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Probing the water permeability of ROMK1 and amphotericin B channels using *Xenopus* oocytes

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Abstract

Water permeability of ion channels in the plasma membrane of *Xenopus* oocytes was studied by simultaneously measuring the membrane conductance under two-electrode voltage-clamp and the cell size by video-imaging technique. The basal level of osmotic water permeability of oocyte plasma membrane was $15.9 \pm 0.98\,\mu m/s$ (SE, $n = 5$). Extracellular application of pore-forming antibiotic amphotericin B at $5\,\mu M$ developed macroscopic conductance of $995 \pm 70\,\mu S$ ($n = 5$) and increased the osmotic water permeability of cell membrane by $44.9 \pm 4.1\,\mu m/s$. Meanwhile, after expressing ROMK1 channels, originally cloned from kidney, virtually no increase in the water permeability was observed even at the conductance level as high as $1113 \pm 47\,\mu S$ ($n = 5$). This result suggests that even though potassium channels, like any others, are considered to be water-filled pores, K^+-selective ion-transporting pathway remains virtually water-impermeable in physiological conditions, such as in kidney epithelia where huge water transport takes place at both apical and basolateral sides. © 1998 Elsevier Science B.V.

Keywords: ROMK1; Amphotericin B; Ion channel; Water permeability

1. Introduction

Water permeability of cellular membranes is of exclusive importance in physiology of secreting and absorbing epithelia. Molecular mechanisms of water permeation have been attributed to membrane proteins, water channels, which have been found in all the ''expected'' locations, such as kidney and colon [1]. On the other hand, ionic channels are commonly believed to be water-filled pores [2] and also found to be highly expressed in the same cells. For example, the kidney epithelium contains not only water channels, like aquaporins [3,4], but also heavily expresses potassium channels (ROMK: [5]), sodium channels [6] and chloride channels [7,8] as well as numerous transporters. Since a water-filled pore should be expected to pass certain amount of water as well, the question arises as to whether ionic channels contribute significantly to water permeation in water-transporting tissues. In fact, recently, CFTR Cl^- channels have been shown to be water-permeable [9]. However, no study has reported about water permeability of cation-selective channels. The present study is the first attempt to assess this problem in a cloned potassium channel ROMK1, in comparison with the water permeability in an anion-selective pore formed by polyene antibiotic amphotericin B.

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2. Materials and methods

2.1. Molecular biology

The cDNA of ROMK1 was a generous gift from Dr. Y. Kurachi (Osaka University, Japan). mRNAs were synthesized by linearization with Kpn I followed by transcription with T3 polymerase using the mCAP™ mRNA capping kit (Stratagene, USA). The transcript concentration was estimated spectrophotometrically, and aliquots were stored at −80°C. Stage V and VI *Xenopus* oocytes were defolliculated by collagenase (Sigma, type I) treatment (1 mg/ml for 2–4 h) in Ca-free ND 96 solution containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.4. Cells were injected with 5–100 nl (Drummond ''Nanoject'', Drummond Scientific, Broomall, PA) of mRNA solution (0.5 ng/nl) and incubated at 19°C in normal ND 96 medium containing (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4 and supplemented with gentamicin (100 μg/ml).

2.2. Electrophysiology

By the two-microelectrode voltage-clamp technique, whole-cell currents were recorded in oocytes 2–4 days after injecting mRNA. Oocytes were impaled with two 3 M KCl-filled micropipettes (tip resistance, 0.5–1 MΩ) that served as voltage recording and current injecting electrodes. When necessary, a grounded shield was placed between the micropipettes to reduce capacitative coupling.

Cell-attached single-channel recordings were performed on oocytes after manual removal of the vitelline envelope as described by Methfessel et al. [10]. To nullify the intracellular potential oocytes were exposed to depolarizing bath solution composed of (mM): 100 KCl, 10 EDTA and 10 HEPES (pH 7.3). Patch pipettes were filled with bath solution used for whole-cell measurements.

For electrophysiological experiments, currents were measured using an amplifier (CA-1, Dagan Instruments, Minneapolis) for whole-cell recordings, and an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) for single-channel recordings. Voltage protocols were computer-controlled using an i-486-based personal computer (AST, USA), coupled to a TL-1 or DigiData 1200 (Axon Instruments, Foster City, CA) interface. Currents were filtered at 1 kHz and sampled at 5 kHz. Data acquisition and analysis were done using pCLAMP6 (Axon Instruments, Foster City, CA).

2.3. Volume measurement and osmotic water permeability determination

Oocytes were viewed by transmitted light on an inverted microscope (Nikon, Japan) using a ×2 objective and imaged on a CCD camera (ICD-42AC, Ikegami, Japan). Data were recorded on video-tape for subsequent off-line image analysis using a frame grabber, VISIONplus-AT (Image Technology, Bedford, MA), controlled by an i-486 based computer (AST, USA). Images were collected in every 5 s. Volume changes were calculated from changes in the cross-sectional area A using relationship $V/V_0 = (A/A_0)^{3/2}$, where V_0 and A_0 are the initial volume (cm^3) and area (cm^2), V and A are their corresponding values at time t.

Isotonic solutions contained (mM): 46 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 100 mannitol, 5 HEPES, pH 7.4 for amphotericin B experiments and 50 KCl, 100 mannitol, 1 $MgCl_2$, 5 HEPES, pH 7.4 for ROMK1 experiments. In both cases, hypotonic challenge was performed by removing 100 mM mannitol from an isotonic solution. This procedure allowed to keep the ionic strength and Cl^- concentration constant. Bathing solution was continuously perfused at a rate of 2.5–3 ml/min through a recording chamber of 0.15 ml total volume. Normally, cells attached spontaneously to the bottom of the chamber and did not move upon perfusion. When necessary, a glass micropipette (same as for voltage clamp but without impaling) was used to prevent the cell movement.

Under two-electrode voltage-clamp conditions, cell volume can be largely affected by changes in intracellular osmolality due to ionic current flowing through the membrane. It can be shown that an inward current of 16 μA would cause an increase in intracellular osmolality by about 40 mosmol/kg in approx. 100 s. Therefore, in all experiments, the holding voltage was kept at the reversal potential level. This was particularly important for amphotericin B experiments, where the reversal potential varied time-dependently.

Osmotic water permeability was obtained from the relationship:

$$P_f = \left[\frac{V_0}{(SV_w\Delta\Pi)}\right]\left[\frac{d(V/V_0)}{dt}\right], \quad (1)$$

where S is the oocyte surface area (cm^2), V_w the partial molar volume of water ($18\,cm^3/mol$), $\Delta\Pi$ the osmotic gradient (100 mosmol/kg in our experimental conditions); and $d(V/V_0)/dt$ the rate of relative cell volume change.

The oocyte volume under the video-system was calibrated with certified particle-size standards (glass microspheres, Duke Scientific corporation, CA).

Experiments were performed at room temperature (23–25°C). Data are presented as the mean ± SE. The number of experiments (n) was five, except where indicated.

3. Results

3.1. Effect of amphotericin B on electric conductance and water permeability of Xenopus oocytes

First we attempted to observe a change in water permeability of *Xenopus* oocytes after forming amphotericin B channels. This antibiotic at 5 μM induced large currents with very little inactivation at high positive and negative voltages (Fig. 1(A)) and linear I–V relationships independent of osmotic pres-

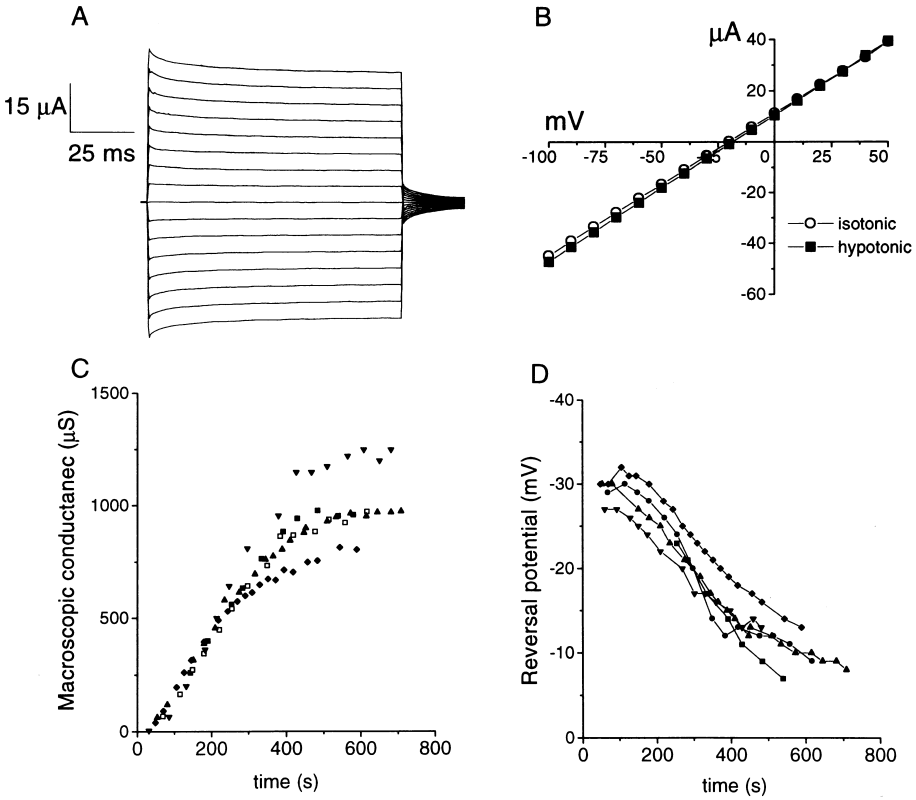

Fig. 1. Effect of amphotericin B on the macroscopic conductance of *Xenopus* oocytes. (A) Representative current traces measured 5 min after addition of 5 μM of amphotericin B. Holding potential was −30 mV. Traces elicited by voltage steps from +50 to −100 mV in 10-mV increments in hypotonic conditions. (B) I–V curves of data in A (filled squares). Current amplitudes recorded 10 ms after the beginning of the voltage step are plotted to eliminate artifacts due to capacitative transients. Open circles represent the data for the same oocyte perfused with isotonic bath solution. (C) Time course of macroscopic conductance change. The plotted values were obtained from the slopes of I–V curves (in response to a ramp pulse from −100 to +50 mV at 1 mV/ms rate) in the range from −25 to −5 mV. The data for five independent experiments (shown in Fig. 2, hypotonic conditions) are plotted. (D) Time course of reversal potential change. The values were obtained from the same I–V curves as in C. The data from five independent experiments (shown in Fig. 2, hypotonic conditions) are plotted.

sure (Fig. 1(B)). The steady-state current level reached within 400–600 s (Fig. 1(C)). The reversal potential was not constant. For small currents at the beginning of the experiments, it was about −30 mV, decreasing down to approx. −10 mV at the steady state (Fig. 1(D)). This is consistent with the notion that single-sided action of amphotericin B creates cation-selective pores, while two-sided application induces channels with preferably anion selectivity [11]. Supposedly, an intracellular concentration of antibiotic rises during the experiment, reaching the level, sufficient for formation of symmetrical 16-meric channel. The relative volume of oocyte changed slowly under the 100-mosmol/kg osmotic gradient (Fig. 2) in control conditions. Application of 5 μM amphotericin B drastically increased the rate of volume change (Fig. 2(A)). Note that two-electrode voltage-clamp (TEV) had virtually no effect on the water permeability of oocytes in control conditions and in the presence of amphotericin B (Fig. 2(B)). Application of amphotericin B increased the osmotic water permeability by 44.7 ± 1.6 μm/s in intact oocytes and by 44.9 ± 4.1 μm/s for the same cells under two-electrode voltage-clamp. Evidently, massive formation of water-filled pores on the oocyte plasma membrane caused such a marked change in oocyte water permeability. Fig. 3 shows osmotic water permeability calculated from the rate of relative volume change. Control values were consistent with those measured previously at room temperature (9–18 μm/s at 22–25°C: [12–14]), but higher than those at 10°C (3–5 μm/s: [9,12]).

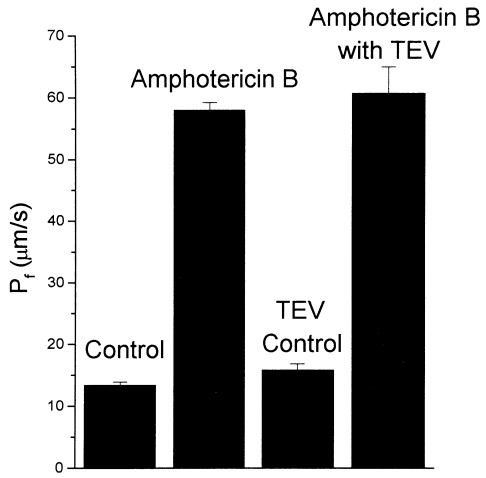

Fig. 3. Osmotic water permeability of control and amphotericin B-treated oocytes with or without TEV. The linear part of curves in Fig. 2 immediately after hypotonic challenge was used to calculate control values of P_f. Late linear portion of kinetic curves (approx. 400 s after addition of an antibiotic) was used for P_f calculations of amphotericin-treated cells. Data represent the mean ± SE ($n = 5$).

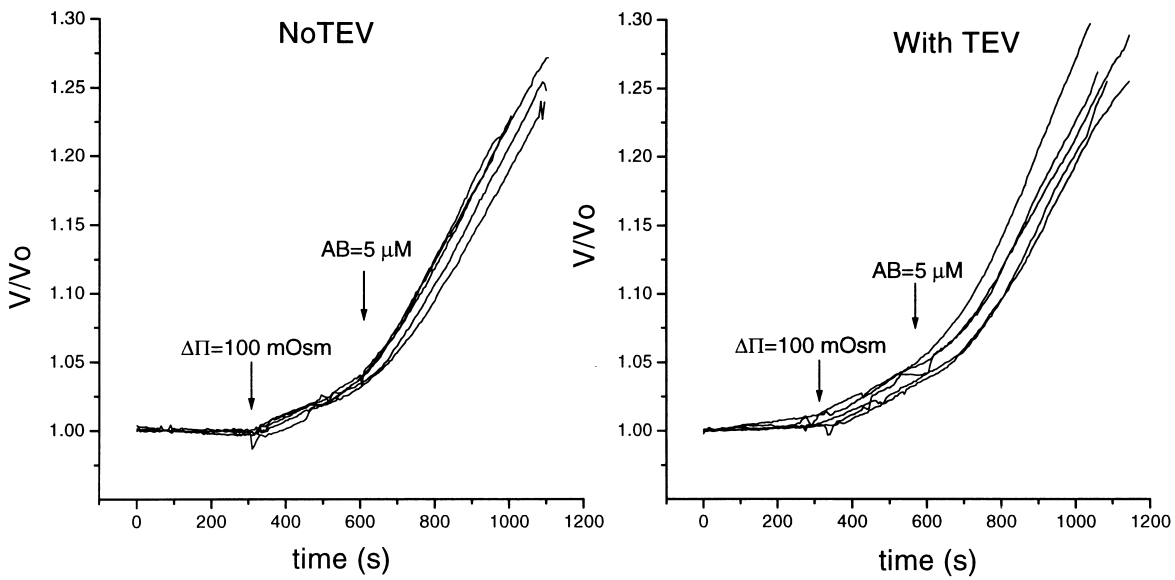

Fig. 2. Time course of relative volume change without (A) and with two-electrode voltage-clamp (TEV: B). Arrows indicate the moment of hypoosmotic challenge ($\Delta \Pi = 100$ mosmol/kg) and subsequent application of amphotericin B (AB). The results of five independent experiments are superimposed.

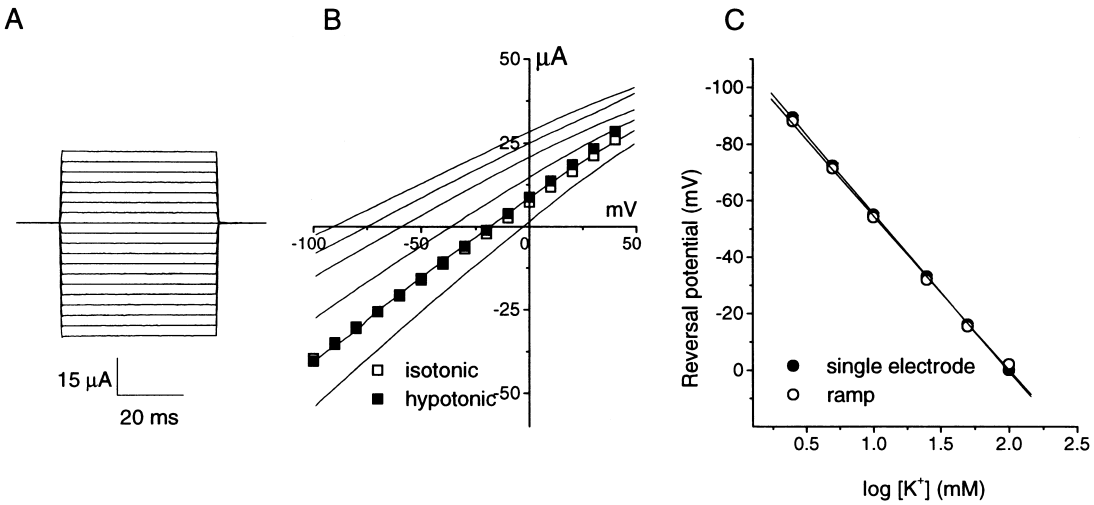

Fig. 4. Macroscopic currents of oocytes expressing ROMK1 channels. (A) Representative current traces recorded in oocytes three days after injection of 50 nl of ROMK1 mRNA in isotonic conditions. Holding potential was −30 mV. Traces elicited by steps from +40 to −140 mV in 10-mV increments. (B) I–V curves of data in A (open squares). Current amplitudes recorded 10 ms after the beginning of the voltage step are plotted to eliminate artifacts due to capacitative transients. Filled squares represent the data for the same oocyte after perfusion of the cell with hypotonic bath solution. Currents in response to voltage ramp from −100 to +50 mV were obtained at $[K^+]$ of 2.5, 5, 10, 25, 50 and 100 mM (from top to bottom) with $[Na^+]$ adjusted to keep $[K^+] + [Na^+] = 100$ mM in the bath containing 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4. (C) Reversal potentials at various K^+ concentrations. Filled circles correspond to the membrane potential measured by impalement with a single microelectrode (slope 55.9 ± 0.3 mV, $n = 6$); open circles are reversal potentials obtained from ramp-clamp measurements (slope 54.4 ± 1.0 mV, $n = 3$).

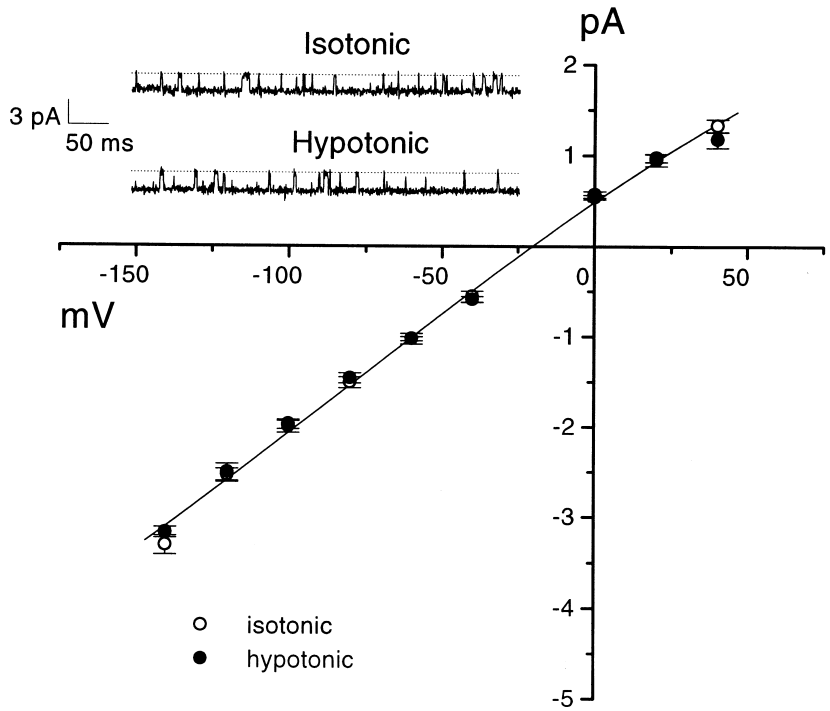

Fig. 5. Single-channel I–V relationships for ROMK1 channel in isotonic (open circles, $n = 7$) and hypotonic (filled circles, $n = 5$) conditions. Pipette solutions were same as isotonic and hypotonic bath solutions used for macroscopic current measurements in Fig. 4. Insets show representative single-channel recordings at −100 mV. Dashed lines denote the closed state.

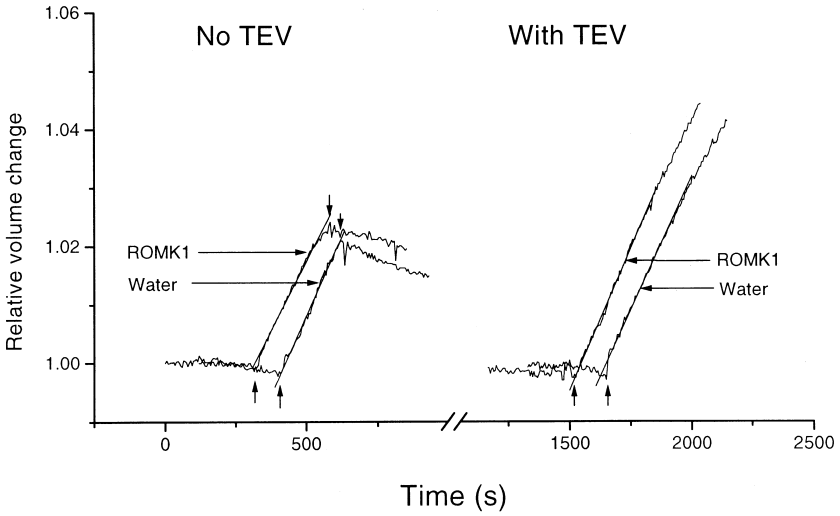

Fig. 6. Time course of relative volume change without (left) and with two-electrode voltage clamp (TEV: right) in a control water-injected oocyte and ROMK1-expressing oocyte. First the rate of volume change was assessed by perfusing the oocyte with hypotonic (100 mosmol/kg gradient, composition is same as in Fig. 4) solution for approx. 3 min, then the cell was returned to isotonic solution for 15 min for recovery and impaled with two electrodes for subsequent simultaneous measurement of conductance (by voltage ramps from −100 to +50 mV at 1 mV/ms rate) and volume changes under similar hypotonic challenge. Upward and downward arrows indicate the moments of the application of hypotonic and isotonic bath solutions, respectively. Solid lines are linear fits used for water permeability calculations. Time scale corresponds to ROMK1 expressing cell. The trace for water-injected oocyte is shifted leftward by 100 s for better viewing.

3.2. Macroscopic conductance and water permeability of oocytes expressing ROMK1 channels

ROMK1-expressing oocytes exhibited large macroscopic currents with virtually no inactivation (Fig. 4(A)). I–V curves exhibited slight inward rectification and were independent of osmotic pressure (Fig. 4(B)). Reversal potentials observed at a variety of K^+ concentrations indicated that the current was highly selective to K^+ over Na^+ (Fig. 4(C)). The current was largely blocked by 5 mM Ba^{2+} ($n = 4$, data not shown). Single ROMK1 channels had a conductance of 25.1 ± 0.5 pS in isotonic and 23.9 ± 0.7 pS in hypotonic pipette solution. I–V relationships and gating properties of the single channel were apparently independent of osmotic pressure (Fig. 5).

Fig. 6 illustrates typical experiments in which cells (control water-injected and ROMK1-expressing oocytes) were first assayed with 100 mosmol/kg osmotic challenge without microelectrodes, and then the same cells were impaled with two microelectrodes and tested again with hypoosmotic stress. It should be noted that the expression level of ROMK1 channels in this experiments was higher than that shown in Fig. 4, because the injected amount of mRNA was doubled. Clamp performance, however, was less successful because of too low membrane resistance of cells. Therefore, only small part of I–V relationship around the reversal potential (±20 mV) was used for macroscopic conductance measurements

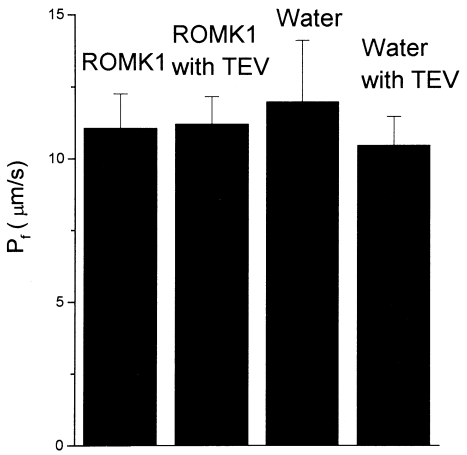

Fig. 7. Osmotic water permeability of control water-injected and ROMK1-expressing oocytes. The linear rising portions of curves in Fig. 6 under hypotonic conditions were used to calculate P_f values. The data represent the mean ± SE ($n = 5$–6).

in this set of experiments. As can be seen in Fig. 7, oocytes expressing high amount of ROMK1 potassium channels (with macroscopic conductance of $1113 \pm 47\,\mu S$) behaved in a manner very similar to water-injected cells under hypotonic conditions. Osmotic water permeability of ROMK1-expressing cells was practically unchanged. TEV had little, if any, effect on mean P_f values.

4. Discussion

Amphotericin B is an important drug widely used in antifungal therapy. Its physiological activity is related to the formation of small pores permeable to water and small ions. The properties of this pore have been studied both in artificial lipid bilayers and in biological cell membranes [15]. In our experiments, a concentration of $5\,\mu M$ was enough to induce large currents in the oocyte plasma membrane associated with a marked increase in osmotic water permeability. Given the single-channel conductance value of 0.2 pS (extrapolated to 50 mM NaCl from the data of [16]) and the macroscopic conductance of $995\,\mu S$, we can calculate the single-channel water permeability of $4 \times 10^{-14}\,cm^3/s$, which is close to the value obtained from lipid bilayer measurements for two-sided action of the antibiotic ($4.5 \times 10^{-14}\,cm^3/s$: [11]).

In contrast, expression of ROMK1 potassium channel did not significantly affect the membrane water permeability. This result may be consistent with the fact that the thick ascending limb of Henle, where highest expression of ROMK2 is known [17], has an extremely low water permeability. Also, this result suggests that ROMK-related potassium channels may not participate significantly in huge water transport which takes place in the other part of renal tubular membrane.

Indeed, following estimation of the current and channel densities supports this inference. In ROMK1-expressing oocytes, from the macroscopic conductance of $1113\,\mu S$, the single-channel conductance of 25 pS and the oocyte surface area of $4 \times 10^6\,\mu m^2$ (from cell sizing) or $20 \times 10^6\,\mu m^2$ (from capacitance measurements: [10]), we can estimate the conductance density to be $5.6–22.4\,mS/cm^2$ and the channel density to be 2–10 channels per μm^2. The macroscopic potassium conductance of isolated early distal tubule epithelial cells was reported to be between 4.2 nS [18] and 12.9 nS [19] in high-K^+ solutions with $[K^+]$ two times as higher as that used in our experiments. From the membrane capacitance of 10 pF (lower limit of typical values [20]), we can estimate the conductance density to be 0.42–$1.3\,mS/cm^3$ in the tubular cells. For the same cells, cell-attached patches were found to contain 2–6 K^+-selective channels with the amplitude and gating, resembling ROMK-type ones [18]. Implying that a ''typical'' pipette of $2–3\,M\Omega$ isolates an area of $5–20\,\mu m^2$ [21], the channel density can be estimated as 0.1–1.2 per μm^2. From this we assume that the expression level in our experiments is comparable to, or even higher than, that naturally occurring in kidney epithelial cells. Osmotic water permeability of renal epithelial cells can be as high as hundreds and thousands of $\mu m/s$. For instance, membrane vesicles derived from rabbit and rat proximal tubules have P_f of 166 and 760 $\mu m/s$, respectively [22], whereas the apical side of rabbit proximal straight tubules exhibits the P_f value 2750–4490 $\mu m/s$ [23]. Cortical collecting ducts, where ROMK channels were shown to be heavily expressed [17,24], have osmotic water permeability of 20–30 $\mu m/s$ in the absence, and 400–1000 $\mu m/s$ in the presence of antidiuretic hormone [22]. At the same time, the difference in P_f for ROMK1-expressing and control water injected oocytes was less than $1\,\mu m/s$ which is negligible compared to tremendous values found in kidney epithelia.

In contrast to no water permeability of the ROMK1 potassium channel, the CFTR chloride channel, the structure of which belongs to the ABC transporter superfamily, has been reported to permeate water [9]. Also, glucose uniporters [25] and sodium–glucose cotransporter SGLT1 [26,27], have recently been found to be water permeable. Further investigations are needed to know what differences in the protein structure are responsible for the difference in water permeability of channels and transporters.

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