^+ transport balances of rat hepatocytes as a function of hypertonic stress. Pflugers Arch. 441, 12–24.

[9] Böhmer, C., Wagner, C.A., Beck, S., Moschen, I., Melzig, J., Werner, A., Lin, J.T., Lang, F. and Wehner, F. (2000) The shrinkage-activated Na^+ conductance of rat hepatocytes and its possible correlation to rENaC. Cell. Physiol. Biochem. 10, 187–194.

[10] Böhmer, C. and Wehner, F. (2001) The epithelial Na^+ channel (ENaC) is related to the hypertonicity-induced Na^+ conductance in rat hepatocytes. FEBS Lett. 494, 125–128.

[11] Chan, H.C. and Nelson, D.J. (1992) Chloride-dependent cation conductance activated during cellular shrinkage. Science 257, 669–671.

[12] Nelson, D.J., Tien, X.Y., Xie, W.W., Brasitus, T.A., Kaetzel, M.A. and Dedman, J.R. (1996) Shrinkage activates a nonselective conductance: Involvement of a Walker-motif protein and PKC. Am. J. Physiol. 270, C179–C191.

[13] Koch, J.P. and Korbmacher, C. (1999) Osmotic shrinkage activates nonselective cation (NSC) channels in various cell types. J. Membr. Biol. 168, 131–139.

[14] Volk, T., Frömter, E. and Korbmacher, C. (1995) Hypertonicity activates nonselective cation channels in mouse cortical collecting duct cells. Proc. Natl. Acad. Sci. USA 92, 8478–8482.

[15] Lawonn, P., Hoffmann, E.K., Hougaard, C. and Wehner, F. (2003) A cell shrinkage-induced non-selective cation conductance with a novel pharmacology in Ehrlich–Lettre-ascites tumour cells. FEBS Lett. 539, 115–119.

[16] Harteneck, C., Plant, T.D. and Schultz, G. (2000) From worm to man: Three subfamilies of TRP channels. Trends Neurosci. 23, 159–166.

[17] Montell, C. (2001) Physiology, phylogeny, and functions of the TRP superfamily of cation channels. Sci. STKE 90, 1–17.

[18] Liedtke, W., Tobin, D.M., Bargmann, C.I. and Friedman, J.M. (2003) Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 2, 14531–14536.

[19] Voets, T. and Nilius, B. (2003) TRPs make sense. J. Membr. Biol. 192, 1–8.

[20] Shen, M.R., Chou, C.Y., Hsu, K.F. and Ellory, J.C. (2002) Osmotic shrinkage of human cervical cancer cells induces an extracellular Cl-dependent nonselective cation channel, which requires p38 MAPK. J. Biol. Chem. 277, 45776–45784.