Modulation of the Ca^{2+} -Activated Large Conductance K^+ Channel by Intracellular pH in Human Renal Proximal Tubule Cells

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Abstract: The Ca^{2+} -activated and voltage-sensitive large conductance K^+ channel (BK channel) with a slope conductance of about 300 pS is present in the surface membrane of cultured human renal proximal tubule epithelial cells (RPTECs). In this study we examined the effects of cytoplasmic pH (pH_i) on activity and gating kinetics of the BK channel by using the inside-out configuration of the patch-clamp technique. At a constant cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), membrane depolarization raised channel open probability (P_o), and lowering pH_i shifted the P_o –membrane potential (V_m) relationship to the positive voltage direction. However, the value of the gating charge was not affected by changes in pH_i , suggesting that the effects of pH_i on P_o were

not due to an alternation of the voltage sensitivity. At constant V_m , lowering pH_i suppressed the $[\text{Ca}^{2+}]_i$ -dependent channel activation and shifted the P_o – $[\text{Ca}^{2+}]_i$ relationship in the direction of higher $[\text{Ca}^{2+}]_i$ with a reduction of maximal P_o . Furthermore, both the mean open and mean closed times of the BK channels at pH_i 6.3 in the presence of 10^{-4} M $[\text{Ca}^{2+}]_i$ were shorter than those at pH_i 7.3 in the presence of 10^{-5} M $[\text{Ca}^{2+}]_i$, even though these two different conditions gave a similar P_o . The data indicate that cytoplasmic H^+ suppresses P_o of the BK channel in RPTECs, which involves the mechanism independent of Ca^{2+} activation. Our preliminary kinetic analysis also supported this notion. [Japanese Journal of Physiology, 52, 267–276, 2002]

Key words: kidney, intracellular pH, channel activity, gating kinetics.

The properties of many Ca^{2+} -activated and voltage-sensitive large conductance (big) K^+ channels (BK channels) have been reported in the cell membranes of a variety of tissues [1, 2]. An elevation of cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and depolarization of the membrane commonly stimulates activity of the BK channels [1–3]. It has already been proved by the use of cloned BK channels that the channel protein possesses the Ca^{2+} -binding sites [4, 5] and the voltage-sensing domain [6, 7]. Besides Ca^{2+} and voltage, other factors such as ATP [8–11], pH [8, 9, 12–21], and Mg^{2+} [19, 22–24] were also reported to modulate BK channels. Among these factors, intracellular pH (pH_i) is one of the significant factors that affect channel activity [8, 9, 12–21], i.e., channel activity is in-

hibited by intracellular acidification and stimulated by its alkalization. Although the mechanism for pH_i effects on channel activity was not precisely understood, two different mechanisms have been proposed. One is that an increased cytoplasmic H^+ can compete with Ca^{2+} in binding to the same sites, thereby inhibiting channel activity [14–16], whereas the other is that the pH_i -mediated change in channel activity is not competitive with Ca^{2+} [17]. Moreover, it was demonstrated that the conductance of some BK channels was reduced by lowering pH_i and enhanced by raising it [9, 15, 20].

In the renal tubules, BK channels have been reported in cultured rabbit proximal tubule cells [25], cultured rabbit medullary thick ascending limb [14,

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26], rabbit connecting tubule [27], and rat cortical collecting ducts [8, 28]. Furthermore, we recently demonstrated the presence of a BK channel with ATP-sensitivity in cultured human renal proximal tubule epithelial cells (RPTECs) [29]. Among these channels, pH_i -sensitive activity was shown in rabbit medullary thick ascending limb [14] and rat cortical collecting ducts [8]. However, pH_i -sensitivity of the BK channel in human renal tubules has not been reported. In the present study, we investigated the effect of pH_i on the BK channel in RPTECs by using the patch-clamp technique, and found that its activity was also pH_i -sensitive. Here demonstrate the characteristics and the possible mechanism for the pH_i -sensitivity of this BK channel with analyses of gating kinetics.

MATERIALS AND METHODS

Cell culture. Secondary cultures of RPTECs from normal human kidneys were purchased from Clonetics (Walkersville, MD, USA). RPTECs were incubated in renal epithelial cell growth medium (REGM) (Clonetics) as reported previously [29]. In the experiments, cells were isolated from the 70 to 80% confluency at passages 3–6 with 0.025% trypsin and 0.01% EDTA and incubated on collagen-coated cover glasses (Iwaki, Tokyo, Japan) with REGM for 3 to 10 h before use. The cover glass to which the cells adhered was then transferred into the control bath solution in a heating chamber (TC-324B, Warner, Hamden, CT, USA). All experiments were performed at approximately 30°C with the heating chamber.

Solutions. The pipette solution for inside-out patches contained (in mM) 145 KCl; 3 MgCl_2 ; 1 ethylene glycol-bis(β -amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA); and 5 N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES), pH adjusted to 7.3. The control bath solution contained (in mM) 140 NaCl; 5 KCl; 1 MgCl_2 ; 1 CaCl_2 ; 5 glucose; and 5 HEPES, pH adjusted to 7.3. Experimental bath solutions contained (in mM) 145 KCl; 1 MgCl_2 ; and 5 HEPES with 10^{-6} – 10^{-3} M free Ca^{2+} . The solutions with 10^{-5} – 10^{-3} M free Ca^{2+} concentration were prepared by adding CaCl_2 without EGTA, and the solution with 10^{-6} M Ca^{2+} was made by adding an adequate amount of CaCl_2 and EGTA (10 mM), as reported previously [29]. Experimental bath solutions with pH adjusted to 6.3, 6.8, 7.3, and 7.8 were made by adding HCl or KOH. The free Ca^{2+} concentration and pH were checked simultaneously with a Ca^{2+} -electrode (CL-125B, TOA, Tokyo, Japan) and a pH-electrode (GST-5311C, TOA), respectively, at approxi-

mately 30°C.

Patch-clamp technique and data analyses.

Experiments were carried out with the patch-clamp technique applied to single RPTECs, and channel recordings were performed in excised inside-out patches. Patch-clamp electrodes were made of borosilicate glass capillaries (Warner, Hamden, CT, USA) on a two-step puller (Model PP-830, Narishige, Japan), and filled with the pipette solution described above. The electrode resistance ranged from 4 to 6 M Ω . Electric currents measured with a patch-clamp amplifier (AXOPATCH 200B, Axon, Foster City, CA, USA) were stored on a DAT recorder (RD-120TE, TEAC, Tokyo, Japan). The current records were then low-pass filtered (3611 Multifunction Filter, NF, Tokyo, Japan) at 1 to 3 kHz and analyzed by means of the pCLAMP8 program system (Axon). Values for the potential represent the voltages of the membrane potential (V_m). Currents flowing from the cytoplasmic to the extracellular side were defined as outward and shown as upward deflections in figures.

Channel activity was determined by the mean channel open probability (P_o), which was calculated as

$$P_o = (1/N) \cdot \sum_{n=1}^N (n \cdot t_n) \quad (1)$$

where N is the number of maximally activated channels with $[\text{Ca}^{2+}]_i$ of 10^{-3} M. t_n ($t_1, t_2, \dots, t_N$) is the fractional time spent at each current level.

P_o and V_m data obtained from inside-out patches at each $[\text{Ca}^{2+}]_i$ were fitted by the Boltzmann curve as described below, using a least-squares regression analysis [30].

$$P_o = P_{\max} \cdot (1 + \exp[\alpha F(V_{1/2} - V_m)/RT])^{-1} \quad (2)$$

where F , R , and T have their usual meaning, $P_{\max}$ is the maximal P_o , V_m is the membrane potential tested, $V_{1/2}$ is the membrane potential at which P_o is half-maximal, and α is the equivalent number of charges moving across the transmembrane potential during an open–close transition (the gating charge or Boltzmann constant).

The P_o and $[\text{Ca}^{2+}]_i$ data at constant V_m obtained from inside-out patches were best fitted by the Hill equation described below.

$$P_o = P_{\max} \cdot [1 + (K_{d1}/[\text{Ca}^{2+}]_i)^{N_1}] \quad (3)$$

where K_{d1} and N_1 are the dissociation constant and the Hill coefficient for Ca^{2+} , respectively.

The linearized relationships between the data of P_o and $[\text{Ca}^{2+}]_i$ or pH_i were best fitted by the linearized Hill equation described below.

$$\log[P_o \cdot (P_{\max} - P_o)] = N_n \cdot X - N_n \cdot \log K_{dn} \quad (4)$$

where X is $\log[Ca^{2+}]_i$ or pH_i . K_{dn} and N_n are the dissociation constant and the Hill coefficient for Ca^{2+} (K_{d2} and N_2) and H^+ (K_{d3} and N_3), respectively.

Single channel recordings for more than 60 s were used for the analysis of dwell time distributions. Open and closed time histograms were best fitted with the Simplex least-squares fitting method by the pCLAMP8 program system (Axon).

Analysis of channel gating. For analyzing the open and closed dwell-time data, we developed a gating model (Fig. 8) with a simplified general two-tiered gating kinetic model described by Rothberg and Magleby [31, 32]. At constant V_m , the original two-tiered kinetic model involved five states in open and closed tiers [31, 32]. We reduced the states in the model to two open and four closed states by omitting those with very low occupancy probability and/or very short mean lifetime in the experimental condition used for the kinetic analysis (Fig. 7).

For estimating the rate constants, we employed an iterative simulation method similar to that in Magleby and Weiss [33]. In brief, the simulation program generated an artificial single channel current record according to the proposed gating scheme. The simulated currents were then filtered, sampled, and binned into the dwell-time histograms in a manner identical to that used to analyze the experimental currents. Therefore any effects of filtering and sampling (such as missed events) are essentially identical for the experimental and simulated data, and they thus cancel out. The most likely rate constants in the proposed gating scheme were determined by a comparison of experimental and simulated distributions. For our purpose, we manually set the values of some rate constants with reference to the values reported by Rothberg and Magleby [31, 32] and taking other (P_o and $P_{\max}$) data into account.

Statistics. The data are expressed as means $\pm$ SE. Statistical significance was determined by using the Student's t -test or ANOVA in conjunction with the Tukey t -test [34]. A p value of less than 0.05 was considered significant.

RESULTS

Effects of pH_i on current-voltage (I - V) relationships of the BK channel in RPTECs

Figure 1A shows actual current recordings of a BK channel in RPTECs in response to step voltage in various pH_i conditions at constant $[Ca^{2+}]_i$ (10^{-4} M). It is apparent that P_o of the channel is higher in positive

voltage than in the negative one at every pH_i condition. Furthermore, P_o in the acid pH_i (6.8) was lower and P_o in the alkaline pH_i (7.8) was higher than that in the control pH_i (7.3), especially in the conditions at negative voltage. However, there is no appreciable difference in current amplitude at constant V_m , even when pH_i was altered. The I - V relationships in the conditions at constant $[Ca^{2+}]_i$ (10^{-4} M) with pH_i 6.3, 6.8, 7.3, and 7.8 are shown in Fig. 1B. From these relationships, the values of channel conductance (pS) at pH_i 6.3 (closed circle), 6.8 (closed square), 7.3 (closed triangle), and 7.8 (closed diamond) were 291.7 ± 11.7 ($n=4$), 296.9 ± 10.3 ($n=4$), 300.8 ± 10.0 ($n=4$), and 303.3 ± 12.5 ($n=4$), respectively. Varying pH_i induced no significant change in channel conductance of this BK channel at the range we used.

Effects of pH_i on P_o - V_m relationships in various $[Ca^{2+}]_i$

As mentioned above, P_o of the BK channel in RPTECs was sensitive to pH_i . Thus in the next we examined the effects of varying pH_i on P_o - V_m relationships in the conditions with various $[Ca^{2+}]_i$. Figure 2 represents the P_o - V_m relationships at pH_i of 6.3 (closed circle), 6.8 (closed square), 7.3 (closed triangle), and 7.8 (closed diamond) in conditions with $[Ca^{2+}]_i$ of 10^{-5} M (A), 10^{-4} M (B), and 10^{-3} M (C). The P_o data were best fitted by the Boltzmann equation (Eq. 2). Cytoplasmic acidification shifted P_o - V_m relationships toward the positive direction in each $[Ca^{2+}]_i$ condition. These data indicate that cytoplasmic acidification suppresses P_o of the BK channel. Although we tested the effects of pH_i on P_o - V_m relationships in the presence of 10^{-6} M $[Ca^{2+}]_i$, P_o obtained in this $[Ca^{2+}]_i$ was too low to analyze the data in acid conditions. Thus we did not represent these data in Fig. 2.

The voltage of half-maximum activation ($V_{1/2}$) obtained from individual relationships represented in Fig. 2 are shown in Fig. 3A. Lowering pH_i increased $V_{1/2}$ in individual $[Ca^{2+}]_i$ levels (10^{-5} M: open circle; 10^{-4} M: open square; and 10^{-3} M: open triangle), which amounts to about 27.6 mV per pH unit (range, 18.0–35.5 mV). Figure 3B shows the values of the gating charge (α) obtained from individual relationships represented in Fig. 2. The values of α in response to changing pH_i from 6.3 to 7.8 at 10^{-5} M $[Ca^{2+}]_i$ (open circle) ranged from 1.05 to 1.24, at 10^{-4} M $[Ca^{2+}]_i$ (open square) from 1.08 to 1.53, and at 10^{-3} M $[Ca^{2+}]_i$ (open triangle) from 0.76 to 1.00. From these data, α was not significantly altered by changes in pH_i at constant $[Ca^{2+}]_i$, suggesting that pH_i -mediated changes in P_o of the BK channel were

Fig. 1. A: Single channel recordings of a BK channel with various pH_i in an inside-out patch. The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl containing 10⁻⁴ M [Ca²⁺]_i with three different pH_i represented atop the figure. The membrane potential (V_m) in mV is indicated atop each trace. The current levels of the channel are indicated by short bars (left), and closed channel states are indicated by dotted lines and C. **B: Current-voltage (I-V) relationships of the BK channel with various pH_i in inside-out patches.** The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl containing 10⁻⁴ M [Ca²⁺]_i with four different pH_i (●, 6.3; ■, 6.8; ▲, 7.3; ◆, 7.8; *n*=4). The curves through the mean values were drawn by hand.

not affected by voltage. Furthermore, the values of α in individual [Ca²⁺]_i at each pH_i were not varied, which is similar to our previous report [29].

Effects of pH_i on $P_{\max}$ at constant V_m

Figure 4A represents the P_o -[Ca²⁺]_i relationships fitted by Eq. 3 in pH_i of 6.3 (closed circle), 6.8 (closed square), 7.3 (closed triangle), and 7.8 (closed diamond) at V_m of -30 mV. This setting of V_m showed the marked difference of P_o induced by changes in [Ca²⁺]_i and pH_i. The [Ca²⁺]_i-dependency of P_o is sigmoidal, reaching a maximal value ($P_{\max}$) as [Ca²⁺]_i is increased. Note that $P_{\max}$ is pH_i-dependent; that is, the acidification moderately decreases $P_{\max}$ (Fig. 4B). H⁺ inhibited the channel activity under the condition that Ca²⁺ binding should be saturated (a full occupation), suggesting that H⁺ might also affect the channel activity via a mechanism independent of Ca²⁺ activation.

Linearized relationships between P_o and [Ca²⁺]_i or pH_i at constant V_m

The linearized Hill plots between P_o and [Ca²⁺]_i were best fitted by Eq. 4 with logarithmic scales in both ordinate and abscissa, as shown in Fig. 5A. These data were obtained from mean values of P_o at constant V_m of -30 mV in Fig. 4A. The values of K_{d2} in pH_i 6.3 (closed circle), 6.8 (closed square), 7.3 (closed triangle), and 7.8 (closed diamond) were 10^{-4.11}, 10^{-4.46}, 10^{-4.80}, and 10^{-5.03}, respectively. These data indicate that a lowering of the pH_i increases the dissociation constant for Ca²⁺. However, the Hill coefficient (N_2), which represents the lower limit of the number of Ca²⁺-binding involved in channel activation, was not modified by pH_i in the range between 6.3 and 7.8 (N_2 =1.34 at pH_i 6.3, N_2 =1.27 at pH_i 6.8, N_2 =1.30 at pH_i 7.3, and N_2 =1.59 at pH_i 7.8). Therefore it is suggested that a lowering of the pH_i

Fig. 2. Effects of pH_i on P_o - V_m relationships of the BK channel in inside-out patches. The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl containing 10^{-5} M $[\text{Ca}^{2+}]_i$ (A), 10^{-4} M $[\text{Ca}^{2+}]_i$ (B), and 10^{-3} M $[\text{Ca}^{2+}]_i$ (C) with four different pH_i (●, 6.3; ■, 6.8; ▲, 7.3; ◆, 7.8; $n \geq 4$). The lines were fitted by Eq. 2.

shifted the P_o - $[\text{Ca}^{2+}]_i$ relationship in the right direction without changing the number of Ca^{2+} ions participating in the channel opening.

With the same data adopted in Fig. 5A, Hill plots between P_o and pH_i were represented in Fig. 5B, in which the data were also fitted by Eq. 4. The values of $\log K_{d3}$ in the presence of 10^{-5} (open circle), 10^{-4} (open square), and 10^{-3} M (open triangle) $[\text{Ca}^{2+}]_i$ were 7.66, 6.20, and 5.12, respectively. N_3 , which represents the lower limit of the number of H^+ ions involved in channel closing, in the presence of 10^{-5} ,

Fig. 3. Effects of pH_i on voltage-sensitivity of the BK channel in inside-out patches. The plots of $V_{1/2}$ (A) and α (B) against pH_i were obtained from individual relationships represented in Fig. 2. The data were determined by the use of bath solutions with three different $[\text{Ca}^{2+}]_i$ (○, 10^{-5} M; □, 10^{-4} M; △, 10^{-3} M).

Fig. 4. A: Relationships between P_o and $[\text{Ca}^{2+}]_i$ at V_m of -30 mV in various pH_i . The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl with four different pH_i (●, 6.3; ■, 6.8; ▲, 7.3; ◆, 7.8; $n \geq 4$). The lines were best fitted by Eq. 3. B: Effects of pH_i on $P_{\max}$ at V_m of -30 mV. The plots of $P_{\max}$ against pH_i were obtained from individual relationships represented in A.

Fig. 5. A: Linearized Hill plots between P_o and $[Ca^{2+}]_i$ at V_m of -30 mV. The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl with four different pH_i (●, 6.3; ■, 6.8; ▲, 7.3; ◆, 7.8; $n \geq 4$). The lines were best fitted by Eq. 4. **B: Linearized Hill plots between P_o and pH_i at V_m of -30 mV.** The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl containing three different $[Ca^{2+}]_i$ (○, 10^{-5} M; □, 10^{-4} M; △, 10^{-3} M). The lines were fitted by Eq. 4 with the use of P_{max} obtained from A.

10^{-4} , and 10^{-3} M $[Ca^{2+}]_i$ was 0.83, 0.98, and 1.15, respectively. Since the values of N_3 obtained in individual $[Ca^{2+}]_i$ conditions are close to one, a minimum one H^+ would be involved in the closing reaction of the BK channel.

Effects of pH_i and $[Ca^{2+}]_i$ on mean dwell time of the BK channels

We characterized H^+ -mediated channel gating by using the mean dwell time of single BK channels. Figure 6 shows P_o (closed square) and mean open (open circle) and closed times (closed circle) of single channels ($n=4$) at pH_i 7.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ (control), at pH_i 6.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ (low pH group), and at pH_i 7.3 in the presence of 10^{-5} M $[Ca^{2+}]_i$ (low Ca^{2+} group). The data were obtained from channel recordings filtered at 3 kHz and digitized at 10 kHz with V_m held at -15 mV. The values of P_o in the control, the low pH, and the low Ca^{2+} groups were 0.89 ± 0.05 , 0.69 ± 0.28 , and 0.67 ± 0.34 , respectively. Note that P_o in the low pH group was almost the same as in the low Ca^{2+} group. The mean

Fig. 6. Effects of lowering pH_i or $[Ca^{2+}]_i$ on P_o and the mean dwell time of the BK channels in inside-out patches. P_o (■), mean open time (○), and mean closed time (●) were obtained from single channel recordings at V_m of -15 mV ($n=4$) in various $[Ca^{2+}]_i$ and pH_i indicated in the abscissa. The pipette solution contained 145 mM KCl. The bath solutions used were 145 mM KCl with indicated $[Ca^{2+}]_i$ and pH_i .

open times (ms) in the control, the low pH, and the low Ca^{2+} groups were 4.28 ± 0.87 , 3.27 ± 0.93 , and 3.87 ± 1.37 , whereas the mean closed times (ms) were 2.35 ± 1.30 , 3.42 ± 2.01 , and 4.86 ± 3.39 , respectively. These data suggest that lowering pH_i from 7.3 to 6.3 at constant $[Ca^{2+}]_i$ suppresses P_o by shortening the mean open time and extending the mean closed time, whereas lowering $[Ca^{2+}]_i$ from 10^{-4} to 10^{-5} M at constant pH_i suppresses P_o by mainly extending the mean closed time. Therefore it is suggested that the mechanism for channel inhibition induced by lowering pH_i would be different from that by lowering $[Ca^{2+}]_i$.

Effects of pH_i and $[Ca^{2+}]_i$ on gating kinetics of the BK channels

We examined transition rates in a presumptive gating scheme for the BK channel to indicate the effects of H^+ on gating kinetics. Figure 7 shows open (left panels) and closed time histograms (right panels) of a single channel at pH_i 7.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ (control: A), at pH_i 6.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ (low pH: B), and at pH_i 7.3 in the presence of 10^{-5} M $[Ca^{2+}]_i$ (low Ca^{2+} : C). These histograms were obtained from channel recordings filtered at 1 kHz and digitized at 5 kHz with V_m held at -15 mV. The open and closed times were plotted on the abscissa in a logarithmic scale, and the square root

Fig. 7. Effects of lowering pH_i or $[Ca^{2+}]_i$ on dwell-time histograms of the BK channel in an inside-out patch. The dwell-time histograms were obtained from single channel recordings at pH_i 7.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ (A), at pH_i 6.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ (B), and at pH_i 7.3 in the presence of 10^{-5} M $[Ca^{2+}]_i$ (C), with V_m held at -15 mV. The pipette and bath solutions both contained 145 mM KCl. The open (left panel of A, B, and C) and closed times (right panel of A, B, and C) were plotted on the abscissa in a logarithmic scale, and the square root values of the number of observations were plotted on the ordinate. Each histogram was fitted by the Simplex least-squares fitting method (see text for details).

values of the number of observations were plotted on the ordinate. The open and closed time histograms were best fitted by the sum of one and three exponentials using the Simplex least-squares fitting method. Table 1 shows the values of P_o and the time constants of open (τ_o) and closed (τ_c) time distributions represented in Fig. 7. The one showing the most remarkable change by lowering pH_i from 7.3 to 6.3 was shortening τ_o , whereas lowering $[Ca^{2+}]_i$ from 10^{-4} to 10^{-5} M mainly extended τ_{c1} and τ_{c2} .

Next, by applying the gating model (Fig. 8) to the dwell-time distribution data (Fig. 7), we estimated the rate constants under each condition. The estimated values of rate constants and the open and closed time distributions reproduced by the gating model were represented in Table 2. Our preliminary kinetic analysis showed that H^+ and Ca^{2+} affected the rate constants in specific ways. Lowering $[Ca^{2+}]_i$ from 10^{-4} to 10^{-5} M decreased k_{O12} , k_{C21} , and k_{C32} , whereas the de-

Table 1. Values of P_o and time constants of open (τ_o) and closed (τ_c) time distributions of a BK channel shown in Fig. 7.

pH_i $[Ca^{2+}]_i$ (M)	7.3 10^{-4}	6.3 10^{-4}	7.3 10^{-5}
P_o	0.84	0.73	0.69
τ_o (ms)	9.1	6.6	8.2
τ_{c1} (ms)	1.2 (0.87)	1.4 (0.83)	2.0 (0.83)
τ_{c2} (ms)	7.8 (0.12)	7.5 (0.16)	16.3 (0.16)
τ_{c3} (ms)	99.0 (0.007)	69.0 (0.009)	110.0 (0.016)

The proportion of each component of closed time distributions were represented in parentheses under each value of time constant. The data were obtained at V_m of -15 mV and filtered at 1 kHz and digitized at 5 kHz.

Fig. 8. Schematic representation of a gating model of the BK channel.

Table 2. Values of rate constants (s^{-1}) between kinetic states in the kinetic model (Fig. 8), and the kinetic parameters (P_o , τ_o and τ_c) reproduced with 1 kHz filtered and 5 kHz digitized.

pH _i [Ca ²⁺] _i (M)	7.3 10 ⁻⁴	6.3 10 ⁻⁴	7.3 10 ⁻⁵
k_{O12}	2,000	500	1,200
k_{O21}	120	120	120
α_1	400	440	400
β_1	200	200	200
α_2	120	130	120
β_2	2,000	2,000	2,000
k_{C12}	2,000	2,000	2,000
k_{C21}	2,000	1,800	800
k_{C23}	150	150	150
k_{C32}	150	150	90
k_{C34}	9	9	9
k_{C43}	11	11	11
P_o	0.77	0.63	0.54
τ_o (ms)	9.1	6.4	8.2
τ_{C1} (ms)	1.1 (0.87)	1.2 (0.84)	1.7 (0.76)
τ_{C2} (ms)	7.4 (0.12)	7.4 (0.15)	13.3 (0.21)
τ_{C3} (ms)	99.0 (0.007)	96.0 (0.009)	110.0 (0.027)

crease of k_{C43} was not significant (Table 2). Note that these transitions were Ca²⁺-dependent steps in the original two-tiered kinetic model [31]. This is consistent with many previous reports that the transitions between closed (C–C) and open (O–O) states were Ca²⁺-dependent. On the other hand, lowering pH_i from 7.3 to 6.3 induced marked reduction in k_{O12} and a slight reduction only in k_{C21} among the closed-state transitions. These results suggested that H⁺ shortened the mean open time by preventing transition from the shorter open state O₁ (mean lifetime: ~ 0.4 ms) to the longer open state O₂ (mean lifetime: ~ 4 ms), whereas it also elongated the mean lifetime of O₁ to ~ 1.1 ms, from ~ 0.4 ms. Thus H⁺ and Ca²⁺ affected the different points of the gating model in different ways. How-

ever, we could not easily conclude that the channel involved an H⁺-specific regulatory mechanism independent of Ca²⁺ activation because the affinity of H⁺ to the H⁺ site might be different in each state, as Laurido *et al.* [17] concluded.

DISCUSSION

pH_i-sensitivity of the BK channels has been reported in several tissues, including renal tubule cells such as rabbit medullary thick ascending limb [14] and rat cortical collecting duct cells [8]. Namely, lowering pH_i suppressed channel activity, and raising pH_i enhanced it. However, mechanisms for the pH_i-sensitivity of BK channels proposed in the previous papers were varied in individual tissues. In this study we demonstrated that the BK channel in RPTECs was also pH_i-sensitive. Furthermore, we attempted to explore the mechanism of pH_i-sensitivity of this BK channel with kinetic analysis and to characterize the pH_i-mediated channel gating.

It had been shown in some reports that lowering pH_i reduced the conductance of BK channels and that raising pH_i enhanced it besides the pH_i effect on channel activity [9, 15, 20]. However, changes in pH_i induced no significant effect on the conductance of the BK channels in RPTECs (Fig. 1B), suggesting that a cytoplasmic H⁺ dose not interfere with K⁺ conduction through the pore at the pH range we used (between pH 6.3 and 7.8). These results are consistent with other previous papers [12, 14, 16–19, 21]. The difference of the pH_i effects on channel conductance may be due to the molecular variant in individual BK channels.

Lowering pH_i shifted the P_o – V_m relationship toward the right direction (Fig. 2) and $V_{1/2}$ toward more positive values, which amounts to about 27.6 mV per pH unit (Fig. 3A) at constant [Ca²⁺]_i. This value is similar to those obtained from BK channels in rat skeletal muscle (about 23.5 mV per pH unit [17]) and in the epithelium of choroid plexus (about 30 mV per pH unit [13]). Thus P_o of the BK channel in RPTECs is regulated by pH_i in addition to V_m and Ca²⁺. Changes in pH_i, however, have no significant effect on values of the gating charge of the BK channel (α , about 1.0), as shown in Fig. 3B. This suggests that the inhibitory effect of pH_i on P_o is independent of voltage-sensitivity of the channel, which is similar to BK channels in several animals [13, 17, 21].

The effect of changes in pH_i on the Ca²⁺-sensitivity of BK channels was reported in various tissues [14–18, 21]. Regarding this mechanism, several investigators have proposed that Ca²⁺ and H⁺ could compete for binding to the Ca²⁺-binding sites [14–16, 18].

In contrast, Laurido *et al.* [17] have revealed that H^+ inhibition in BK channels in rat skeletal muscle is well explained by a kinetics model in which H^+ and Ca^{2+} are assumed to be noncompetitive. In this study we examined the changes in mean dwell times in response to lowering pH_i or $[Ca^{2+}]_i$ to clarify whether the H^+ -induced channel inhibition resulted from the competitive inhibition for Ca^{2+} . As the results show in Fig. 6, both mean open and mean closed times of the BK channels at pH_i 6.3 in the presence of 10^{-4} M $[Ca^{2+}]_i$ were shorter than those at pH_i 7.3 in the presence of 10^{-5} M $[Ca^{2+}]_i$, even though these two different conditions gave the similar P_o . These results indicate that the kinetics of channel inhibition induced by lowering pH_i would be different from that induced by simply lowering $[Ca^{2+}]_i$, which is consistent with the notion concluded by Laurido *et al.* [17]. Furthermore, the Hill coefficient for H^+ -mediated changes in the BK channel was close to 1.0 (Fig. 5B). Taken together, it is strongly suggested that H^+ might inhibit the BK channel by affecting at least one site distinct from the Ca^{2+} -binding sites in the BK channel in RPTECs.

The precise mechanism of pH_i -sensitivity in the BK channel is still unclear. Laurido *et al.* [17] proposed a mechanism that H^+ bound to an allosteric site and inhibited the channel activity by reducing the affinity of the Ca^{2+} binding site. On the other hand, a recent report using mSlo3, a member of the BK channel family sensitive to pH_i rather than to Ca^{2+} , provided evidence that the pH_i -sensitivity of this channel is independent of Ca^{2+} -sensitivity [35]. Furthermore, it has also been demonstrated in a cloned pH_i -sensitive K^+ channel, K_{ir} 1.1 (ROMK), that an intracellular lysine close to the M1 segment, composing the pore region, has been identified as the structural element necessary for pH_i -dependent gating [36, 37]. These data suggest a possibility that pH_i -sensitive K^+ channels usually have the specific pH_i -sensitive site in the channel protein. Although the mechanism of pH_i -sensitivity in the BK channel of RPTECs is still unknown, our conclusion that H^+ regulates the activity of this BK channel by binding to the site distinct from Ca^{2+} sites is supported by findings in the above recent reports [35–37]. It is quite likely that H^+ inhibited the channel activity mainly by the allosteric reduction of the Ca^{2+} binding affinity. However, it is also worthy to note our novel finding that the cytoplasmic acidification moderately decreased P_{max} in the BK channel of RPTECs. This suggested a possibility that H^+ affected the channel activity partially via a mechanism independent of Ca^{2+} activation, and that the point affected by H^+ might be the open–closed equilibrium.

In the previous paper we have demonstrated that the BK channel in RPTECs requires high $[Ca^{2+}]_i$ to open, and the channel opening is prevented by cytosolic ATP [29]. Furthermore, the present study has demonstrated that lowering pH_i inhibits channel activity and raising pH_i enhances it. Despite the several regulatory factors mentioned above, the BK channel current is not observed in cell-attached patches under normal conditions, since normal $[Ca^{2+}]_i$ is too low to open this channel. The channel may open and participate in maintaining membrane potential when the cells fall into some pathological conditions with an elevation of $[Ca^{2+}]_i$ and a lack of cytosolic ATP. In these conditions, pH_i would affect the membrane potential maintained with the BK channels, though the physiological role of the pH -sensitivity of this channel has not been determined.

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