Hypertonic activation of phospholemman in solitary rat hepatocytes in primary culture

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Abstract Under hypertonic conditions, solitary rat hepatocytes in primary culture shrink and subsequently exhibit a distinct regulatory volume increase (RVI). Reverse-transcribed polymerase chain reaction and 5′ and 3′ RACE (rapid amplification of cDNA ends) techniques reveal that these cells express phospholemman (PLM). In whole-cell recordings, the hypertonic activation of a channel is observed that resembles PLM with respect to unitary conductance (600–700 pS), gating pattern, and non-selectivity for Na^+ over K^+. In *Xenopus* oocytes expressing hepatocyte PLM, hypertonic stress induces a non-selective cation conductance and noise analysis reveals the activation of a channel with a unitary conductance of approximately 700 pS. These results suggest a role of PLM in the RVI of rat hepatocytes.

Key words: Liver; Cell-volume regulation; Phospholemman; Cation channel

1. Introduction

Phospholemman (PLM) is a small membrane protein of 15 kDa which was first purified, cloned, and sequenced from dog heart [1]. The mature protein consists of 72 amino acids, it has a single trans-membrane domain, and its name denotes its high degree of phosphorylation which (under most physiological conditions) appears to be mediated by protein kinases A and C [1–3]. When expressed in *Xenopus* oocytes, PLM induces anion currents that are activated by large hyperpolarizations of membrane voltage and most likely it does so by inducing endogenous oocyte channels [3,4]. On the other hand, when reconstituted in lipid bilayers PLM could be clearly shown to form functional ion channels by itself which exhibited rather slow gating kinetics and a unitary conductance as high as 730 pS [5]. It was also found that PLM (in lipid bilayers) exhibits two different modes of operation, namely a cation-selective one (500 pS) and an anion-selective one (730 pS) and that the protein appears to spontaneously switch between these modes [6,7]. Interestingly, PLM appears to be involved in processes mediating cell-volume regulation: Over-expression of PLM in HEK293 cells significantly increased the membrane currents elicited by hypotonic stress [7] as well as the release of Cl^- (I^-) and taurine during regulatory volume decrease (RVD) [8] and this osmolyte release could be markedly reduced by use of PLM-specific antisense oligonucleotides [9].

In most systems studied so far, the initial event in the regulatory volume increase (RVI) in response to cell shrinkage is an uptake of extracellular Na^+ (plus Cl^- and HCO_3^-) [10,11]. In rat hepatocytes, this Na^+ uptake is mainly achieved by the activation of a cation conductance which exhibits a relatively low selectivity for Na^+ over K^+ (namely a $P_{Na}/P_K = 1.4$) [12–14]. Given the permeability ratio close to unity found for PLM [5] and the role that the protein appears to play in volume regulation in some systems, we wondered whether PLM could be a functional component of the RVI of rat hepatocytes as well.

2. Materials and methods

2.1. Cell culture

Isolation and primary culture of rat hepatocytes have been reported in detail in previous publications from this laboratory [12,15].

2.2. Quantification of cell volumes

Changes of cell volumes in response to hypertonic stress were determined by use of the fluorescent dye calcein on a confocal laser-scanning device as was reported previously [12,13,16]. Briefly, cells were optically sectioned in 0.9 μm steps following the *z*-axis and from the measured areas of slices obtained (8–12 per cycle) cell volumes were computed.

2.3. Reverse-transcribed polymerase chain reaction (RT-PCR) and 5′ and 3′ RACE techniques

Total RNA was isolated using the RNeasy Kit from Qiagen or Tri reagent (Sigma) according to the supplier's protocol. Reverse transcription of total RNA (approximately 0.5 μg/reaction) was performed using an oligo dT_{18} primer and AMV reverse transcriptase (Qiagen). 5′- and 3′-RACE (rapid amplification of cDNA ends) experiments were carried out according to the RACE system from Gibco BRL (Life Technologies). Sequencing of relevant PCR fragments was performed with the ABIPrism BigDye Terminator Cycle Sequencing Ready Reaction Kit. The gene-specific primers for the amplification of PLM (forward: 5′-CTCTCCATGGCCAGTGCAGAAGCTC-3′; reverse: 5′-GCGGATGGAGCTGCGGAAAGTTC-3′) were derived from rat heart [17].

2.4. Patch-clamp techniques

Patch-clamp set-up and recording techniques were described in detail in a previous report [18]. Patch pipettes were pulled from 1.5 mm outside diameter hematocrit glass capillaries (Heraeus, Osterode, Germany) on a horizontal puller (DMZ-Universal Puller, Zeitz-Instrumente, Munich, Germany). They were filled with a Cl^--free solution containing (in mmol/l): Na-gluconate, 18; K-gluconate, 100; $MgSO_4$, 1.1; Na-HEPES, 2; HEPES, 3; EGTA, 1; sucrose, 50. pH was ad-

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justed to 7.2 by addition of 4 M NaOH. Pipettes had resistances of 2.5–4.5 MΩ when immersed in the (Cl^--free) hepatocyte control solution (see Section 2.7). Seals were in the range of 3–5 GΩ. The slow whole-cell configuration was achieved by use of amphotericin B (240 μg/ml). Membrane currents were measured with an Axopatch 200 amplifier in combination with a Digidata 1200 interface (Axon Instruments, Foster City, CA, USA). Data were filtered at 1 kHz and stored and analyzed on a PC by use of the pClamp 6 software package (Axon Instruments).

2.5. Oocyte preparation, injection, and recording techniques

Preparation and injection procedures on *Xenopus laevis* oocytes were as previously described [19]. Rat PLM cDNA subcloned into a pSP64p(A) vector (Promega) was a kind gift from the Wyeth-Ayerst Research Division of the American Home Products Corporation (Parsippany, NJ, USA). The plasmid was linearized with EcoRI and cRNA was synthesized using a kit from Ambion. 5 ng PLM cRNA in 50 nl of water were injected per cell and standard two-electrode voltage-clamp techniques were applied at 24–36 h after injection [14,19]. Water-injected oocytes served as controls.

2.6. Noise analysis

Fluctuation analysis of the hypertonicity-induced currents in oocytes was performed with a fast Fourier transforming DSP-board which automatically subtracted the DC component of the currents and averaged 10 sweeps of noise spectra per run [20,21].

2.7. Solutions

For the quantification of cell volumes, the normosmotic hepatocyte control solution of 300 mosmol/l contained (in mmol/l): NaCl, 144; KCl, 2.7; NaH_2PO_4, 0.4; Na-HEPES, 2.5; HEPES, 2.5; $CaCl_2$, 1.8; $MgCl_2$, 1.1; glucose, 5.6. pH was adjusted to 7.4 by addition of 4 M NaOH. Osmolarity was increased by addition of 100 mosmol/l sucrose. Experimental solutions were continuously gassed with O_2; experiments were performed at 36°C.

In the patch-clamp measurements, the extracellular (and intracellular) Cl^- (see also Section 2.4) was replaced by gluconate or SO_4^{2-} to isolate cation conductances. In addition, 0.5 mmol/l quinine was present in the extracellular solution to block K^+ conductance [13,22]. Rapid ion substitutions (during the short-lived activations of PLM) were achieved by means of a custom-made double-barreled flow-pipe that was positioned close to individual cells by use of a micromanipulator. Experiments were performed at room temperature (20–22°C).

The control oocyte solution of 220 mosmol/l contained: NaCl, 100; KCl, 2; $CaCl_2$, 1; $MgCl_2$, 1; HEPES/Tris, 10. The pH was 7.5, solutions were gassed with O_2, and experiments were performed at room temperature. Osmolarity was increased by addition of 250 mosmol/l sucrose.

All chemicals were of the highest grade available.

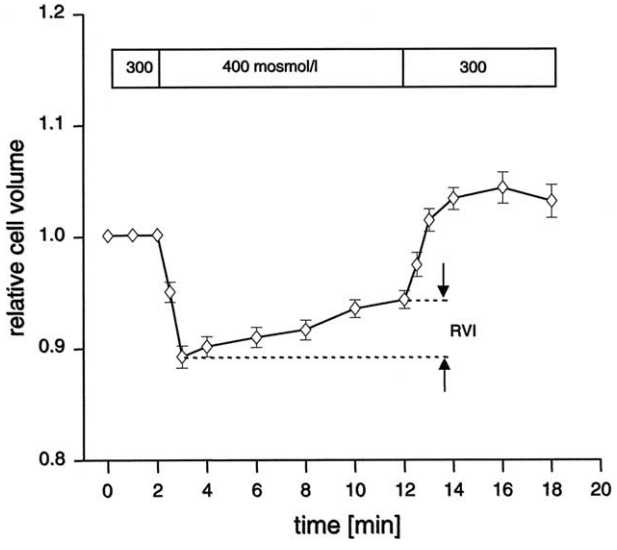

Fig. 1. Changes of cell volumes of solitary rat hepatocytes in response to hypertonic stress. $n = 27$ for each data point. See text for details.

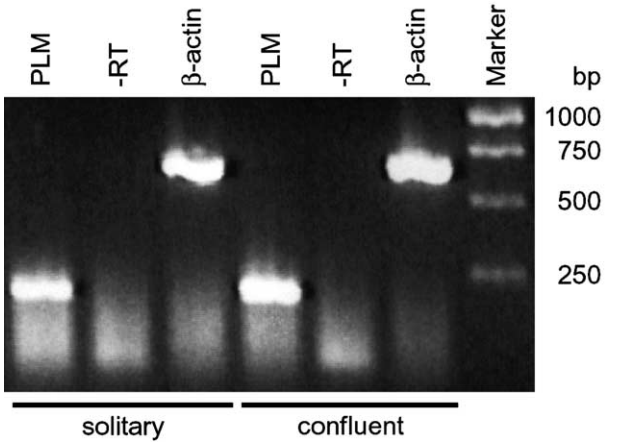

Fig. 2. PLM expression in solitary rat hepatocytes and confluent monolayers determined by means of RT-PCR on total RNA. A β-actin related fragment was amplified as a positive control [30]; omission of the reverse transcriptase yielded no product (negative control). The agarose gel (2%, stained with ethidiumbromide) was loaded with a 1 kb DNA ladder (Promega) and the PCR reactions as indicated.

2.8. Statistical analysis

Means ± S.E.M. are presented with n denoting the number of cells tested. In every series of experiments, measurements were performed on at least four different cell or oocyte preparations.

3. Results and discussion

3.1. RVI of solitary rat hepatocytes

When grown in confluent primary culture, rat hepatocytes exhibit a significant RVI upon cell shrinkage [12,13,16] that closely resembles the in vivo response [23,24]. A quantitative analysis of membrane currents under these conditions, however, is complicated by the high degree of electric cell-to-cell coupling [13,15]. In sharp contrast, cells that are freshly isolated and readily accessible to patch-clamp techniques [25,26] remain continuously shrunken under hypertonic conditions with no detectable RVI at all [27,28]. For these reasons we decided to employ solitary cells in primary culture for the present study and, in the first series of measurements, the volume response to hypertonic stress was tested. Increasing extracellular osmolarity from 300 to 400 mosmol/l led to an initial decrease of cell volumes to 89.1 ± 1.0% of control within 1 min ($n = 27$, $P < 0.001$; Fig. 1). This period of passive cell shrinkage was then followed by a slowly developing partial volume recovery to 93.4 ± 0.8% after 10 min. When referred to the initial cell shrinkage this is equivalent to an RVI value of 41.9 ± 2.9% ($P < 0.001$). Upon changing back to 300 mosmol/l, cell volume transiently increased and then returned towards control levels.

These values as well as the time course of volume changes are similar to those determined in confluent monolayer culture [12,13,16]. There was, however, a major difference with respect to the amiloride sensitivity of RVI. In the continuous presence of 10^{-5} mol/l amiloride (which inhibits some 90% of RVI in monolayers in a highly reproducible fashion [13]), in

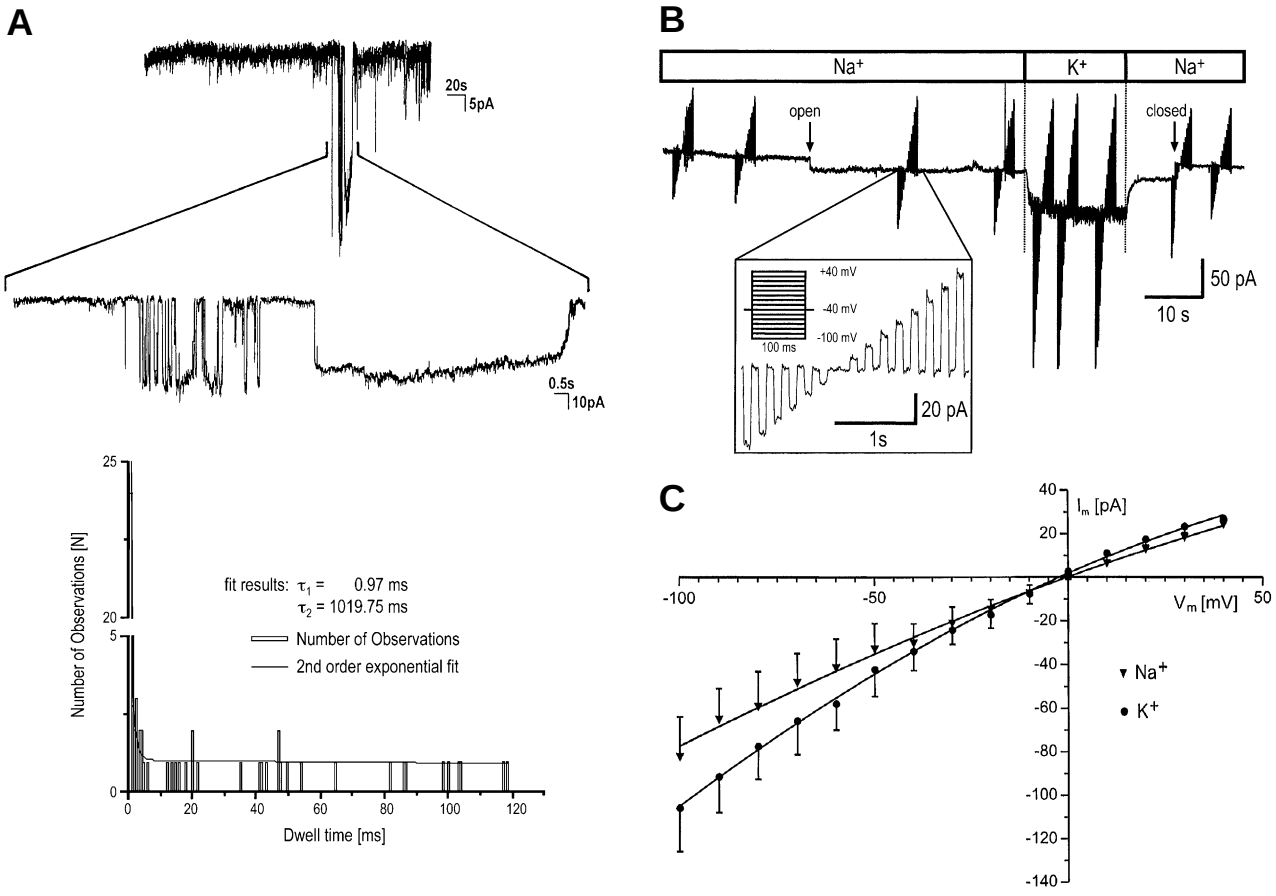

Fig. 3. Hypertonicity-induced membrane currents in solitary rat hepatocytes. 0.5 mmol/l quinine, Cl^--free solutions. A: Typical recording of membrane currents and dwell-time distribution of channel events at $V_h = -60$ mV. B: Determination of current-to-voltage relationships and ion substitutions. During a prolonged opening of the channel, extracellular Na^+ was completely exchanged for K^+. Voltage protocol and membrane currents are shown enlarged in the inset. C: Summary of current-to-voltage relations obtained in the presence of extracellular Na^+ and K^+.

only 17 out of 35 solitary cells, there was a significant reduction of RVI (to $8.7 \pm 4.2\%$; $P < 0.01$) whereas, in the second group, the RVI was comparable to control conditions (namely, $35.8 \pm 5.6\%$). These findings suggest that, in some 50% of solitary cells, a mechanism of RVI alternative to amiloride-sensitive cation channels is employed.

3.2. Solitary rat hepatocytes express PLM

A possible expression of PLM in solitary rat hepatocytes was tested on the mRNA level by carrying out RT-PCR experiments on total RNA preparations. As is illustrated in Fig. 2, a single band of about 220 bp was obtained by means of these techniques, which is very close to the expected length of the PCR fragment amplified, namely 213 bp. Interestingly, PLM was also detectable in confluent monolayers.

To obtain the full length sequence of PLM mRNA, 5′ and 3′ RACE experiments were performed. A nucleotide sequence of 526 bp was obtained including the 279 bp open reading frame of the PLM-precursor protein of 92 amino acids. Sequence alignments with the PLM from rat heart (SwissProt O08589) revealed 100% identity on the peptide level (and similarities of 94.4 and 90.3% to the PLM from dog and human heart, respectively [1,17]). Taken together, these experiments clearly show that solitary rat hepatocytes in primary culture do express PLM.

3.3. Hypertonic stress induces a 600–700 pS non-selective cation channel

The effects of hypertonic stress on membrane conductance were monitored in slow whole-cell patch-clamp recordings. Experiments were performed in Cl^--free solutions to isolate cation conductances. In addition, rat hepatocyte K^+ conductance was blocked by 0.5 mmol/l quinine [13,16,22]. In nine out of 24 measurements under these conditions, the activation of a single channel could be observed upon change to hypertonic conditions that was of remarkable size – and thus became detectable in this configuration (Fig. 3A). The unitary conductance of the channel was 693 ± 112 pS, which is close to the value of 500 pS calculated for the cationic mode of PLM when reconstituted into lipid bilayers [5]. Except for occasional openings that lasted for time periods of several seconds, the dwell-time histogram could be fitted with a second-order exponential with relaxation constants of $\tau_1 = 1.2 \pm 0.3$ ms and $\tau_2 = 1422 \pm 523$ ms ($n = 4$; Fig. 3A).

To obtain information about the cation selectivity of the channel, current-to-voltage relationships were determined in ion-substitution experiments. Due to the observed gating characteristics of the channel these measurements were only feasible during the rare periods of prolonged channel openings. In the control solution, slope conductances at 0 mV

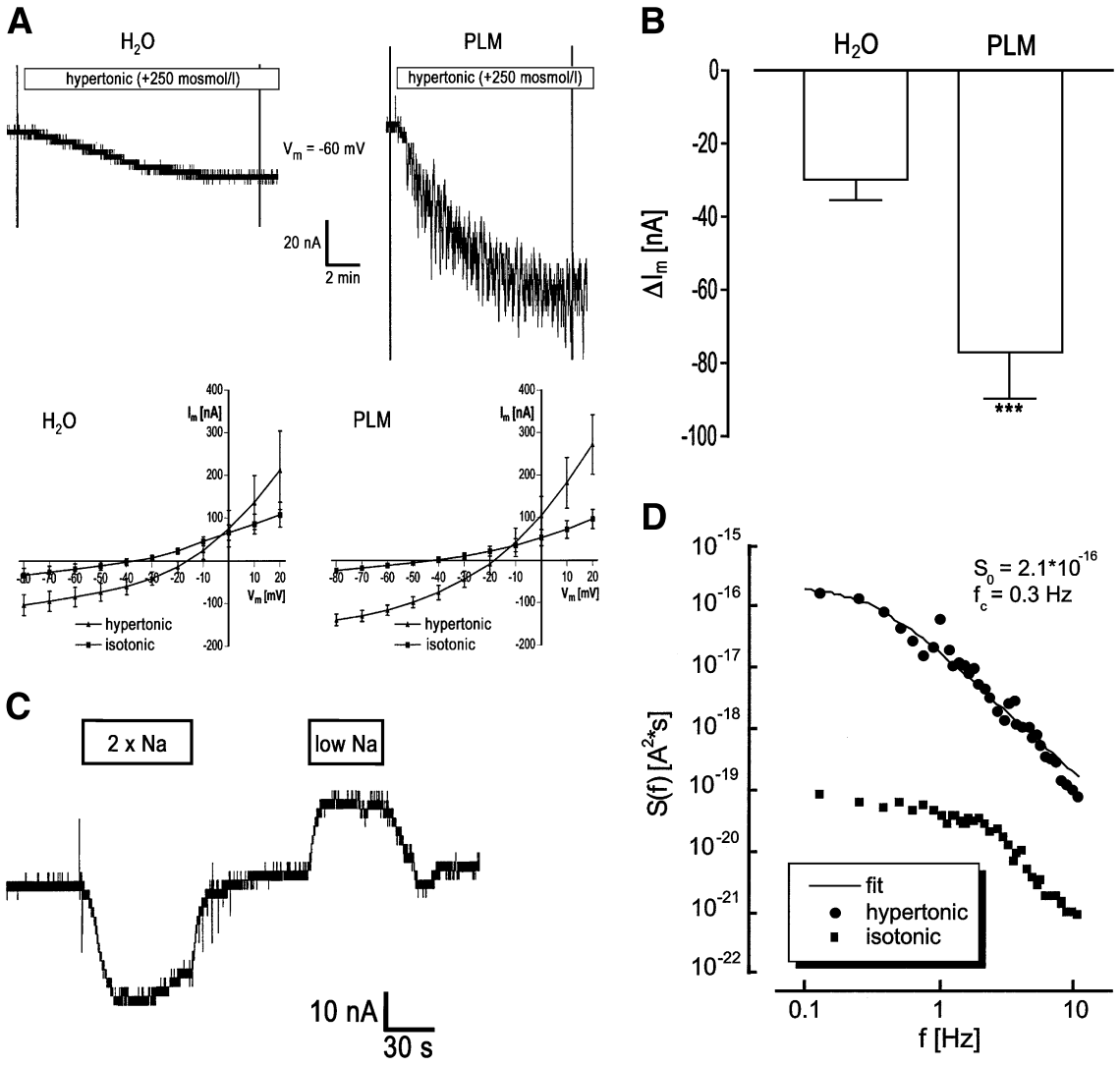

Fig. 4. Hypertonicity-induced membrane currents in *Xenopus* oocytes injected with water (H_2O) or cRNA (PLM) as indicated. $V_h = -60$ mV. A: Typical recordings. Note the significant increase in current noise in the PLM-expressing cell. B: Summary of hypertonicity-induced currents (hypertonic *minus* isotonic control). C: Typical Na^+-substitution experiment performed on a PLM-expressing oocyte. For the times indicated, the extracellular NaCl concentration was either doubled (from 96 to 192 mmol/l) or reduced to 1 mmol/l. D: Noise analysis of membrane currents in a cell heterologously expressing PLM. See text for details.

equaled 625 ± 82 pS and currents reversed at 0.2 ± 1.4 mV ($n = 4$; Fig. 3B,C). When extracellular Na^+ was completely exchanged for K^+, slope conductances were increased to 718 ± 97 pS ($P < 0.05$). Zero-current voltages, however, were only shifted by -2.6 ± 1.6 mV, which was not statistically significant. These latter findings clearly show that the hypertonicity-induced channel – like PLM – does not discriminate between Na^+ and K^+.

3.4. *Expression of PLM in* Xenopus *oocytes: ion substitutions and noise analysis*

The next series of measurements was conducted on *Xenopus laevis* oocytes injected with PLM cRNA, or water as the control. As is depicted in Fig. 4A,B, hypertonic stress (+250 mosmol/l) led to an inward current that equaled -74.5 ± 12.0 nA ($n = 14$, $P < 0.001$) at -60 mV. This current is significantly higher than the one determined in water-injected cells, namely -30.7 ± 5.0 nA ($n = 20$), with $P < 0.001$. In addition, in the group of PLM-expressing oocytes, hypertonicity induced a remarkable increase in current noise (see Fig. 4A).

Fig. 4C depicts experiments in which, after complete hypertonic activation of currents, the extracellular concentration of NaCl was rapidly changed from 96 mmol/l to 192 and 1 mmol/l in PLM-expressing oocytes. These maneuvers led to changes in current by -36.3 ± 5.2 nA ($n = 5$, $P < 0.001$) and 23.5 ± 3.6 nA ($P < 0.001$), respectively, that coincided with shifts in zero-current voltages by 12.0 ± 2.0 mV ($P < 0.001$) and -12.5 ± 2.4 mV ($P < 0.001$; data not shown). Because in the above measurements Na^+ and Cl^- were changed by equal amounts, these results clearly show that the hypertonicity-induced conductance expressed in oocytes operates in a cation rather than in an anion-selective mode.

In Fig. 4D the current noise in PLM-expressing oocytes was quantified. Noise spectra of current fluctuations in the range of 0.125–10 Hz are shown. As is obvious from the figure, hypertonic stress increased noise by more than three decades to a value that equaled $5.88 \times 10^{-17} \pm 2.1 \times 10^{-17}$ A^2 s on aver-

age ($n = 8$) [20,21]. In addition, a Lorentzian component with a cut-off frequency of 0.35 ± 0.04 Hz appeared. With the open dwell-time data obtained from the patch-clamp recordings on hepatocytes (see Section 3.3) and on the basis of the mean inward currents in oocytes (see above) one would expect the hypertonicity-induced noise to be described by a Lorentzian with two components [20,21]. The short dwell time should induce a noise component with a cut-off frequency at 0.009 Hz, which would not be detectable because of the technical limitations of the setup. The second, longer dwell time would result in a Lorentzian with a cut-off frequency at 0.36 Hz, which closely matches the observed component in our fluctuation analysis. With the plausible assumption that this Lorentzian is indeed attributed to PLM channels one could calculate a single-channel conductance of 671 ± 256 pS, which is very close to the single-channel conductance observed in solitary rat hepatocytes.

3.5. Conclusions

Solitary rat hepatocytes in primary culture exhibit a volume response to hypertonic shrinkage that is similar to that found in confluent monolayers. In only some 50% of the cells tested, however, this RVI is inhibited by the Na^+/cation-channel blocker amiloride. Solitary cells express PLM and, in 40%, hypertonic stress activates a channel that resembles PLM with respect to its unitary conductance, its gating pattern, and its non-selectivity for Na^+ over K^+. In *Xenopus* oocytes expressing PLM, hypertonic stress induces a conductance which is non-selective for Na^+ over K^+ and noise analysis reveals the activation of a channel with characteristics that appear to be very similar to those found in hepatocytes. Taken together, these results strongly suggest a role of PLM in the RVI of solitary rat hepatocytes in culture.

In the confluent monolayer system, hypertonic stress could be shown to induce a cation channel [12] that is blocked by amiloride and its derivative EIPA [29], and that is functionally related to the α-subunit of the epithelial Na^+ channel ENaC [14]. This cation channel exhibits a P_{Na}/P_K of 1.4 [14] and it is likely to be correlated to a 6 pS channel that is observed in cell-attached patches (Lawonn and Wehner, unpublished observation). Also of note, in a quantitative study performed over the range of 9–50% hypertonicity, it could recently be shown that the amounts of Na^+ uptake via amiloride-sensitive cation channels, Na^+/H^+ antiport, and Na^+–K^+–$2Cl^-$ symport is in excellent agreement with the actual increases of cell Na^+ plus the amounts of Na^+ extrusion by Na^+/K^+-ATPase [16]. In other words, there does not appear to be an additional component that may reflect a contribution of PLM.

This apparent discrepancy may be interpreted in terms of the actual state of rat hepatocyte (re)-differentiation under the conditions used in this study, which do not permit the formation of cell-to-cell interactions. Also of interest in this context is the observation that the RVI of only some 50% of cells could be blocked by amiloride, which is in sharp contrast to the 100% responsiveness of hepatocytes found in the confluent monolayer system [12,13,16]. This could reflect a synergistic role of amiloride-sensitive channels and PLM in the RVI of rat hepatocytes when re-differentiating. Given the significant role that mechanisms of cell-volume regulation play in the proliferation and differentiation of cells [10,11], a possible employment of PLM in these processes that actually depends on the status of differentiation of a cell would be of special interest.

Of note, PLM appears to be involved in the mechanisms of RVD [7–9] and RVI as well. This could mean that it functions as a last line of defense so that a cell under strong anisotonic conditions just opens a large osmotic shunt to survive. If this were the case, it would also explain why hepatocytes still express PLM when grown to confluence (Fig. 2) although more sophisticated mechanisms of cell-volume regulation have become available under these conditions [13,16].

The regulatory role that PLM appears to play in some systems [3,4] as well as its high degree of phosphorylation per se [1–3] should also be considered. As an additional function, the protein may well be part of the signaling machinery that actually tunes the ion transporters responsible for cell-volume regulation. This notion is supported by the strong similarities between PLM and other members of the FXYD family of single-span transmembrane proteins like CHIF, Mat-8, and the γ-subunit of Na^+/K^+-ATPase that could be clearly shown to function as regulators of membrane transporters [7].

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