pH-dependent modulation of the cloned renal K⁺ channel, ROMK

CARMEL M. MCNICHOLAS,1 GORDON G. MACGREGOR,2 LEON D. ISLAS,1 YINHAI YANG,2 STEVEN C. HEBERT,3 AND GERHARD GIEBISCH1

1Department of Cellular and Molecular Physiology, Yale University
School of Medicine, New Haven, Connecticut 06520; 2Department of Cardiology, Children's Hospital, Boston, Massachusetts 02115; and 3Division of Nephrology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2372

McNicholas, Carmel M., Gordon G. MacGregor, Leon D. Islas, Yinhai Yang, Steven C. Hebert, and Gerhard Giebisch. pH-dependent modulation of the cloned renal K⁺ channel, ROMK. Am. J. Physiol. 275 (Renal Physiol. 44): F972–F981, 1998.—pH is an important modulator of the low-conductance ATP-sensitive K⁺ channel of the distal nephron. To examine the mechanism of interaction of protons with the channel-forming protein, we expressed the cloned renal K⁺ channel, ROMK (Kir1.1x), in Xenopus oocytes and examined the response to varied concentrations of protons both in the presence and in the absence of ATP. Initial experiments were performed on inside-out patches in the absence of ATP in Mg⁺²-free solution, which prevents channel rundown. A steep sigmoidal relationship was shown between bath pH and channel activity. We calculated values for pKₐ = 7.18 and 7.04 and Hill coefficients = 3.1 and 3.3, for ROMK1 and ROMK2, respectively. Intracellular acidification (pH 7.2) also increased the Mg-ATP binding affinity of ROMK2, resulting in a leftward shift of the relationship between ATP concentration and the reduction in channel activity. The K_{1/2} for Mg-ATP decreased from 2.4 mM at pH 7.4 to ~0.5 mM at pH 7.2. Mutation of lysine-61 to methionine in ROMK2, which abolishes pH sensitivity, modulated but did not eliminate the effect of pH on ATP inhibition of channel activity. We previously demonstrated that the putative phosphate loop in the carboxy terminus of ROMK2 is involved in ATP binding and channel inhibition [C. M. McNicholas, Y. Yang, G. Giebisch, and S. C. Hebert. Am. J. Physiol. 271 (Renal Fluid Electrolyte Physiol. 40): F275–F285, 1996]. Conceivably, therefore, protonation of the histidine residue within this region could alter net charge (i.e., positive shift) and increase affinity for the negatively charged nucleotide.

METHODS

Preparation of oocytes for patch-clamp experiments. Stage V–VI oocytes were isolated from Xenopus laevis frogs by partial ovariectomy under tricaine methanesulfonate anesthesia. Oocytes were then defolliculated by treatment with 2 mg/ml collagenase (Sigma Chemical) in zero-Ca²⁺ hypotonic solution (in mmol/l: 82.5 NaCl, 2.0 KCl, 1.8 MgCl₂, and 5.0 HEPES, pH 7.4) combined with gentle agitation for 1 h. Following this incubation, oocytes were washed in ND96 media (in mmol/l: 96 NaCl, 2.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, and 5.0 HEPES, pH 7.4). Thereafter, oocytes were maintained in supplemented ND96 media (in mmol/l: 96 NaCl, 2.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, and 5.0 HEPES, pH 7.4; plus 50 mg/ml gentamicin and 2.5 mmol/l sodium pyruvate). Twenty-four hours after defolliculation, oocytes were injected with 50 nl (5 ng) of ROMK cRNA prepared as described previously (11). Experiments were performed on days 3–6 after injection.

Oocytes were placed in a hypertonic solution (in mmol/l: 220 N-methylglucamine, 220 aspartic acid, 2 MgCl₂, 10 EGTA, 10 HEPES, pH 7.4, at room temperature) and allowed to shrink for 2–5 min. The vitelline membrane (VM) appears as a transparent sphere around the oocyte that can easily be removed using fine watchmaker’s forceps. Nylon mesh placed beneath the oocyte immobilizes and thereby facilitates VM removal. Following this, oocytes were carefully transferred into a chamber (volume 500 µl) mounted on an inverted microscope (Olympus IMT-2) and positioned so that the animal pole was freely accessible to patch pipettes. A 5- to 10-min period was allowed for equilibration.

Experimental media. Standard pipette solutions contained (in mmol/l): 150 KCl, 1 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.4, and were used throughout the entire study. Bath solutions were composed of the following (in mmol/l): +Mg²⁺ solution contained 150 KCl, 1 MgCl₂, 5 EGTA, and 5 HEPES, pH 7.4;
"Mg\(^{2+}\)-free" solution contained 150 KCl, 5 EDTA, and 5 HEPES, pH 7.4. Stock solutions of nucleotide (10 mM) (Sigma) were made in standard \(^{+}\)Mg\(^{2+}\)/N\(^{-}\) bath solution and were maintained at 4°C. Protein kinase A (PKA; Promega) was diluted to 50 mM in bath solution. All solutions were titrated to the desired pH using KOH/HCl as required.

Patch-clamp technique. Single channel recordings were obtained from membrane patches in the inside-out configuration. Recording pipettes were constructed from boro-silicate glass capillaries (Dagan, Minneapolis, MN) using a Narishige PP83 microelectrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and were not fire polished. The pipettes were partially filled with standard pipette solution and had tip resistances of 2–5 MΩ. Experiments were performed at room temperature (20–22°C). Rapid solution changes were achieved for inside-out experiments using a multi-lane flow system (model SF-77; Warner Instrument, Hamden, CT).

Single channel currents were recorded with a patch-clamp amplifier (model EPC-7, List Electronics, Darmstadt, Germany; or model PC-505, Warner Instrument), low-pass filtered at 1 kHz using an 8-pole Bessel filter (Frequency Devices, Haverhill, MA), and then digital signals were stored on videotape after pulse code modulation (Sony model PCM-501ES). For analysis, data were redigitized (4 kHz) and transferred to a PC after analog-to-digital conversion (Digi-data 1200; Axon Instruments, Foster City, CA) and then analyzed using pCLAMP6 (Axon Instruments) software systems. There was no further filtering of data for analysis. For Figs. 6 and 7, data were transferred to Atari 1040 ST computer (Instrutech, Port Washington, NY) and analyzed using the TAC program system (Instrutech).

Analysis of channel recordings. Data were sampled for the entire course of an experiment. For the majority of experiments, patches contained more than one channel and in many instances more than five channels. To calculate accurately the total number of open channels (N), the channel activity from individual experiments was plotted out to allow visualization of open-closed channel transitions. To calculate the number of channels in a patch, we have adopted a method described previously (19). Lowering pH to 6.6 completely and reversibly inhibited ROMK activity. The total number of channels, N, can thus be accurately counted. N\(_o\), the product of N and open probability P\(_o\), is measured using pCLAMP6 and NPO software as described below. This permits derivation of N\(_o\)/N, the single channel open probability. N\(_o\)/N could not be measured accurately with the ROMK2 K61M mutant, because this channel is not sensitive to pH (4); thus we measured mean patch current.

Because of the limitations of the pCLAMP6 (Axon Instruments) analysis system, which only allows five current levels to be analyzed at any given time, we elected to use the events list mode of the Fetchan suite of programs rather than calculating open probability from amplitude histograms. Because of the nature of multi-channel patches of this kind, accurate, sharply defined peaks in the amplitude histogram are difficult to obtain, especially when more than five channels are present. These continuous channel recordings can be segmented during analysis if more than five channels are present. Comparison of the Fetchan file displayed on the computer screen with the data hardcopy allows one to ensure that the correct number of channels are being taken into account. We generated events list files from the channel recordings, which measure channel amplitude (50% threshold) and duration of channel open/closed states. The parameters obtained are stored in a binary file format (extension "*.EVL"), which can be read by the NPO analysis program (Dr. Jiliang Sui, Dept. Physiology and Biophysics, Mt. Sinai School of Medicine, NY. Program available at http://www.axonet.com/pub/userware/popen). Using the NPO program, we calculated the open probability (N\(_o\)/N) for the number of channel levels determined during FETCHAN analysis generation of EVL files. At this time the data were binned at 0.5-s intervals. These data were then transferred to Excel (Microsoft) and, where appropriate (i.e., if >5 channel levels were present), corrected for the total number of channels in the patch, thereby deriving N\(_o\). Since we knew the precise number of channels (N) in the patch, we then calculated N\(_o\)/N, i.e., single channel open probability.

For dose-response relationships, we exposed patches to test solutions at various pH values for at least 30 s, or until a plateau was reached, and thereafter for another 20- to 30-s period to obtain an estimate of the response. At pH 6.6, the time to maximal effect was 32 ± 7 s (n = 10). We calculate the average N\(_o\)/N over a 20- to 30-s period after exposure to a given pH. This was true for all values with the exception to pH 6.6. Here we calculate the average N\(_o\)/N for 5–10 s after reaching maximal inhibition, because prolonged exposure to acidic pH leads to an irreversible inhibition of channel activity (to be discussed further in RESULTS).

In all traces shown, unless otherwise stated, recordings were made from inside-out patches [holding potential (V\(_{\text{h}}\)] = −50 mV]. Data are presented as means ± SE. Normalized P\(_o\) (expressed as percentage of control) were obtained by dividing the N\(_o\)/N value per given proton concentration with that obtained at maximal activity (P\(_{\text{omax}}\)). For data shown in Figs. 5 and 12, data were transferred to Excel 5.0. The Student's t-test was used to compare data, and P < 0.05 was considered significant.

Method of mutagenesis and preparation of cRNA. Site-directed mutagenesis to produce mutant ROMK2 clones R2H206G and R2G204D was performed as described previously (22). cDNAs were propagated in the vector pSPORT in Escherichia coli DH5α. To prepare cRNA, the plasmid was linearized with NotI, and transcripts were generated with T7 RNA polymerase in the presence of capped GTP as described previously (11).

RESULTS

Effect of intracellular acidification on channel activity in the absence of ATP. Figures 1 and 2 show the effects of changing intracellular pH on ROMK1 and ROMK2, respectively. Intracellular pH was initially altered in the absence of ATP. We have previously demonstrated that ROMK channel activity can be maintained for prolonged periods in the absence of ATP, provided Mg\(^{2+}\) was omitted from the bath solution (21, 22). Patches of membrane expressing either ROMK1 or ROMK2 were excised from the cell, and the intracellular surface was perfused with solutions at a variety of pH values ranging from pH 7.8 to 6.6.

Figure 1A shows that acidification inhibits ROMK1 channel activity in a concentration-dependent fashion. The apparent pK of proton-mediated inhibition of ROMK1 occurred at pH 7.18. The Hill coefficient of the relationship between bath pH and channel activity was 3.1. This Hill value, greater than 1, suggests that closure of ROMK1 involves the cooperative action of more than one proton. An example of a continuous channel recording from a patch containing four channels is shown in Fig. 1B. Channel recordings were
A similar response to pH was observed for ROMK2. The pH channel activity relationship obtained for ROMK2 is shown in Fig. 2, together with an example of a continuous channel recording from an inside-out patch expressing ROMK2 channels. The pK and Hill coefficient (7.04 and 3.3) values were similar to those obtained for ROMK1. From these data, we conclude that alternative splicing at the amino terminus of the ROMK protein has no significant effect on the response of channel activity to intracellular pH.

Fig. 1. Effect of pH on ROMK1. A: dose-response relationship of the response of ROMK1 channel activity to variation in intracellular H⁺ concentration. Data are means of 3 separate experiments in which NPo/N was measured at holding potential (−Vp) = −50 mV. Relationship between intracellular pH (pHi) and channel activity is described by a modified Hill equation NPo = 1/[1 + ([H]/K)n], where [H] is the bath proton concentration, n = Hill coefficient, and pK = −log10K with a Hill coefficient of 3.1, and apparent half-maximal inhibition (pK) was observed at pH 7.18. Data are means ± SE. B: continuous recording from an inside-out patch expressing ROMK1 channel activity. Bath ("cytosolic") pH was changed at the points indicated. Recordings were made in symmetrical KCl (150 mM, ATP and Mg²⁺-free) at −Vp = −50 mV. A subconductance state emerges when the patch is exposed to pH 7.0 solutions (marked by the asterisk). C, zero current level when all channels are closed.

Fig. 2. Effect of pH on ROMK2. A: dose-response relationship of the response of ROMK2 channel activity to variation in intracellular H⁺ concentration. Data are generated from 25 separate experiments in which NPo/N was measured at −Vp = −50 mV. Relationship between pH and channel activity is described by equation in legend of Fig. 1, with a Hill coefficient of 3.3, and apparent half-maximal inhibition (pK) was observed at pH 7.04. Data are means ± SE. B: continuous recording from an inside-out patch expressing ROMK2 channel activity. Bath (cytosolic) pH was changed at the points indicated. Recordings were made in symmetrical KCl (150 mM, no ATP or Mg²⁺) at −Vp = −50 mV. Reversibility of the effect of acidification is demonstrated as channel activity recovers when bath solutions are switched to pH 7.4.
We also observed subconductance states of ROMK2 in 63% of patches (n = 16) during exposure to acidic bath solutions. Similar to ROMK1, the subconductance state emerged after exposure to pH 6.6, generally preceding the final channel closure and lasting for only a few seconds. An example of such a subconductance state is shown in Fig. 3, where following exposure to pH 6.6, two channels are present, one fully open and another in a subconductance state (Fig. 3A). This particular subconductance state remained active for many seconds and occasionally transitioned into a fully open state for brief periods as shown Fig. 3B. As shown in Fig. 3C, the subconductance was 39% of the fully open channel. A more thorough characterization of the subconductance properties of ROMK channels is being presently undertaken in our laboratory (15).

Reversibility of proton-induced inhibition. The loss of channel activity observed following exposure of the membrane patch to pH 6.6 was fully reversible, providing there was a prompt exchange of solution back to control pH 7.4. More prolonged exposure to acid media resulted in inability to recover channel activity, despite pH increases to 7.4 or 7.8 or exposure to solutions containing ATP and PKA. Figure 4 shows the results of 11 experiments in which we exposed the patch to acidic solution and assessed the reversibility from proton-induced inhibition. In 10 experiments, channel activity returned to a value not significantly different from control, and channel activity in the remaining experiment only partially recovered. The pH 6.6 bath solution reduced channel activity to 8.7 ± 2.9% (n = 11) of the control value, and channel activity increased to 91.5 ± 5.8% of the initial control value upon return to the pH 7.4 bath. A similar reversibility of the proton-induced decrease of channel activity was observed for ROMK1 (data not shown).

Figure 5 shows a single experiment in which channel activity is monitored throughout an entire pH pulse protocol. Data are expressed as percentage of control with the initial exposure to a pH 7.4 (Mg2+/ATP-free) solution. In this series of experiments, we analyzed data in the following manner. Patch current was measured each second throughout the protocol that consisted of the following steps: 1) obtaining a control value in Mg2+-free solutions at pH 7.4; 2) switching to another perfusion lane in which the same basic solution was flowing, which was titrated to a lower pH (6.6); and 3) upon obtaining a maximal response to acidic pH, the patch was returned to the control perfusion, and recovery from inhibition was monitored. The currents measured were compared with those obtained in the control period, expressed as percentage of control, and plotted as shown in Fig. 5. For the experiment shown in Fig. 5, the rate of response to acidification could be fit to a
single exponential with a time constant \((t_{\text{inact}})\) of 18.9 s. A similar response was observed in 11 other experiments, and an average time constant \((t_{\text{inact}})\) of 18.6 ± 4.4 s \((n = 12)\) was calculated. Recovery from acidification could also be fit to a single exponential and for the example given in Fig. 5, the time constant \((t_{\text{act}})\) was 7.3 s with an average value of 7.3 ± 1.6 s \((n = 11)\). There was no significant difference between \(t_{\text{inact}}\) and \(t_{\text{act}}\) (paired Student’s t-test).

Acidification does not affect current amplitude. Previous studies have shown the response to pH at a macroscopic level \((8, 25, 28)\), therefore an effect on channel open probability versus conductance cannot be distinguished. Cytosolic acidification has been shown to induce a decrease in single channel current amplitude in cardiac myocyte \((5, 12, 13)\) and skeletal muscle \(K_{\text{ATP}}\) channels \((1, 6, 26, 29)\). We assessed whether single channel current amplitude was affected by changing intracellular pH. With the exception of infrequently observed subconductance states, there was no alteration in current amplitude at a single holding potential over a wide range of pH values (Table 1).

**Effect of acidification on single channel kinetics.** We assessed the effect of manipulation of cytosolic pH on single channel kinetics. Figure 6 shows the mean open and closed times for ROMK2 at three different pH values. At pH 7.4, ROMK2 has a high single channel open probability \((0.93 ± 0.01, n = 25)\) exhibited by a long mean open time \((t_{o} = 30.2 \text{ ms})\) and markedly shorter closed time \((t_{c} = 1.2 \text{ ms})\). Similar values were obtained for pH 7.8 \((t_{o} = 32.7 \text{ ms}; t_{c} = 1.2 \text{ ms})\). During exposure to pH 7.0 solutions, \(t_{o} = 29.9 \text{ ms}\) and \(t_{c} = 1.2 \text{ ms}\), not different from values obtained for pH 7.4. The low \(P_{o}\) obtained in acidic solutions is derived from a prolonged closed state, which is demonstrated in Fig. 7.

**Table 1.** Average current amplitudes for ROMK2 at different pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>Amplitude, pA</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.8</td>
<td>2.09 ± 0.02</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>7.4</td>
<td>2.11 ± 0.03</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>7.2</td>
<td>2.15 ± 0.03</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>7.0</td>
<td>2.11 ± 0.02</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>6.8</td>
<td>2.19 ± 0.07</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>6.6</td>
<td>2.18 ± 0.02</td>
<td>6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\) = no. of experiments. Note: \(-V_{p} = -50 \text{ mV}\). Statistical comparison made against values obtained at pH 7.4 for each pH shown. NS, no significant difference.
When ROMK2 was exposed to pH 6.6, random prolonged closed states are initially seen (τ_c1 = 310 ms); however, between these stretches of inactivation, τ_c3 (1.1 ms) remain close to the pH 7.4 values. After several seconds, the channel enters into a prolonged closed state. In general, we restored control solutions within 10–15 s of observing this final closure, as prolonging this inactive state lessens the ability to recover channel activity. We routinely calculate NP_o for pH 6.6 as the apparent maximal level of inhibition. The mean NP_o calculated for ROMK2 at pH 6.6 (0.050 ± 0.025) may underestimate the "true" NP_o, because we routinely switch back to the control (pH 7.4) solution as soon as any prolonged level of inhibition is achieved.

Effect of acidification on channel activity in the presence of ATP. Alteration of cytosolic pH modulates the effect of ATP inhibition of K_ATP channels in several tissues (1, 5, 6, 10, 13, 14, 23, 29, 30). To investigate the effect of cytoplasmic acidification on nucleotide-mediated inhibition of ROMK2 channel activity, we exposed membrane patches to a range of Mg-ATP-containing solutions at two different pH values. PKA was added to the bath solution to minimize rundown during these experiments (22). The relation between channel activity and Mg-ATP concentration at pH 7.2 is shown in Fig. 8. We also show on the graph the data previously obtained for Mg-ATP-dependent inhibition of ROMK2 channel activity at pH 7.4 (22). We initially recorded the baseline "control" channel activity in Mg^2+/ATP-free solutions and then changed the bath solution to pH 7.2. This maneuver resulted in a decrease in NP_o/N from 0.94 ± 0.021 (n = 6) to 0.70 ± 0.08 (n = 9). Application of 0.5 mM Mg-ATP (pH 7.2) resulted in a significant decrease (P < 0.05) in channel activity to NP_o/N = 0.41 ± 0.10 (n = 11). This is in contrast to the virtual absence of effect of Mg-ATP concentrations below 1.0 mM at pH 7.4 (see dashed line in Fig. 8). To verify that the results obtained at pH 7.2 compared with previous results at pH 7.4 were not due to differences in oocytes or other conditions, we performed additional experiments at pH 7.4. We confirmed that channel activity is maintained at pH 7.4 in the presence of 0.5 mM Mg-ATP by performing five additional experiments. These data points were added to the 10 previous observations (22) and plotted in Fig. 8. There was no significant difference between the previous values compared with the new data; thus 0.5 mM Mg-ATP had no significant inhibitory effect on ROMK2 channel activity at pH 7.4. We were unable to obtain data below 0.5 mM Mg-ATP, because of channel rundown. Increasing Mg-ATP above 1.0 mM resulted in a further attenuation of channel activity. We have shown that ATP activates ROMK because of PKA-dependent phosphorylation events (21, 22). This suggests that the complex inhibitory effects shown in Fig. 8 may result from the interaction of Mg-ATP-dependent phosphorylation-activation and Mg-ATP-dependent inhibition processes.

To assess whether Mg-ATP affected proton-mediated inhibition of ROMK2, the experiments shown in Fig. 9 were performed. These results are compared with those shown in Fig. 2A, where that curve is reproduced as a dashed line in Fig. 9. The effect of cytoplasmic pH was determined at a constant concentration of Mg-ATP of 0.5 mM, since this concentration of ATP significantly inhibited ROMK2 channel activity at pH 7.2 (Fig. 8). Unlike in ATP-free solutions, these data were not well fit to the Hill equation; however, we estimated the half-maximal inhibitory concentration to be between 7.2–7.3. Thus the apparent K_i is shifted ~0.2 pH units compared with that obtained at zero Mg-ATP (pK = 7.04, Fig. 2A).
In this series of experiments, we were unable to obtain patch current was recovered after ATP was washed off. Patches), respectively. In this instance, 91.6% of the patches) with a recovery to 99.5% of control after ATP.

The rapid activation of channel activity (Fig. 10, A) is apparent other than infrequent, brief bursts of channel openings (Fig. 10, B). This is in contrast to another mutation within the Walker A domain of the cloned renal $\mathrm{K}^+$ channel (ROMK) at the half-maximal inhibitory pH of $\sim 7.4$. For wild-type ROMK2 (ROMK2-WT), there was no change in single channel current amplitude for a given holding potential at various pH values (Fig. 10B). For comparison, the pH effects on wild-type ROMK2 activity are reproduced from Fig. 2A (dashed line in Fig. 11). It is evident that mutation of His206 shifts the pH activity curve to the right. This is in contrast to another mutation within the Walker region, G204D, which had no significant effect on pH sensitivity (pK = 7.2, data not shown).

**DISCUSSION**

We examined the effect of intracellular pH on two isoforms of the cloned renal $\mathrm{K}^+$ channel (ROMK) at the single channel level and examined the relationship between pH- and ATP-binding inhibition on channel activity. Cytosolic pH reduced ROMK channel activity
in a concentration-dependent manner as described previously by others (4, 7, 8, 25, 28). Acidification reduced both ROMK1 and ROMK2 channel activity in the absence of Mg-ATP, and this inhibition was completely reversible on return to control pH (Figs. 1–5). It is unlikely that this pH-dependent inhibition of ROMK channel activity is due to rundown, since we have previously shown (21, 22) that recovery from rundown requires PKA-dependent phosphorylation in the presence of Mg-ATP. Moreover, we demonstrate that recovery from acidification-induced channel inhibition is reversible in Mg-ATP-free solutions (Figs. 3–5).

No differences were seen in the pK for pH-mediated inhibition of ROMK1 and ROMK2, indicating that differences in the amino termini apparently do not affect the general characteristics of pH inhibition of ROMK channels. The kinetics of pH-mediated inhibition of ROMK channel activity do not appear to be due to alterations in open probability ($P_o$), at least over the range of pH 7.8–7.0 (Fig. 6); rather, channels close. More specifically, as shown in Fig. 6, while the channels remain open there is no change in kinetic parameters during a burst of activity, and alteration in $P_o$ occurs following channel closure. According to Figs. 1 and 2, ~60–80% of the inhibition of ROMK channel activity occurs over pH values from 7.4 to 7.0, and thus, over this range, the reduction in channel activity is due primarily to reduction in the number of active channels, i.e., complete channel closure. Occasionally, we observed a subconductance state of channel activity, most often preceding the final channel closure. This is the only incident of a decrease in current amplitude. Below pH 7.0, low channel activity appears to be a combination of both reduction in the number of open channels together with a modest effect on open probability (Fig. 7). These results are similar to those reported by Choe et al. (4), where decreases in intracellular pH inhibited channel activity by inducing very long closures while leaving the basic kinetic properties and the current amplitude of the still open channel essentially unchanged. Moreover, it is apparent from inspection of Fig. 7 that prior to the final closure of individual channels, open times and closed times remain similar to channels exposed to pH 7.4. In both the present study as well as in experiments reported in Ref. 4, prolonged exposure to acidic solutions prevented recovery of channel activity upon return to pH 7.4.

We examined the time course of response and recovery to a reduction in bath pH (Fig. 12). The best fit to these data suggests a single binding-site model, with equal time constants for inactivation and activation, a pH-sensitive (proton) “on rate” ($k_{on}$), and a pH-insensitive (proton) “off rate” ($k_{off}$). Experimental data in Fig. 12 are shown as a dashed line, and the solid line is the time course for response and recovery predicted from the following

\[ n \xrightarrow{k_{on}} nH \]

\[ nH(t) = nH_0 e^{-k_{off}[H] + k_{on}K} \]

where $nH$ represents the fraction of channels with one proton bound. Proton association is described by

\[ k_{on} = \frac{k_{on0}}{1 + [H]^m} \]

and proton dissociation is described by

\[ k_{off} = \frac{k_{off0}}{1 + [H]^m} \]

Fig. 11. Effect of pH on ROMK2 Walker-site mutant, R2H206G. Dose-response relationship of R2H206G channel activity to variation in intracellular H\(^+\) concentration in Mg\(^2\)/ATP-free solutions. Data are generated from 6 separate experiments in which NPo/N was measured at $-V_p = -50$ mV. Data are presented as NPo/N and are means ± SE. Dashed line is a reproduction of the effect of pH on wild-type ROMK2 (WT) taken from Fig. 2A.

Fig. 12. Time course and binding-site model for ROMK2. A: time course of the response of ROMK2 to a decrease in bath pH to 7.0. Experiments were performed in Mg\(^2\)/ATP-free solutions from an inside-out patch containing 12 channels. Experimental data are shown as a dashed line, and the solid curve is the time course for response and recovery as predicted from the equations in the DISCUSSION. B: time course data reproduced from Fig. 5 from a patch containing at least 27 channels, where the solid curve is the prediction from the same model at a pH of 6.6.
Proton dissociation is described by
\[ nH(t) = nH_\infty + \left[ 1 - e^{-(k_{on}[H]+k_{off})t} \right] \]
where \( t \) = time (s), \( k_{on} = 0.01 \text{ s}^{-1} \), and \( k_{off} = 150,000 \text{ s}^{-1}.\text{M}^{-1} \), and
\[ nH_\infty = \frac{k_{on}[H]}{k_{on}[H]+k_{off}} \]
At this point our data do not justify a more complex model. Although our pH response time course data are best fit using a single binding-site model, the Hill coefficient for pH-induced reduction in channel activity shown in Figs. 2 and 3 is greater than 3, suggesting cooperative binding sites for multiple protons. Moreover, from our time course experiments, we cannot rule out that ROMK has more than one proton-binding site. For example, one could hypothesize that the channel protein has multiple binding sites exhibiting different time constants. Assuming that one site has faster time constant than the other, the response to pH could be fit with a single exponential. This, however, does not exclude other cooperative proton binding sites, reflected in a Hill coefficient greater than one. One possibility would be a simple proton binding to each of the ROMK channel in a tetrameric model. Clearly, further studies will be required to assess the precise number of protons required to bind to ROMK to induce channel inhibition. The macroscopic currents recorded with the two-electrode voltage clamp (TEVC) technique also exhibited a steep dependence on intracellular pH (4). However, in these experiments (4), the apparent \( pK_a \) was 0.5 pH units more acidic than in the present study. Furthermore, the Hill coefficient for the TEVC currents was close to 5, in contrast to the value of 3 reported here for excised patches.

We had previously shown that His^{206} in the Walker site in the carboxy terminus of ROMK was involved in Mg-ATP-binding inhibition of ROMK channel activity (22). The persistence of pH-mediated reduction in channel activity, as shown in Figs. 10 and 11, suggests that this His^{206} is not critical for pH-sensing; however, mutation of this residue to Gly markedly shifts the curve of pH sensitivity to the right. Furthermore, our results demonstrate that the pH-dependent enhanced sensitivity to ATP is partially retained in ROMK2K61M (4), an otherwise pH-insensitive mutant. These data indicate that the pH-dependent modulation of ROMK is complex and may not be entirely attributable to a single residue (8). Clearly, further studies beyond the scope of this study will be required to assess whether titration of His^{206} in the Walker A site is involved in this process. Of note, another pH modulatory site on ROMK2 has also been described at location T51, where the charge at this site causes a shift in the pH sensitivity of the channel (4).

The results presented in this study indicate quite clearly that 1) pH modulates ROMK channel activity, and that 2) reductions in internal pH markedly alter channel response to Mg-ATP. A similar enhanced ATP sensitivity of the low-conductance ATP-sensitive K^+ channel of the CCD was described previously (30). Furthermore, decreasing pH enhanced the ATP sensitivity of K^+ transport by pancreatic secretory granule membrane (10). This may provide a mechanism during metabolic compromise in which lower concentrations of ATP would continue to inhibit ROMK channel activity. During periods of metabolic compromise, the reduction in ATP could lead to an increase in channel activity and promote K^+ leak from cells; however, reduction in cytosolic pH would permit the reduced ATP to inhibit this K^+ leak pathway. This could be of significant advantage to the thick ascending limb of Henle’s loop where inhibition of this K^+ channel would abolish sodium chloride transport and consequently reduce the oxygen and metabolic requirement for this cell. A similar mechanism may be operative in the principal cell in the CCD.

We thank Drs. H. Sackin, M. Koettgen, and W.-H. Wang for discussion and Dr. L. G. Palmer for generously providing the ROMK2 mutant, R2K61M.

This work was supported by grants from the National Kidney Foundation (to C. M. McNicholas) and by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-09432 (to Y. Yang), DK-17433 (to G. Giebisch), and DK-37605 (to S. C. Hebert).

Address for reprint requests: G. Giebisch, Dept. of Cellular and Molecular Physiology, Yale Univ. School of Medicine, 333 Cedar St., New Haven, CT 06520-8026.

Received 11 September 1997; accepted in final form 3 September 1998.

REFERENCES