The epithelial Na^+ channel (ENaC) is related to the hypertonicity-induced Na^+ conductance in rat hepatocytes

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Abstract The epithelial Na^+ channel (ENaC) is composed of the subunits α, β, and γ [Canessa et al., Nature 367 (1994) 463–467] and typically exhibits a high affinity to amiloride [Canessa et al., Nature 361 (1993) 467–470]. When expressed in *Xenopus* oocytes, conflicting results were reported concerning the osmosensitivity of the channel [Ji et al., Am. J. Physiol. 275 (1998) C1182–C1190; Hawayda and Subramanyam, J. Gen. Physiol. 112 (1998) 97–111; Rossier, J. Gen. Physiol. 112 (1998) 95–96]. Rat hepatocytes were the first system in which amiloride-sensitive sodium currents in response to hypertonic stress were reported [Wehner et al., J. Gen. Physiol. 105 (1995) 507–535; Wehner et al., Physiologist 40 (1997) A-4]. Moreover, all three ENaC subunits are expressed in these cells [Böhmer et al., Cell. Physiol. Biochem. 10 (2000) 187–194]. Here, we injected specific antisense oligonucleotides directed against α-rENaC into single rat hepatocytes in confluent primary culture and found an inhibition of hypertonicity-induced Na^+ currents by 70%. This is the first direct evidence for a role of the ENaC in cell volume regulation.

Key words: Liver; Cell volume regulation; Na^+ conductance; Amiloride; Epithelial Na^+ channel; Antisense DNA

1. Introduction

Volume control is essential for the survival of cells when confronted with changes of intra- or extracellular osmolarity [5,6]. In rat hepatocytes, hyperosmotic conditions and cell shrinkage trigger the activation of a Na^+ current [3] that leads to a significant influx of Na^+ into the cell [7]. Via Na^+ accumulation, which is the initial event of regulatory volume increase in these cells, an osmotic gradient is established mediating compensatory influx of water. The sensitivity of the volume-activated Na^+ current in rat hepatocytes to amiloride ($IC_{50} = 6.0$ μmol/l) [4] suggested a molecular link to the (amiloride-sensitive) epithelial Na^+ channel (ENaC) [8]. This notion is also supported by the mechanosensitivity of several members of the Mec/ENaC gene superfamily found in *Caenorhabditis elegans* [9]. To prove or disprove whether ENaC mediates the volume-activated Na^+ current in rat hepatocytes we inhibited the expression of the α-ENaC subunit (which could be shown to function as the crucial element of all heteromultimeric ENaCs reported so far [8]) by transfection with specific antisense oligo-DNA. It was found that the knock-out of α-ENaC reduced hyperosmotically induced Na^+ currents to 30% of control. These results clearly indicate that α-ENaC is a functional component of the volume-activated Na^+ channel in rat hepatocytes.

2. Materials and methods

2.1. Primary culture of hepatocytes

Isolation and primary culture of rat hepatocytes were done as previously described [10,11]. Briefly, after heparinization male Wistar rats (220–280 g body weight) were exsanguinated under urethane anesthesia by in situ perfusion of the liver with nominally Ca^{2+}-free Krebs–Henseleit solution (approved by Regierungspräsident Arnsberg and the Institute Animal Care Committee). After removal, the liver was perfused for 20 min with 0.05% collagenase A, dissolved in the same buffer. After isolation, cells were plated on collagen-coated, gas-permeable dishes (Petriperm; Bachofer, Reutlingen, Germany) and cultured in Dulbecco's modified Eagle's medium fortified with 10% fetal bovine serum, 2 mmol/l glutamine, 100 U/ml penicillin–100 μg/ml streptomycin, 1 μmol/l dexamethasone, 10 nmol/l triiodothyronine/thyroxine (T_3/T_4) and 5 μg/ml bovine insulin at 37°C in 5% CO_2–air. Cells formed confluent monolayers within 24 h and were used from days 1–3 after preparation.

2.2. Electrophysiology on hepatocytes

The electrophysiological set-up and impalement techniques have been described in detail previously [3,11]. For determination of membrane currents, a modification of the continuous two-electrode voltage-clamp was used and cells were impaled with dual-channel theta-glass microelectrodes (Thick-Septum-Theta; WPI, New Haven, CT, USA). Membrane voltage was measured through one channel and holding currents were applied through the other. To avoid space-clamp problems due to the high intercellular coupling in the monolayer, gap-junctional communication was blocked by 40 μmol/l 18-β-glycyrrhetinic acid [12,13]. Within the experimental time frame used in this study, the effects of 18-β-glycyrrhetinic acid on cell coupling were fully reversible and the compound did not significantly change membrane voltage.

2.3. Microinjection of hepatocytes

All oligonucleotides used were synthesized by Biognostik (Göttingen, Germany): (i) antisense oligo-DNA directed against α-rENaC, (ii) control oligo-DNA composed of the same number of nucleotides but with no consensus sequence in the rat genome sequenced so far, (iii) control oligos labeled with the dye fluorescein. Oligonucleotides were microinjected in volumes <1 pl by use of a semiautomatic system (Transjector 5246 and Micromanipulator 5171; Eppendorf, Hamburg, Germany). The micropipettes for injection were produced on a horizontal puller (DMZ-Universal Puller; Zeitz Instrumente, Munich, Germany) from 1 mm o.d. glass capillaries (WPI).

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2.4. Microfluorometry

The injection procedure was optimized and the spatial (and temporal) distribution of fluorescein-labelled control oligo-DNA in the cell monolayer was monitored in pilot experiments by means of confocal laser scanning microscopy. A standard microscope (Diaphot; Nikon, Düsseldorf, Germany) with a ×20 objective was equipped with a confocal laser scanning device (MRC-600; Bio-Rad, Hemel Hempsted, UK). Fluorescence was excited by use of the 488 nm band of an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA).

2.5. Oocyte preparation, injection and electrophysiology

Xenopus laevis oocytes were prepared as described in a previous report from this laboratory [14]. All oocytes used were injected with 5 ng each of α-, β- and γ-rENaC cRNA. Cells were then injected with 50 nl of water, or 50 nl of a solution of either 10 mmol/l control oligo-DNA or anti-α-rENaC oligo-DNA. 24–36 h after injection, standard two-electrode voltage-clamp techniques were applied for quantification of membrane currents [14]. In the experiments, a holding voltage of −60 mV was used and ENaC currents were monitored by superfusing the cells for 2 min with 10^{-5} mol/l amiloride.

2.6. Experimental solutions

The hepatocyte normosmotic control solution (300 mosmol/l, pH 7.4) contained (in mmol/l): NaCl, 144; KCl, 2.7; NaH_2PO_4, 0.4; $CaCl_2$, 1.8; $MgCl_2$, 1.1; glucose, 5.6; Na-HEPES, 2.5; HEPES, 2.5; pH was adjusted by addition of 1 M NaOH. The increase of osmolarity to 400 mosmol/l was achieved by addition of 100 mmol/l sucrose to the superfusate.

The standard oocyte solution (pH 7.5) contained (in mmol/l): NaCl, 100; KCl, 2; $CaCl_2$, 1; $MgCl_2$, 1; HEPES/Tris, 10.

2.7. Statistical analysis

Means ± S.E.M. are presented with n denoting the number of cells tested. $P < 0.05$ was considered significant.

3. Results

3.1. Capacity of α-rENaC antisense DNA

To ensure the potency of the anti-α-ENaC oligo-DNA used we performed pilot experiments on *X. laevis* oocytes heterologously expressing α-, β-, γ-rENaC. Channel activity was monitored by application of 50 μmol/l amiloride pulses and the resultant inhibitions of Na^+ inward currents in two-electrode voltage-clamp recordings [1,4]. Under control conditions (i.e. in oocytes injected with an equivalent amount of water instead of oligo-DNA-containing solution), amiloride-sensitive currents in α-, β-, γ-rENaC-expressing cells equalled 361 ± 85 nA ($n = 8$) at −60 mV. In cells injected with anti-α-ENaC oligo-DNA (expected to hybridize with its complementary mRNA target sequence and thus to inhibit ribosomal processing of the α-ENaC subunit), amiloride-sensitive currents were significantly reduced to 8 ± 2 nA ($n = 9$, $P < 0.001$; Fig. 1). As an additional control, oocytes were coinjected with oligo-DNA composed of the same number of nucleotide bases but with no consensus sequence in the rat genome sequenced so far. Under these conditions, amiloride-sensitive currents equalled 290 ± 110 nA ($n = 9$; Fig. 1) which is not significantly different from α-, β-, γ-rENaC-expressing cells coinjected with water (see above). These experiments verified the proper design of our anti-α-ENaC oligo-DNA and, at the same time, they proved that expression of the functional ENaC is readily inhibited by solely blocking the processing of the channel's α-subunit.

3.2. Introduction of antisense DNA into hepatocytes

We then assessed the relation of α-ENaC to the native

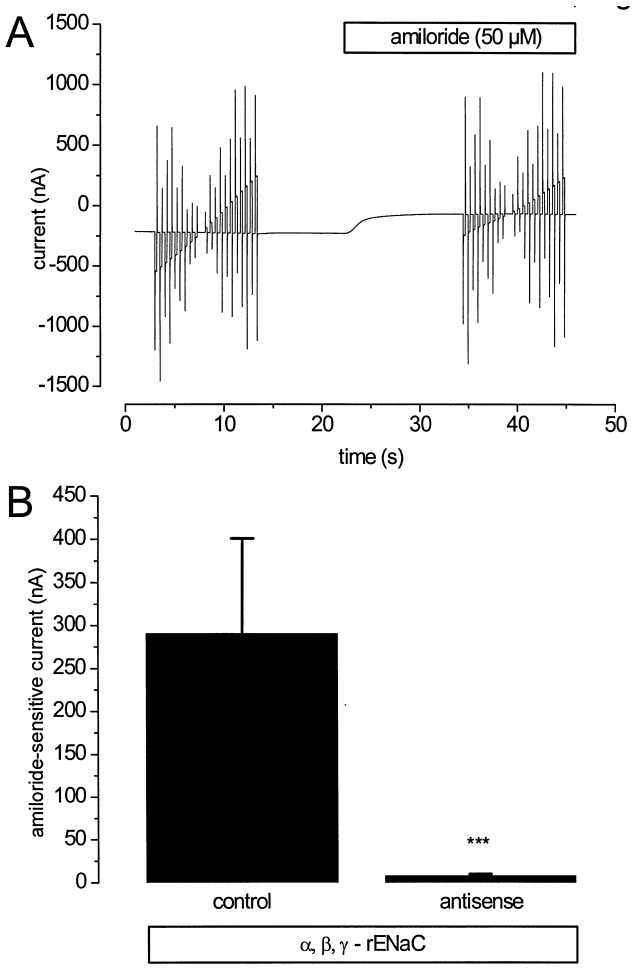

Fig. 1. A: Original recording exemplifying the quantification of amiloride-sensitive currents in two-electrode voltage-clamp experiments on *X. laevis* oocytes. In every experimental group of this series, oocytes expressed α-, β-, γ-rENaC. Membrane currents were determined by application of voltage steps in the range of −150 to +50 mV in 10 mV increments. The holding voltage was −60 mV. For the time indicated, 50 μmol/l amiloride was added to the superfusate. Amiloride-sensitive currents were then computed from the currents obtained under control conditions minus those obtained in the presence of amiloride. B: Amiloride-sensitive currents at −60 mV. In addition to α-, β-, and γ-rENaC cRNA, cells were injected either with control oligo-DNA or with anti-α-rENaC oligo-DNA as indicated. ***Significantly different from cells injected with control oligo-DNA at $P < 0.001$.

osmo-sensitive Na^+ channel in rat hepatocytes. Because electroporation as well as various chemical permeabilization techniques yielded only marginal transfection rates in the confluent monolayers used, oligo-DNA constructs had to be microinjected directly into the cytosol of single hepatocytes. As was determined by means of confocal laser scanning microscopy, cells injected with fluorescein-labelled oligo-DNA still exhibited a high degree of fluorescence and a sharp contrast at their borders after 24 h (data not shown), which represents the time point at which the electrophysiological experiments were performed (see below). Thus, the oligo-DNA was not diffusing into neighboring cells and the construct did not appear to be extruded across the plasmalemma at any significant rate.

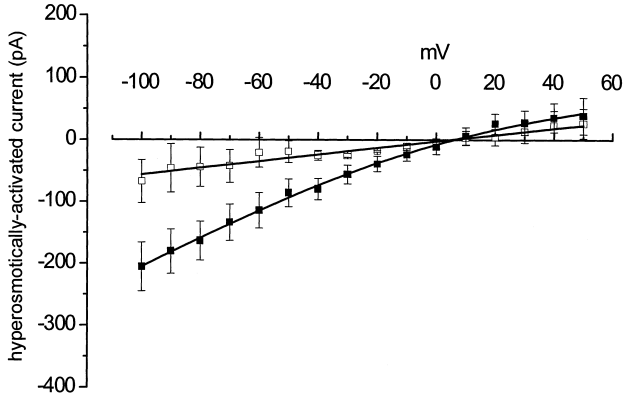

Fig. 2. Current–voltage relations of hypertonicity-induced membrane currents in rat hepatocytes. The differences between currents obtained at 400 mosmol/l and 300 mosmol/l are depicted for cells injected with control oligo-DNA (■) or anti-α-rENaC oligo-DNA (□).

3.3. Effects of antisense DNA on hypertonicity-induced membrane currents

In the electrical recordings, cells were impaled with dual-channel microelectrodes and standard voltage-clamp protocols were applied. Space-clamping of cells was optimized by use of the gap-junctional blocker glycyrrhetinic acid [12,13]. Under these conditions, hypertonic stress (300 → 400 mosmol/l) increased slope conductances (at 0 mV) to 124.4 ± 4.4% of control ($n = 8$) which is indistinguishable from data obtained by means of cable analysis (i.e. 127.1 ± 2.4% [7]). The voltage dependences of the hyperosmotically induced membrane currents (i.e. the differences between the currents at 400 mosmol/l and the currents at 300 mosmol/l) obtained under different experimental conditions are depicted in Fig. 2. As is obvious from the figure, cells injected with anti-α-rENaC oligo-DNA exhibited membrane currents that were reduced to approximately one third of those obtained with control oligo-DNA. Notably, there were no significant differences in the reversal potential (zero current voltage) between the two experimental groups indicating that, indeed, solely the ion conductance under consideration here was affected by the antisense construct. Because the currents reversed close to +6 mV the hypertonicity-induced channel appears to exhibit only a moderate selectivity of Na^+ over K^+ (namely a P_{Na}/P_K of 1.4, calculated on the basis of the known intracellular activities of Na^+ and K^+ under these conditions [7]). At −40 mV, which is the average membrane voltage of rat hepatocytes in confluent monolayers [3,7,11], inward currents in cells injected with control oligo-DNA equalled 80 ± 17 pA ($n = 8$) which is not significantly different from non-injected cells (76 ± 17 pA, $n = 17$). In contrast, hepatocytes microinjected with anti-α-ENaC oligo-DNA exhibited inward currents of 25 ± 17 pA ($n = 6$, $P < 0.01$) which is equivalent to 31% and 33% of the above reference values, respectively.

4. Discussion

A prerequisite for the use of standard voltage-clamp techniques on single rat hepatocytes in confluent monolayer culture is an effective reduction of electric cell-to-cell coupling. In the present study, this was achieved by the gap-junctional blocker 18-β-glycyrrhetinic acid by which cell coupling could be lowered to some 5–10% of control (data not shown). The remaining amount of cell-to-cell coupling, however, still represents a significant *osmotic* shunt between cells, at least when time periods in the order of minutes are considered. Accordingly, it will not be possible to monitor the effects of antisense oligo-DNA injection on α-ENaC expression in single hepatocytes as an impaired capability of cells to regulate their volumes. This would only be feasible in cells that are completely uncoupled from their neighbors.

Likewise, it would be interesting to see if the application of antisense oligo-DNA is, in fact, correlated with a decreased level of α-ENaC mRNA or protein expression. However, with only some 5–15 cells out of a total of 2×10^6 per monolayer being injected these changes (which are to be expected) will not be detectable.

The present study demonstrates that α-ENaC contributes to the hypertonicity-induced sodium current of rat hepatocytes which is the first direct evidence for a contribution of ENaC to cell volume regulation in a native system. This result is in line with the observation that α-ENaC (as well as β- and γ-ENaC) is, in fact, expressed in these cells [4].

The observed differences in amiloride sensitivity, which is higher for ENaC-type channels (typically exhibiting IC_{50} values close to 100 nmol/l [9,15]) than for the hepatocyte channel, might be explained by the fact that the affinity of ENaC for amiloride appears to be significantly modified by mechanical/osmotic stress [2,16]. Moreover, coexpression of α-, β-, γ-rENaC with the serine/threonine kinase hSGK that is activated in hepatocytes under hypertonic conditions [17] could be shown to decrease the sensitivity of the channel to amiloride [4]. These findings together may have more general implications for the analysis of 'low (amiloride) affinity types' of sodium channels and their possible correlation with ENaC. In addition, the relative expression of β- and γ- compared to α-ENaC may be an additional variable in the determination of the channel's pharmacology and the contribution of these subunits to the hypertonicity-induced Na^+ conductance of rat hepatocytes remains to be elucidated.

Interestingly, the hypertonicity-induced membrane currents reported here suggested only a moderate P_{Na}/P_K of the hepatocyte channel whereas ENaCs typically are highly selective for Na^+ (and Li^+) over K^+. On the other hand, the hepatocyte channel is clearly distinct from the non-selective cation channels found in some systems [18] since these channels are clearly not sensitive to amiloride.

With respect to the liver, where cell volume could be clearly shown to function as a *quasi*-second messenger in the control of hepatocyte metabolism [19,20], the novel function of (α-)-ENaC reported in this study will be of considerable physiological (and pathophysiological) significance.

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