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

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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.

Keywords: Hepatocytes; Volume regulation; Cation channel; Flufenamate; Amiloride; Gadolinium; Calcium; Chloride

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.

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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

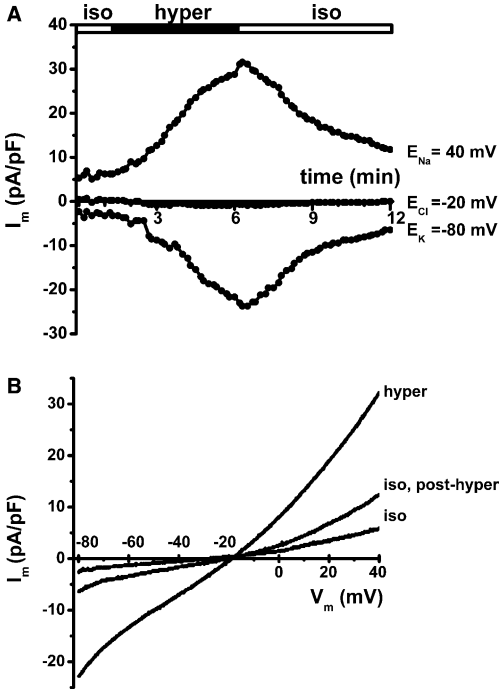

Fig. 1. Effects of hypertonic stress on the membrane currents of a single human hepatocyte in primary culture: (A) whole-cell currents at E_{Na}, E_{Cl}, and E_K, as indicated; (B) complete current-to-voltage relations determined at min 1, 6, and 12, in (A). Representative experiment.