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Na^+ Sensitivity of ROMK1 K^+ Channel: Role of the Na^+/H^+ Antiporter

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Abstract. To examine the extracellular Na^+ sensitivity of a renal inwardly rectifying K^+ channel, we performed electrophysiological experiments on *Xenopus* oocytes or a human kidney cell line, HEK293, in which we had expressed the cloned renal K^+ channel, ROMK1 (Kir1.1). When extracellular Na^+ was removed, the whole-cell ROMK1 currents were markedly suppressed in both the oocytes and HEK293 cells. Single-channel ROMK1 activities recorded in the cell-attached patch on the oocyte were not affected by removal of Na^+ from the pipette solution. However, macro-patch ROMK1 currents recorded on the oocyte were significantly suppressed by Na^+ removal from the bath solution. A blocker of Na^+/H^+ antiporters, amiloride, largely inhibited the Na^+ removal-induced suppression of whole-cell ROMK1 currents in the oocytes. The pH-insensitive K80M mutant of ROMK1 was much less sensitive to Na^+ removal. Na^+ removal was found to induce a significant decrease in intracellular pH in the oocytes using H^+-selective microelectrodes. Coexpression of ROMK1 with NHE3, which is a Na^+/H^+ antiporter isoform of the kidney apical membrane, conferred increased sensitivity of ROMK1 channels to extracellular Na^+ in both the oocytes and HEK293 cells. Thus, it is concluded that the ROMK1 channel is regulated indirectly by extracellular Na^+, and that the interaction between NHE transporter and ROMK1 channel appears to be involved in the mechanism of Na^+ sensitivity of ROMK1 channel via regulating intracellular pH.

Key words: Na^+ sensor — Inward rectifier K^+ channel — ROMK — Na^+/H^+ antiporter

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Introduction

An intriguing feature of inwardly rectifying K^+ currents (I_{Kir}) in native cells is their sensitivity to extracellular Na^+ ions. Two different types of Na^+ sensitivity, Na^+ block and Na^+-induced upregulation, have been observed upon changing the external Na^+ concentration. First, I_{Kir} of frog skeletal muscle (Standen & Stanfield, 1979), guinea-pig ventricular myocytes (Biermans, Vereecke & Carmeliet, 1987), cat ventricular myocytes (Harvey & Eick, 1989*b*), tunicate egg cells (Ohmori, 1978) and mouse clonal corticotrophs (Dousmanis & Pennefather, 1992) are known to be susceptible to voltage-dependent blockage by extracellular Na^+. Second, in tunicate eggs (Ohmori, 1978; Fukushima, 1982), cat ventricular myocytes (Harvey & Eick, 1989*a*) and rat spinal cord astrocytes (Ransom, Sontheimer & Janigro, 1996), I_{Kir} has been reported to be downregulated by a decrease in the extracellular Na^+ concentration, implying a mechanism which is independent of Na^+-dependent permeation and block. The recent cloning of several subfamilies of inward rectifiers opens the molecular mechanism of this sensitivity to external Na^+ to investigation.

The first inward rectifier K^+ channels to be identified were an ATP-insensitive inward rectifier K^+ channel family, IRK (Kir2.0), originally cloned from a mouse macrophage cell line (Kubo et al., 1993) and an ATP-sensitive, weakly inward rectifier K^+ channel family, ROMK (Kir1.0), cloned from the rat kidney (Ho et al., 1993; for review *see* Hebert, 1995; Hebert & Wang, 1997). Inactivation of the inward current of IRK1 channel was shown to be induced by Na^+ block (Kubo et al., 1993), whereas Na^+ block was not observed in ROMK2 channels (Zhou, Tate & Palmer, 1994; Choe, Sackin & Palmer, 1998). However, Zhou et al. (1994) presented the data (Fig. 8) which show suppression of the inward currents of ROMK2 by replacement of extracellular Na^+.

Thus, there is a possibility that the ROMK channel is upregulated by extracellular Na^+. To address this possibility, in the present study, we studied Na^+ sensitivity of ROMK1 channels that were expressed *Xenopus* oocytes and human HEK293 cells. We found that the ROMK1 channel is actually upregulated by extracellular Na^+. Also, the Na^+ sensitivity was found to be enhanced by coexpression with NHE3, which is a subtype of Na^+/H^+ antiporters initially cloned from the rat kidney (Orlowski, Kandasamy & Shull, 1992) and shown to be expressed in different segments of nephron including the macula densa (Amemiya et al., 1995). We provided evidence that the interaction between NHE transporter and ROMK1 channel is involved in the Na^+ sensor mechanism of ROMK1.

Materials and Methods

Cell Preparation

Stage V and VI *Xenopus* oocytes were defolliculated by treatment (2–4 hr) with 1 mg/ml collagenase (Type I: Sigma Chemical, St. Louis, MO) in Ca^{2+}-free ND 96 solution containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES (pH 7.4), and incubated at 19°C in normal ND 96 medium containing $CaCl_2$ (1.8 mM) and gentamicin (100 μg/ml).

Human embryonic kidney HEK293 cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum, streptomycin (10 μg/ml) and penicillin (40 units/ml) at 37°C in 5% CO_2.

Molecular Biology

The cDNA of rat ROMK1 was provided from Dr. Y. Kurachi (Osaka University, Japan), and the cDNA of rat NHE3 was from Dr. Sh. Wakabayashi (National Cardiovascular Center Research Institute, Osaka, Japan). For *in vitro* transcription the insert was subcloned into pSP64 Poly (A) vector (Promega, Madison, WI). For mammalian cell expression, the ROMK1 insert was subcloned into pcDNA3.1 vector (Invitrogen, Groningen, Netherlands), containing the CMV promoter. The K80M mutation of ROMK1 was introduced using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA).

mRNAs were synthesized by linearization with an appropriate enzyme followed by transcription with T7 or SP6 polymerase using *in vitro* transcription kits (Stratagene or Ambion, Austin, TX). The transcript concentration was spectrophotometrically estimated, and aliquots were stored at −80°C. *Xenopus* oocytes were injected with ROMK1 or NHE3 mRNA or their 1:6 mixture using nano-injector (Drummond "Nanoject", Drummond Scientific, Broomall, PA). For single-channel recordings, two-microelectrode voltage clamp measurements and macropatch recordings, 5–10, 10–50 and 50–100 μl, respectively, of mRNA-containing solution (0.5 ng/nl) were injected.

HEK293 cells cultured at 40–70% confluence in 35-mm dishes were transiently transfected using 8 μl of LIPOFECTAMINE™ (Gibco BRL, Rockville, MD) with 1 μg DNA of each species according to the manufacturer's manual. GFP coexpression (0.1 μg of GFP coding region containing vector added to DNA mixture: a gift from Dr. K. Ueda, Kyoto University, Japan) was used to select the transfected cells.

Electrophysiology

Whole-cell oocyte currents were recorded by the two-microelectrode voltage-clamp technique. Oocytes were impaled with two 3M 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. Control bathing solution contained (mM): 90 NaCl, 10 KCl, 1 $MgCl_2$ and 5 HEPES (pH 7.4). When necessary, NaCl was isotonically replaced with NMDG-Cl or mannitol. The elimination of both Na^+ and Cl^- by replacing with mannitol was precluded in two-microelectrode voltage-clamp due to the large change in series resistance. The bathing solution was continuously perfused at a rate of 2.5–3 ml/min through a recording chamber of 0.15 ml total volume. The pipette solution was the same as the bath solution. The oocyte was kept at a holding potential of −60 mV, and a ramp pulse was applied from −100 to −20 mV at the rate of 1 mV/msec every 10 sec.

Cell-attached macro-patch or single-channel recordings were performed on oocytes after manual removal of the vitelline envelope. Patch pipettes were filled with the above bathing solution. To nullify the intracellular potential for single-channel recordings, oocytes were exposed to depolarizing bath solution composed of (mM): 100 KCl, 10 EDTA and 10 HEPES (pH 7.3). In macro-patch experiments the bath solution was the same as that for the two-microelectrode voltage-clamp. In some experiments outside-out single-channel recordings were carried out using the bath solution containing (mM): 10 KCl, 90 NaF (or 180 mannitol), 1 $MgCl_2$ and 5 HEPES-Tris (pH 7.2) as well as the pipette solution containing (mM): 80 KF, 20 KCl, 10 EDTA and 5 HEPES (pH 7.4). Single-channel recordings were at a variety of holding potentials by shifting the pipette potential (Vp). For macro-patch recordings, Vp was kept at 30 mV, and ramp pulses were applied from −150 to +150 mV at the rate of 1 mV/msec.

Perforated whole-cell recordings were performed in HEK293 cells in control bathing solution containing (mM): 130 NaCl, 10 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$ and 5 HEPES (pH 7.4). Pipettes were filled with the solution containing (mM): 107 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 10 EGTA and 5 HEPES (pH 7.2) *plus* 120 μM nystatin. When necessary, NaCl was isotonically replaced with NMDG-Cl or mannitol. Transfected cells were visualized by GFP fluorescence under an Olympus IX70 microscope with a high pressure mercury light source and epifluorescence optics (U-MWIB block, IX-FLA: Olympus, Tokyo, Japan). The cell was kept at holding potential of ~60 mV, and a ramp-pulse was applied from −100 to +100 mV at the rate of 1 mV/msec every 10 sec. When necessary, step pulses were applied from −100 to +100 mV in 10-mV increments.

Currents were measured using an Oocyte Clamp amplifier (CA-1, Dagan Instruments, Minneapolis, MN) for two-microelectrode voltage-clamp, and an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) for macro-patch and single-channel recordings from oocytes and for HEK293 whole-cell current measurements. Voltage protocols were computer-controlled using an i-486-based personal computer (AST, Irvine, CA), coupled to TL-1 or DigiData 1200 (Axon Instruments) interface. Currents were filtered at 1 kHz and sampled at 5 kHz. Data acquisition and analysis were done using pCLAMP6 (Axon Instruments).

Recording of Intracellular pH

H^+-selective microelectrodes were made and calibrated, as described previously (Okada & Oiki, 1985; Ueda, Oiki & Okada, 1986). The electrodes were first filled with H^+-selective ligand, tri-*n*-dodecylamine (Ammann et al., 1981), and then backfilled with 100 mM KCl buffered with 10 mM HEPES-Tris at pH 7.0. Standard intracellular microelec-

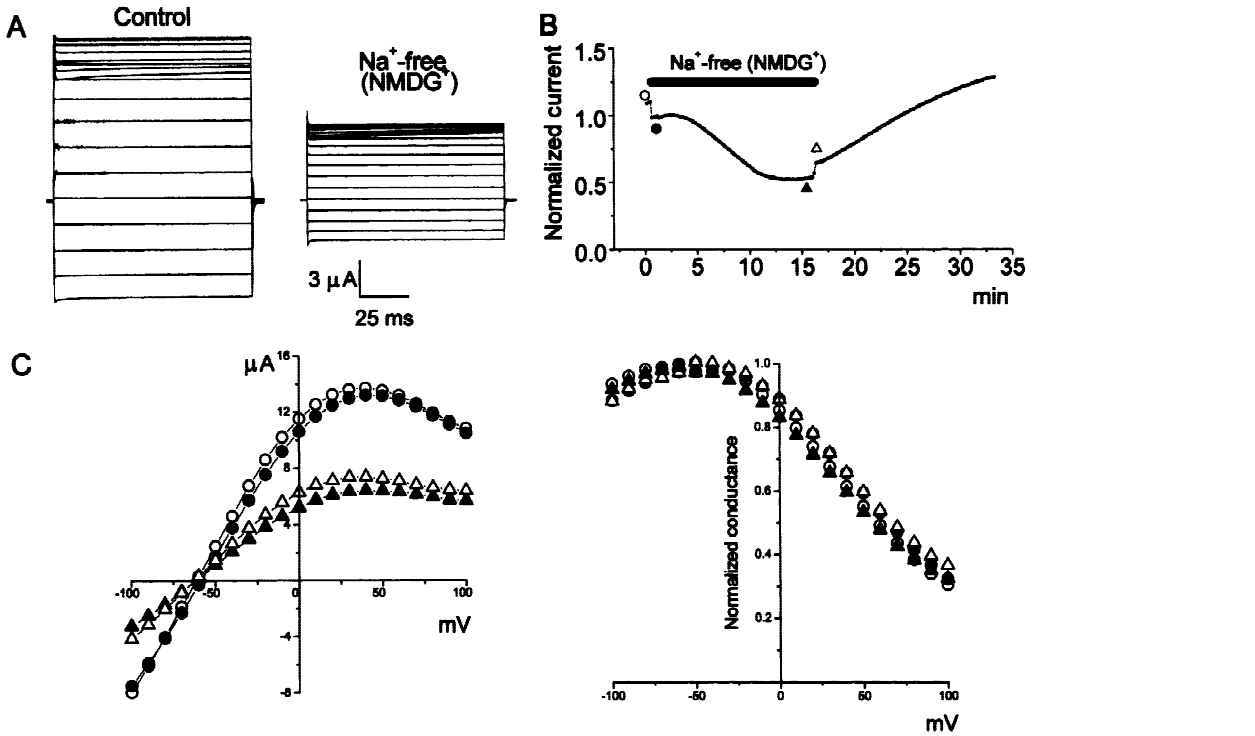

Fig. 1. Inhibitory effect of replacement of extracellular Na^+ with $NMDG^+$ on the macroscopic current of ROMK1 expressed in a *Xenopus* oocyte. (*A*) Representative current traces elicited by voltage steps from −100 to +100 mV in 10-mV increments. The holding potential was −60 mV. Left: initial currents in Na^+-rich solution. Right: currents after 15-min perfusion with Na^+-free NMDG-Cl solution. (*B*) Time course of Na^+ removal-induced inhibition of ROMK1 current. Peak outward currents at −20 mV are plotted as a function of time. Upper bar indicates the duration of Na^+ replacement with $NMDG^+$. (*C*) Current-voltage (*I-V*) relationship (left) and normalized chord conductance (right) as a function of voltage. Current levels were measured at the end of 100 msec step pulses (shown in *A*) applied from a holding potential (−60 mV) to the indicated level. Symbols: open circle, current measured immediately before changing to NMDG-Cl; closed circle, current measured immediately after replacing NaCl with NMDG-Cl; closed triangle, current measured after complete inhibition in NMDG-Cl solution; open triangle, current measured immediately after returning to the normal NaCl solution after maximal inhibition.

trodes filled with 3M KCl were used as a reference. Signals were recorded with an electrometer (model FD223: WPI, Sarasota, FL). The mean slope of the pH sensitivity of the electrodes used was −57.7 ± 2.2 mV ($n = 8$).

Experiments were performed at room temperature (23–25°C). Data are presented as the mean ± SEM with the number of experiments (n). The statistical significance (P) of the data was evaluated by paired or unpaired Student's t-test.

Results

Extracellular Na^+ Removal-Induced Inhibition of Macroscopic K^+ Currents in ROMK1-Expressing Oocytes

Expression of mRNA for ROMK1 resulted in a large current with K^+ selectivity and mild inward rectification in the presence of extracellular Na^+ of 90 mM (Fig. 1*A*: left panel). At negative potentials up to −100 mV no time-dependent inactivation, which is the sign of Na^+ block, was obviously observed. Around 15 min after replacing the bath Na^+ with $NMDG^+$, the K^+ currents were markedly suppressed without alteration of the overall profiles of currents recorded upon pulse applications (Fig. 1*A*: right panel). Figure 1*B* shows the time course of Na^+ removal-induced inhibition of ROMK1 current. The rapid drop of macroscopic current observed upon Na^+ removal is likely due to a change in junction potential and/or surface potential on the oocyte membrane, because the drop was recovered immediately after restoring extracellular Na^+ ions. The mean % inhibition by Na^+ removal was 49 ± 2.7% ($n = 6$) at −20 mV. The currents before and after Na^+ removal had ideal K^+ selectivity (Fig. 1*C*: left panel). The rectification properties of the current did not change, as shown in Fig. 1*C* (right panel).

Lack of Effect of Na^+ Removal from the Pipette Solution on Single-Channel Activities on ROMK1-Expressing Oocytes

Figure 2*A* shows representative single-channel traces recorded from *Xenopus* oocytes under the cell-attached

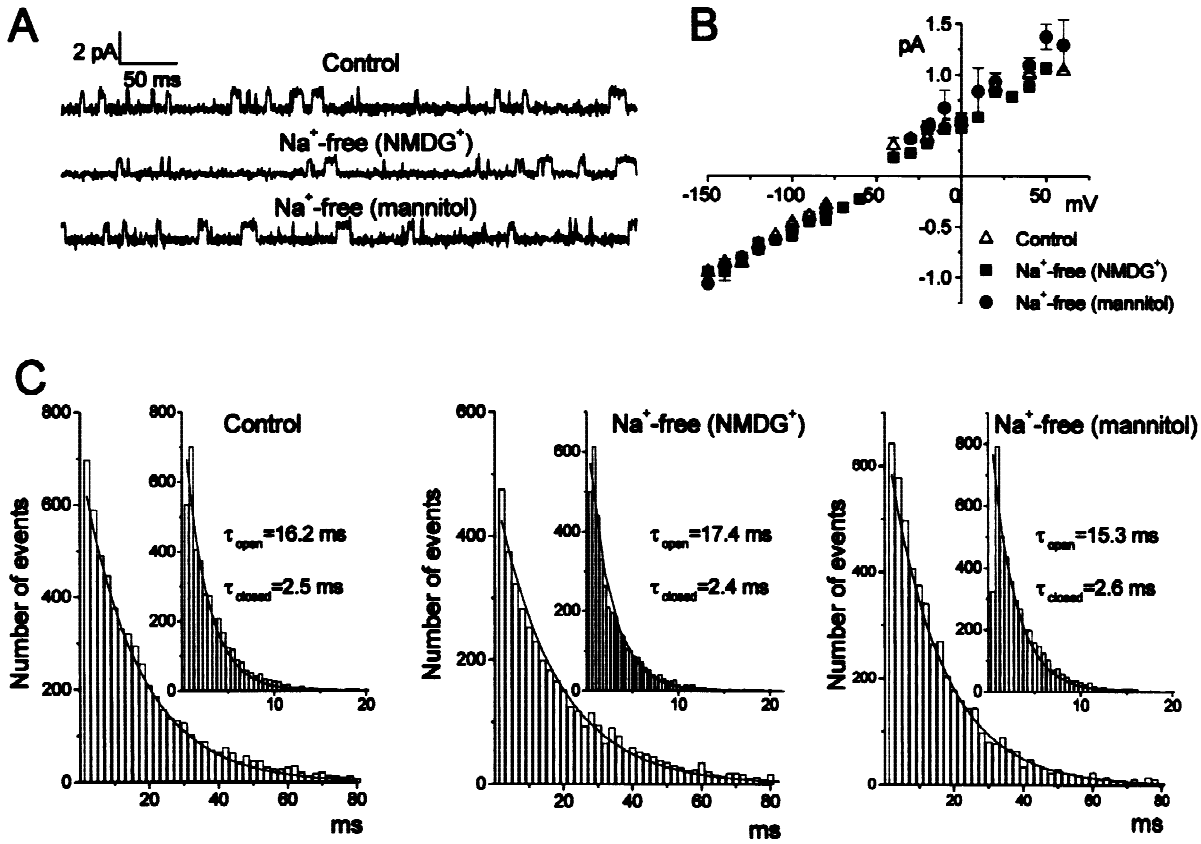

Fig. 2. Lack of effect of Na^+ removal from the pipette solution on single ROMK1 channel activities in the cell-attached patches on *Xenopus* oocytes. (*A*) Representative single-channel records at −150 mV from oocytes expressing ROMK1 channel in cell-attached mode in the presence of NaCl (top trace) and when the NaCl was replaced with 90 mM NMDG-Cl (middle trace) or 180 mM mannitol (bottom trace). (*B*) Single-channel *i-V* curves in the presence of NaCl ($n = 8$), NMDG-Cl ($n = 5$) or mannitol ($n = 4$) in the patch pipette. Each symbol represents the mean value and bar the SEM. (*C*) Gating kinetics of single ROMK1 channel in the presence of NaCl (control: left panel), $NMDG^+$ (middle panel) and mannitol (right panel) in the patch pipette.

patch-clamp configuration in a depolarizing bath solution. Replacing NaCl with NMDG-Cl or mannitol in the pipette solution did not significantly alter the amplitude of single-channel current (i), *i-V* relationships (Fig. 2*B*) and channel fast gating kinetics (Fig. 2*C*). In outside-out patches single ROMK1 channel activities, which were observed before rundown, were also insensitive to removing NaCl from bathing solution by replacing with mannitol (data not shown, $n = 4$). Therefore, it is concluded that single-channel properties of ROMK1 were insensitive to the change in the Na^+ concentration at the external pore entrance of the channel.

Effect of Na^+ Removal from the Bath Solution on ROMK1 Currents in Cell-Attached Macro-Patches on Oocytes

Effects of changes in the external bath Na^+ concentration were observed under cell-attached configuration of patch-clamp while keeping the Na^+ concentration at the external channel pore entrance (exposed to the pipette solution) constant. The macro-patch ROMK1 current exhibited mild rectification under ramp clamp (Fig. 3*A*) and was reversibly depressed by replacing Na^+ with $NMDG^+$ in the bath (not the pipette) solution (Fig. 3*B*). Steady-state inhibition was 37.5 ± 2.1% ($n = 12$) for outward currents at the pipette potential (Vp) of −100 mV and 20.9 ± 2.1% ($n = 10$) for inward curents at Vp of +100 mV. Figure 4 shows effects of NaCl replacement with mannitol on macro-patch currents. Steady-state inhibition was 26.9 ± 5.8% and 17.9 ± 4.2% ($n = 10$) at −Vp of +100 and −100 mV, respectively. These data indicate that the Na^+ sensitivity is not due to the direct action of extracellular Na^+ on the channel but due to an indirect action via changes in some factor in the intracellular milieu.

Na^+/H^+ Antiporter-Mediated Na^+ Sensitivity of ROMK1 Channels in Oocytes

Since the ROMK1 channel is very sensitive to intracellular pH (Tsai et al., 1995; Choe et al., 1997; McNicholas

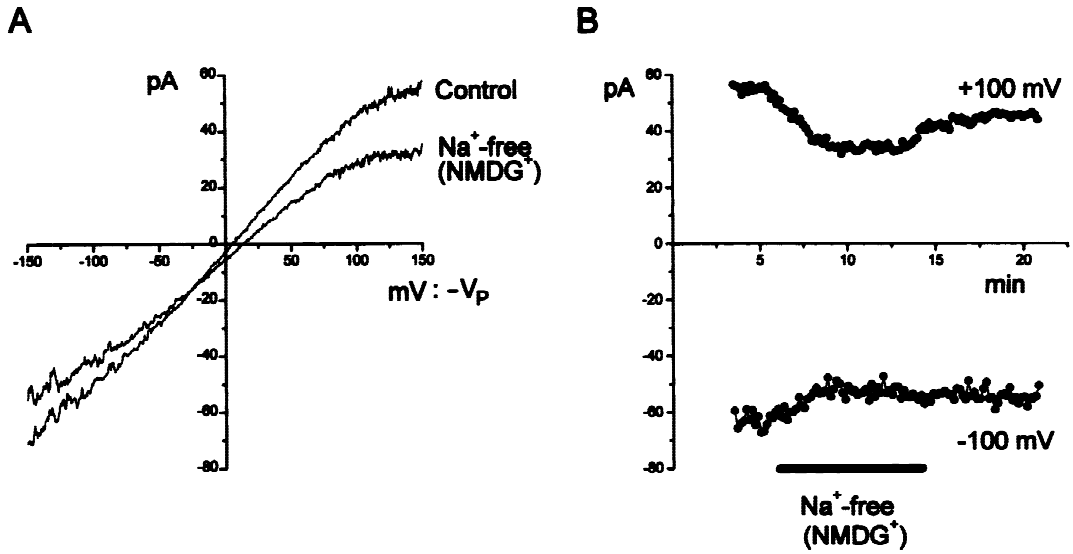

Fig. 3. Inhibitory effect of Na^+ replacement with $NMDG^+$ from bathing (but not pipette) solution on the ROMK1 currents through a macro-patch in the cell-attached mode on a *Xenopus* oocyte. (*A*) *I-V* plots of currents before and 8 min after removal of Na^+ ions. Currents are shown as a function of −Vp, since the membrane potential was not known. However, it is noted that the reversal potential was close to 0 mV to Vp. This fact suggests that the membrane potential was close to the equilibrium potential to K^+ in the oocyte which was highly expressing ROMK1 channels. (*B*) Time course of changes in macro-patch currents recorded at −Vp of −100 and +100 mV every 10 s before, during (at bar) and after Na^+ removal.

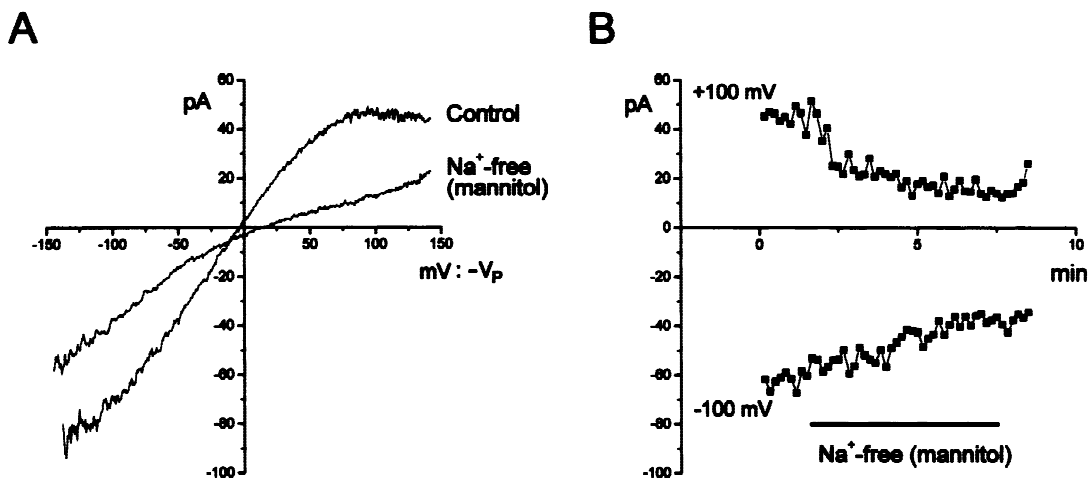

Fig. 4. Inhibitory effect of NaCl replacement with mannitol in the bathing (but not pipette) solution on the ROMK1 currents through a macro-patch in the cell-attached mode on a *Xenopus* oocyte. (*A*) Macropatch *I-V* curves before and 10 min after removal of NaCl. (*B*) Time course of changes in macro-patch currents recorded at −Vp of −100 and +100 mV every10 sec before, during (at bar) and after NaCl removal.

et al., 1998), a candidate for a messenger for coupling of ROMK1 channel to extracellular Na^+ might be intracellular H^+ ions. Actually, the Na^+/H^+ antiporter with sensitivity to amiloride ($K_{0.5}$ = 4 μM) has been functionally characterized for *Xenopus* oocytes (Towle et al., 1991), and the NHE1 subtype of Na^+/H^+ antiporter (X1-NHE) has recently been cloned from *Xenopus* oocytes (Busch, 1997). Indeed, a blocker of the Na^+/H^+ antiporter, amiloride (10 μM), largely slowed the time course of Na^+ removal-induced inhibition (Fig. 5), though did not cancel the phenomenon itself. The mean values of the normalized current at 3 min after amiloride application were 0.928 ± 0.006 (n = 5) and 1.024 ± 0.045 (n = 4; P = 0.045) in the absence and presence of amiloride, respectively. This result suggests that the Na^+ sensitivity is mediated, at least in part by Na^+/H^+ antiporters which are endogenously expressed in oocytes.

Intracellular pH measurements actually demonstrated a significant change in the intracellular pH value upon Na^+ removal. Figure 6 shows an example of the experiments with using H^+-selective microelectrodes. The effect was variable with maximal acidification rang-

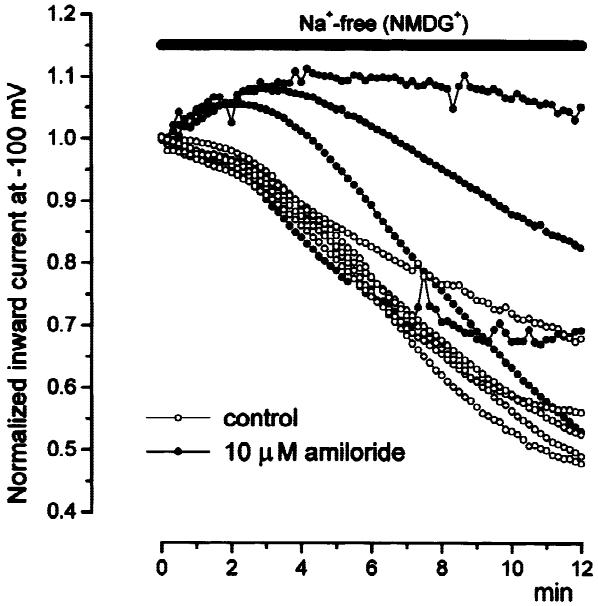

Fig. 5. Effect of amiloride on the time course of whole-cell ROMK1 currents in *Xenopus* oocytes after Na^+ replacement with $NMDG^+$. Currents recorded at −100 mV were normalized to those at zero time. Open circles, normalized currents in the absence of amiloride; filled circles, normalized currents in the presence of 10 μM amiloride.

ing from 0.05 to 0.2 pH units, and the mean value was 0.09 ± 0.05 (n = 8). These data suggest that Na^+-dependent intracellular pH changes regulate the ROMK1 activity in oocytes.

Fakler et al. (1996) established that the steep pH sensitivity of ROMK1 channel is determined by a single amino acid residue, Lys^{80}, located in the N-terminal part close to the first membrane-spanning domain. Expression of the K80M mutant of ROMK1 resulted in large K^+-selective current with weak rectification similar to the wild type. This current also exhibited Na^+-dependent inhibition, which was, however, less prominent than the wild type. Figure 7 shows that the mutation K80M significantly decreased both steady-state inhibition (left panel) and maximal rate of inhibition (right panel).

When ROMK1 and NHE3 were coexpressed, the Na^+-dependent inhibition was largely augmented (Fig. 8*A*). Both the steady-state inhibition (Fig. 8*Ba*) and the inhibition rate (Fig. 8*Bb*) were altered significantly by coexpression with NHE3.

These data indicate Na^+ sensitivity of ROMK1 channel is, at least in part, due to the intracellular pH change mediated by Na^+/H^+ antiporters.

Na^+ Sensitivity of ROMK1 Channels Conferred by Co-expression with NHE3 in Human Kidney HEK293 Cells

Whole-cell recordings showed that HEK293 cells endogenously express rather small currents ranging from 50 to 200 pA at 100 mV with weak outward rectification (*data not shown*, n = 9). Transient transfection of these cells with ROMK1 cDNA gave rise to expression of large K^+-selective currents with amplitude of 2 to 5 nA. Cotransfection with NHE3 did not affect the K^+ current amplitude in the control bath solution. When NaCl was replaced with mannitol in the bathing solution, however, remarkable suppression of ROMK1 current was observed without altering the overall current profiles (Fig. 9*A*) in HEK293 cells transfected with both ROMK1 and NHE3. The inhibitory effect was relatively reversible (Fig. 9*B* and *C*). When ROMK1-expressing HEK293 cells were not cotransfected with NHE3, in contrast, Na^+ sensitivity of ROMK1 channel was much less prominent (Fig. 10*A*: open column) than that in cells coexpressing both ROMK1 and NHE3 (hatched column). Replacement of extracellular Na^+ with $NMDG^+$ also inhibited, though less prominent than the case with mannitol, the macroscopic ROMK1 current by about 15% (open column in Fig. 10*B*). After coexpression with NHE3, more profound inhibition of ROMK1 currents was again conferred (hatched column). These results indicate that Na^+ sensitivity of ROMK1 channel in markedly augmented by NHE3 expression in mammalian cells.

Discussion

Mechanism of Na^+ Sensitivity of ROMK1 Channels

We demonstrate here that elimination of extracellular Na^+ ions produces a prominent decrease in the macro-

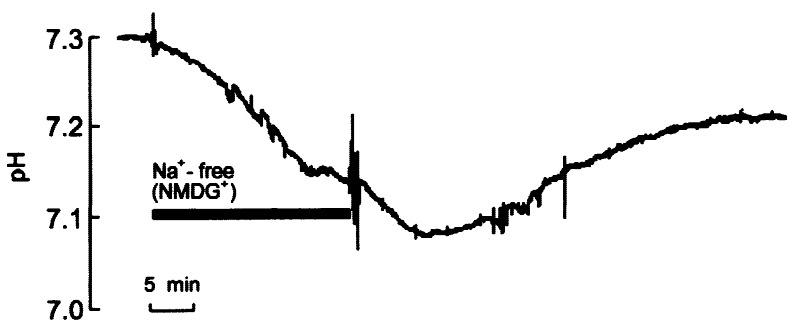

Fig. 6. Intracellular acidification in a *Xenopus* oocyte induced by removal of extracellular Na^+. Representative time course of the intracellular pH change after replacing extracellular Na^+ with $NMDG^+$. The bar indicates the duration of Na^+ elimination.

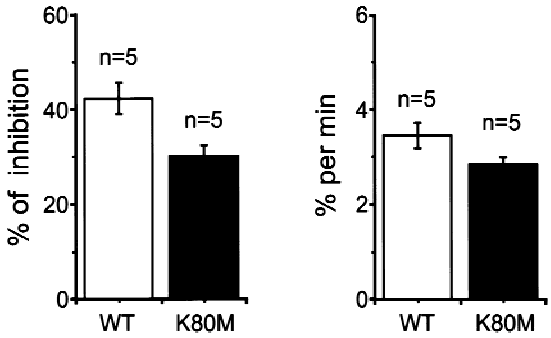

Fig. 7. Effects of the K80M mutation on the Na^+ removal-induced inhibition of ROMK1 currents in *Xenopus* oocytes. The percentage of steady-state inhibition (left panel) and maximal rate of % inhibition (right panel) of currents recorded at −100 mV by Na^+ elimination in oocytes expressing the wild type ROMK1 (open columns) and the K80M mutant (filled columns). The statistical significance (*P*) of the effect of the mutation is 0.00016 for steady-state inhibition and 0.076 for the inhibition rate.

scopic K^+ conductance of heterologously ROMK1-expressing cells. This effect is similar to what was shown for inwardly rectifying K^+ currents of native cells including tunicate eggs (Ohmori, 1978; Fukushima, 1982), cat ventricular myocytes (Harvey & Eick, 1989*a*), and rat spinal cord astrocytes (Ransom et al., 1996) as well as for inward K^+ currents of ROMK2 expressed in *Xenopus* oocytes (Zhou et al., 1994). The effect is not due to Na^+ ion permeation, because the reversal potential did not shift upon removal of extracellular Na^+ (Fig. 1*C*). A direct action of Na^+ on the ROMK1 channel from the extracellular side cannot explain the Na^+ sensitivity, because the single-channel properties were not affected by the presence of Na^+ in the extracellular pipette (not bath) solution in the cell-attached patch membrane (Fig. 2). Cell-attached macro-patch experiments showed that the ROMK1 channel activity was affected by Na^+ removal from the extracellular bath (not pipette) solution (Figs. 3 & 4). This fact indicates that changes in some intracellular factor are responsible for Na^+-dependent channel regulation.

As compared with the IRK family members (Sabirov, Okada & Oiki, 1997), the ROMK members, including ROMK1 and ROMK2 (Tsai et al., 1995; Choe et al., 1997; McNicholas et al., 1998), have much more marked sensitivity to intracellular pH. Thus, there is a possibility that Na^+/H^+ antiporters are involved in the sensitivity of ROMK1 channel to extracellular Na^+. This hypothesis was supported by the inhibiting effect of amiloride (Fig. 5) on the Na^+ removal-induced suppression of ROMK1 currents expressed in *Xenopus* oocytes which are known to express endogenously amiloride-sensitive NHE1 (Busch, 1997). This observation is similar to what was found with dimethylamiloride for the inwardly rectifying K^+ conductance of cat ventricular myocytes (Harvey & Eick, 1989*a*). Direct measurements of intracellular pH changes with H^+-selective microelectrodes provided additional support for the hypothesis (Fig. 6). Removal of extracellular Na^+ did indeed acidify the bulk cytoplasm by around 0.1 pH unit. It is possible that the local pH change in the intracellular microenvironment in the vicinity of the membrane near the location of Na^+/H^+ antiporters and ROMK1 channels may exceed the pH change in the intracellular bulk environment. More di-

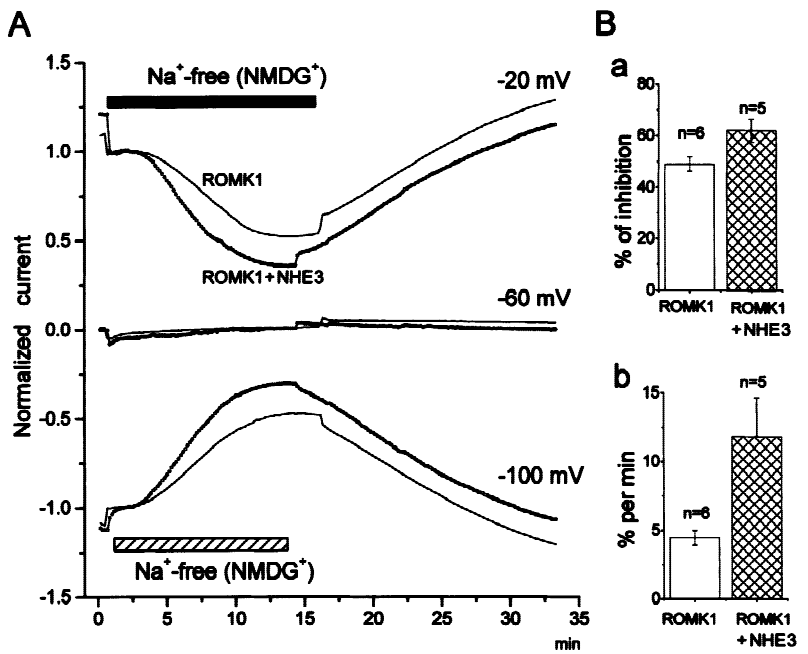

Fig. 8. Effects of coexpression with NHE3 on the Na^+ removal-induced inhibition of ROMK1 currents in *Xenopus* oocytes. (*A*) Time course of Na^+ removal effect on ROMK1 currents with (open circles) and without (solid lines) NHE3 coexpression. The currents recorded at −100, −60 and −20 mV are plotted as a function of time. Upper and lower bars indicate the duration of Na^+ elimination for oocytes expressing ROMK1 and ROMK1 *plus* NHE3, respectively. (*B*) The percent of steady-state inhibition (*a*) and maximal rate of % inhibition (*b*) of currents recorded at −100 mV by Na^+ elimination in oocytes expressing ROMK1 alone (open columns) and together with NHE3 (hatched columns). The statistical significance (*P*) of the effect of NHE3 co-transfection is 0.026 for steady-state inhibition and 0.014 for the inhibition rate.

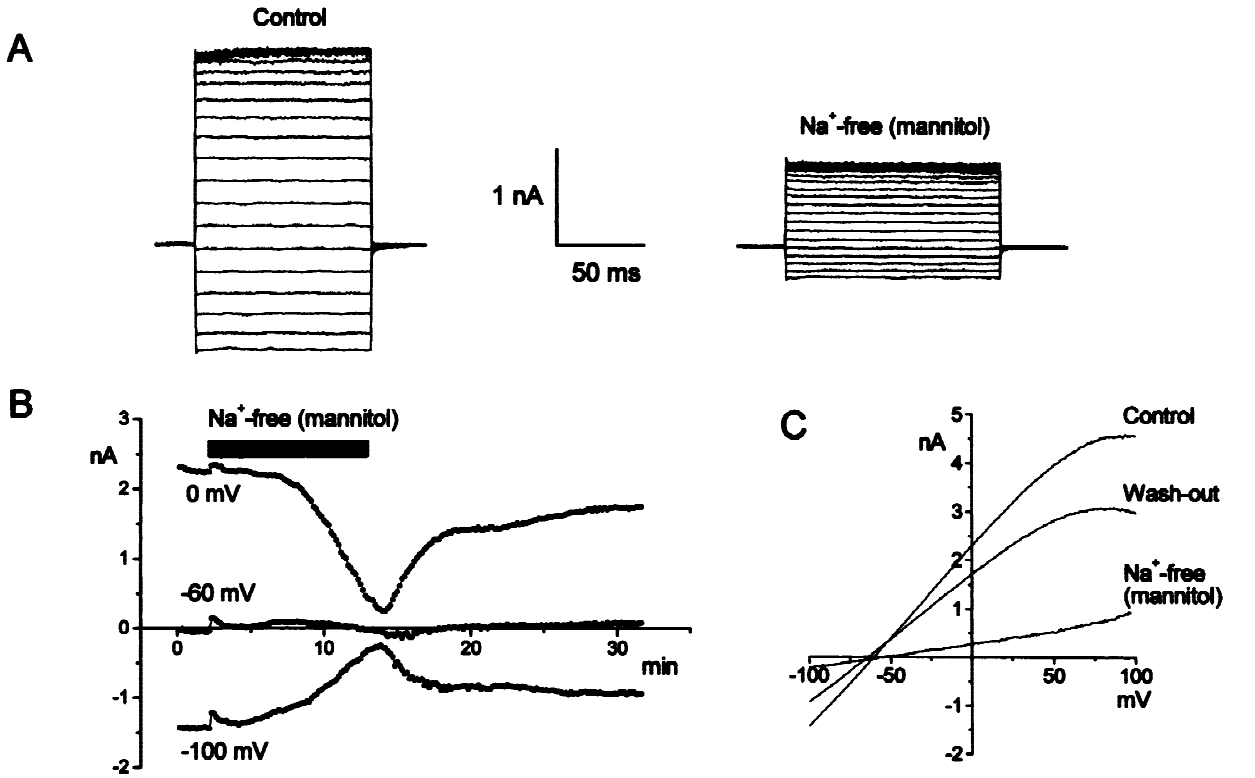

Fig. 9. Inhibitory effect of NaCl replacement with mannitol in the bathing solution on the whole-cell currents in HEK293 cells coexpressing with ROMK1 and NHE3. (*A*) Current traces elicited by voltage steps (−100 to +100 mV in 10-mV increments) from a holding potential of −60 mV. Whole-cell currents were recorded by perforated-patch whole-cell recordings from a cell in normal NaCl bath solution (left trace) and after 20-min perfusion with Na^+-free mannitol bath solution (right traces). (*B*) Time course of the effects of NaCl replacement with mannitol in another HEK293 cell. The bar indicates the duration of NaCl elimination. (*C*) Effects of NaCl replacement with mannitol on ramp *I-V* curves recorded from a HEK293 cell. The data were collected from the maximum and minimum points of *B*.

rect evidence for the involvement of Na^+/H^+ antiporters was obtained by the NHE3 coexpression in *Xenopus* oocytes (Fig. 8) and in HEK293 cells (Figs. 9 and 10). Na^+ sensitivity of ROMK1 channel was largely augmented by coexpression with NHE3, which is a Na^+/H^+antiporter isoform of the kidney apical membrane (Soleimani et al., 1994; Amemiya et al., 1995; Biemensderfer et al., 1997; Yoshioka, Suzuki & Kawakita, 1997; Rutherford et al., 1997). Furthermore, mutation of Lys 80, which is known to be primarily responsible for steep pH sensitivity of ROMK1 (Fakler et al., 1996), significantly diminished the Na^+ sensitivity (Fig. 7), indicating an involvement of proton as a mediator. It should be noted, however, that both amiloride and the K80M mutation did not abolish the inhibition completely. Choe et al. (1997) showed that another amino acid at T51 on ROMK2 (corresponding to T70 on ROMK1) modulates the pH sensitivity of ROMK but that double mutant T51E/K61M of ROMK2 (corresponding to T70E/K80M) had the same insensitivity to pH at K61M. Thus, the partial effect of K80M mutation could not be ascribed to an involvement

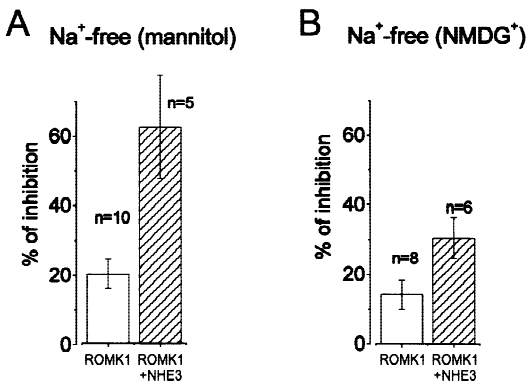

Fig. 10. Effects of coexpression with NHE3 on the Na^+ removal-induced inhibition of whole-cell ROMK1 currents recorded at −100 mV in HEK293 cells. Open columns represent the data from the cells transfected with ROMK1 cDNA alone, and hatched columns the cells cotransfected with cDNAs of ROMK1 and NHE3. (*A*) Steady-state % inhibition by replacement of NaCl with mannitol ($P = 0.003$). (*B*) Steady-state % inhibition by replacement of Na^+ with $NMDG^+$ ($P = 0.037$).

of T70 in pH sensitivity of ROMK1. Therefore, a mechanism other than that involving Na^+/H^+ antiporters (such as that involving Na^+/Ca^{2+}, Na^+-K^+-$2Cl^-$ or Na^+-Cl^- transporters) may also contribute to the Na^+-sensing phenomenon of ROMK1 channels expressed in oocytes.

Since ROMK members primarily represent the renal potassium channels, we expressed ROMK1 in the cell line of human kidney origin, HEK293, and tested the Na^+-sensing property in the mammalian renal cells. Surprisingly, when the ROMK1 channel was expressed alone, its activity was only moderately suppressed by removal of extracellular Na^+ ions (Fig. 10: open columns). However, Na^+ sensitivity of ROMK1 channel became marked after additional expression of NHE3 (Fig. 10: hatched columns). An interesting feature of mammalian expression experiments was that removing both Na^+ and Cl^- by replacing with mannitol (Fig. 10*A*) had on average a more profound effect than removal of Na^+ alone by replacing with $NMDG^+$ (Fig. 10*B*). This result may suggest that, in contrast to oocytes, HEK293 cells may be endogenously expressing some machinery (such as Na^+-K^+-$2Cl^-$ or Na^+-Cl^- transporter) which can participate in modulation of ROMK1 K^+ channels by sensing Cl^-.

The large gradient between the extracellular and intracellular Na^+ concentrations, in addition to the membrane potential, gives energy not only to extrude intracellular H^+ via Na^+/H^+ antiporters but also to extrude cytosolic Ca^{2+} via Na^+/Ca^{2+} antiporters, which are known to be expressed in both *Xenopus* oocytes (Schlief & Heinemann, 1995) and HEK293 cells (Loo & Clarke, 1994). Thus, it is also possible that Na^+ removal-induced rise of intracellular free Ca^{2+} is implicated, in part, in the Na^+ sensitivity of ROMK1 channel. In fact, the Na^+ sensitivity of macula densa K^+ channels was suggested to be largely due to the Na^+/Ca^{2+} exchange mechanism (Hurst et al., 1994). A possible involvement of the cross-talk between Na^+/Ca^{2+} antiporters and ROMK1 channels awaits further studies for the Na^+/H^+ antiporter-independent component of ROMK1 Na^+ sensitivity. However, it should be emphasized that Na^+ sensitivity of ROMK1 in the oocytes in the presence of 2 mM Ca^{2+} in the bath solution was indistinguishable from that in a nominally Ca^{2+}-free solution. Also, our preliminary experiments showed that removal of extracellular Na^+ did not produce a considerable change in the ROMK1 channel activity in NCX1-expressing CCL-39 cells (R.Z. Sabirov, R.R. Azimov, M. Shigekawa and Y. Okada, *unpublished observations*).

Physiological Relevance

ROMK channels localize in the nephron including the macula densa (Lee & Hebert, 1995; Xu et al., 1997) and represent an ATP-regulated, intracellular pH-sensitive, low-conductance, weak inwardly rectifying K^+ channel in the apical membrane of the cortical collecting duct (CCD) and the thick ascending limb (TAL) (Hebert, 1995; Hebert & Wang, 1997). Functional expression of K^+ channels sensitive to intracellular pH and extracellular Na^+ was found in the apical membrane of macula densa cells (Hurst et al., 1994). Functional expression of Na^+/H^+ antiporters was also demonstrated at the apical membrane of macula densa cells (Fowler et al., 1995). Molecular expression of the NHE3 protein was in fact found in the apical membrane of rat renal tubules including the macula densa (Amemiya et al., 1995). Taken together, there is a possibility that Na^+ sensitivity of ROMK conductance due to the cross-talk between ROMK channel and NHE3 is involved in regulation of K^+ secretion in CCD and K^+ recycling in TAL (Wang, Sackin & Giebisch, 1992; Giebisch, 1995), and also in the Na^+ sensing mechanism in macula densa cells (Bell & Lapointe, 1997).

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