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Modulation of Inwardly Rectifying K^+ Channel by Intracellular and Extracellular pH in Bovine Aortic Endothelial Cells

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The effects of intracellular and extracellular pH on the inwardly rectifying K^+ (IRK) channel of the bovine aortic endothelial cells (BAECs) were examined using whole-cell patch-clamp technique. The IRK current, efficiently blocked by Ba^{2+} (200 μM), is the most prominent membrane current in BAECs, which mainly determines the resting membrane potential. The expression of Kir2.1 was observed in BAECs using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Intracellular alkalinization, elicited by the extracellular substitution of NaCl with NH_4Cl (30 mM), significantly augmented the amplitude of IRK current. On the contrary, the amplitude of IRK current was attenuated by the Na-acetate (30 mM)-induced intracellular acidification. The changes in extracellular pH also closely modulated the amplitude of IRK current, which was decreased to 40.2±1.3% of control upon switching the extracellular pH to 4.0 from 7.4. The extracellular pH value for half-maximal inhibition (pK) of IRK current was 5.11. These results demonstrate that the activity of IRK channel in BAECs, probably Kir2.1, was suppressed by proton at both sides of plasma membrane.

Key Words: Inwardly rectifying K^+ channel, Intracellular pH, Extracellular pH

INTRODUCTION

Ionic currents and membrane potential in vascular endothelial cells (ECs) have been postulated to play an important role in the modulation of intracellular calcium and the calcium-dependent release of vasoactive substances including NO (Adams et al, 1989). The major determinant of the resting membrane potential in endothelial cells is a basal K^+ conductance. However, the expression of K^+ channels varies greatly between different EC types and even within the same strain of cultured EC (Kamouchi et al, 1997; Nilius et al, 1997).

The inwardly rectifying K^+ (IRK) channel, especially Kir2 subfamily, is known as the most important channel for the resting membrane potential in macrovascular endothelial cells (Kamouchi et al, 1997). It conducts inward currents at potentials more negative than the K^+ equilibrium potential but permits much smaller currents at potentials positive to that potential. The conductance of single endothelial Kir channels ranges from 23 and 30 pS in symmetrical K^+ solution (Elam & Lansman, 1995; Kamouchi et al, 1997), and the typical features of this channel are the increase in single channel conductance with the square root of the extracellular K^+ concentration (Nilius & Riemann, 1990; Zunkler et al, 1995). Extracellular Ba^{2+}, tetraethylammonium (TEA), tetrabutylammonium (TBA) and Cs^+ all block the Kir2 channels (Voets et al, 1996; von Beckerath et al, 1996).

The Kir2 subfamily has four members, referred to as Kir2.1, Kir2.2, Kir2.3 and Kir2.4 with single channel conductances of 23, 34, 13 and 15 pS, respectively (Kubo et al, 1993; Makhina et al, 1994; Topert et al, 1998). Activity of these K^+ channels is controlled by several intracellular and extracellular factors. One of these factors is the hydrogen ion (Coulter et al, 1995). Among the members of Kir2 subfamily, Kir2.1 is insensitive to changes in the pH, whereas Kir2.3 is inhibited at acidic extracellular pH (Coulter et al, 1995; Fakler & Ruppersberg, 1996; Kamouchi et al, 1997). In Kir2.3 channel, two distinct pH sensors are located on the both sides of the plasma membrane, respectively. With these sensors, Kir2.3 can detect pH changes in both the intra- and extracellular environment (Zhu et al, 1999).

According to the previous reports, the major subtype of IRK in macrovascular endothelial cells is Kir2.1 (Forsyth et al, 1997; Kamouchi et al, 1997). This channel is a strongly inwardly rectifying channel, and has two membrane spanning region and highly conserved TIGYG-H5 motif in the pore region. The M84 site was reported to confer the pH insensitivity of this channel as described above. In this study, however, we observed that the IRK channels in bovine aortic endothelial cells, which is likely

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ABBREVIATIONS: IRK channel, inwardly rectifying K^+ channel; BAECs, bovine aortic endothelial cells; RT-PCR, reverse transcriptase-polymerase chain reaction; NO, nitric oxide; ECs, endothelial cells.

to be Kir2.1, was suppressed either by extracellular or intracellular acidification. Furthermore, intracellular alkalinization augmented the amplitude of IRK current, which implies that IRK channel in BAECs is regulated by the intracellular proton under the physiological conditions.

METHODS

Cell culture

Endothelial cells were isolated from bovine aorta as decribed previously (Park et al, 2002). Briefly, the luminal side of the bovine aorta was subjected to 0.05% collagenase digestion for 10 min. Harvested cells by repeated gentle pipetting were washed twice by centrifugation at 100×g for 5 min in minimal essential medium (MEM) with 10% newborn calf serum, 100 U/ml penicillin and 2 mM glutamine. BAECs were serially passaged after treatment with 0.05% trypsin, and were used in this study between passage 5 and 15.

RT-PCR analysis

Total RNA from BAECs was prepared using a modified guanidium thiocyanate-phenol-chloroform extraction method. Random hexamer-primed complementary DNA (cDNA) synthesis was performed in a final volume of 25 μl, containing 20 units of RNAse inhibitor (Promega, WI, USA) and 200 units of murine leukemia virus reverse transcriptase (Promega) at 37°C for 60 min. Then, PCR amplification of bovine Kir2.1 channel gene was performed in a volume of 50 μl, containing 0.25 units of Taq polymerase (Perkin-Elmer, CT, USA) and 10 pmol of sequence specific oligonucleotide primer pair. Sequence-specific primers for Kir2.1 gene was forward 5'-TTC GTG AAG AAG GAC GGA CA-3' and reverse 5'-TGA TGA AGG CGT CGA TGA TG-3' (388 bp product). The PCR conditions for Kir2.1 gene included an initial denaturing for 5 min, and then 30 cycles as follows: denaturing for 30 sec at 94°C, annealing for 30 sec at 56°C and extending for 1 min at 72°C using GeneAmp (Perkin-Elmer). Amplification of 28S RNA was performed at 24 cycles at the same annealing temperature using sequence-specific primer; forward 5'-TTA AGG TAG CCA AAT GCC TCG-3' and reverse 5'-CCT TGG CTG TGG TTT CGC T-3' (84 bp product). Aliquots of PCR reactions were loaded onto a 1.1% agarose gel containing ethidium bromide, electrophoresed, visualized and exposed to Polaroid film.

Electrophysiological recordings

The modified whole-cell patch clamp was performed using an EPC-9 amplifier (Heka-Electronics, Germany) at room temperature (20~22°C). The membrane potential and ionic currents of BAECs were measured in current-clamp and voltage-clamp mode, respectively. Patch pipettes were fabricated on micropipette puller (PP-83, Narishige, Japan) from borosilicate glass capillary (GC150T-7.5, Warner Instrument Co., CT, USA) and were fire-polished with microforge (MF-83, Narishige) prior to use. When filled with pipette solution, the resistance of these pipettes ranged from 3 to 5 MΩ. Membrane potential and ionic currents were filtered at 3 kHz, and digitized at 1~3 kHz using EPC-9 amplifier and computer software (Pulse v8.40, Heka-Electronics). In most experiments to measure the ionic currents, we used a 'ramp' protocol, which was repeated every 15 sec from a holding potential of 0 mV, and consisted of a 10 ms step to −150 mV followed by a 500 msec linear voltage ramp to +100 mV. In some experiments, we recorded the resting currents at a holding potential of −100 mV.

Measurements of intracellular pH (pH_i)

The intracellular pH (pH_i) was measured using the fluorescent probe, BCECF/AM. The BAECs grown on poly-L-lysine-coated coverslips were incubated with 5 μM BCECF/AM at room temperature for 30 min and then washed twice with standard external solution. The coverslips were attached to the bottom of a perfusion chamber superfused with external solution and alternately excited at 440 and 495 nm using optical filter controller (Lambda 10-2, Sutter Instruments, CA, USA). The fluorescent signal was detected at 530 nm by an intensified charge-coupled device camera that was connected to a personal computer for image acquisition and analysis (Metafluor 4.5, Universal Imaging corp., PA, USA). To obtain a calibration' external and internal H^+ concentration were equilibrated at varying extracellular pH values by the equimolar (140 mM) K^+ solution with 10 μM nigericin.

Solutions and chemicals

The standard external solution contained (in mM); 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, 10 glucose and pH 7.4 with NaOH. To induce the intracellular acidification or alkalinization, equimolar NaCl was substituted with 30 mM sodium acetate and ammonium chloride. The pipette solution contained (mM); 40 KCl, 100 potassium aspartate, 1 $MgCl_2$, 0.5 EGTA, 4 Na_2ATP, 10 HEPES and pH 7.2 with KOH. All materials used were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except BCECF/AM from Molecular Probes (Eugene, OR, USA).

Statistical analysis

All average data are presented as the mean±SEM and n is the number of cells tested. Statistical significance was evaluated by paired or unpaired Student's t-test. The difference between two groups was considered to be significant at $p<0.05$. The pH value for half-maximal inhibition (pK) of IRK current was calculated by fitting the concentration-response curves to the logistic function using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Ba^{2+}-sensitive inwardly rectifying K^+ current

In whole-cell voltage clamp recording, BAEC showed strong inwardly rectifying current elicited by hyperpolarizing voltage steps. At more hyperpolarized pulses than 100 mV, the activation and inactivation kinetics of inward current became faster producing "criss-crossing pattern" by the voltage step pulse protocol (Fig. 1A). Most of BAECs had resting membrane potentials of around −60

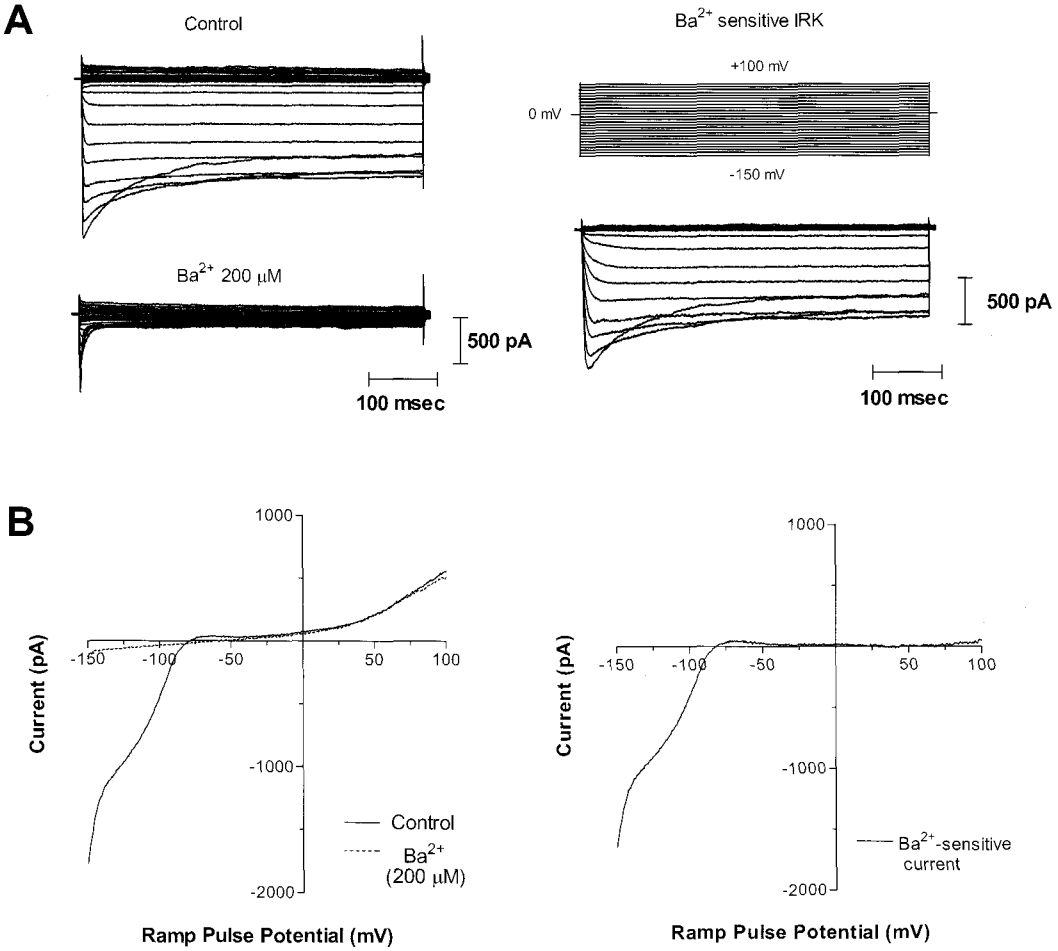

Fig. 1. Ba^{2+}-sensitive inwardly rectifier K^+ (IRK) currents in bovine aortic endothelial cell (BAEC). (A) Current traces from BAEC before and after application of 200 μM Ba^{2+} recorded in response to voltage step pulses from -150 mV to $+100$ mV in 10 mV interval at holding potential of 0 mV. Right traces shows Ba^{2+}-sensitive component determined by subtracting the currents in the presence of Ba^{2+} from controls. (B) Current-voltage relationships measured during the linear ramp pulses from -150 mV to $+100$ mV for 500 msec. The difference current (Ba^{2+}-sensitive current; right traces) was obtained from the subtraction of left traces.

mV (-60.3 ± 6.8 mV; n=32), which was consistent with the report on human capillary endothelial cells (Jow & Numann, 1998). Application of Ba^{2+} (200 μM) to the outside of the cell completely eliminated the inwardly rectifying current except early component of inward current (Fig. 1A). During ramp pulse protocol, Ba^{2+} also selectively block the inward current, and Ba^{2+} sensitive currents were reversed near -80 mV, close to the K^+ equilibrium potential (Fig. 1B). Ba^{2+} did not significantly affect the outward current component, which was known as Cl^- and nonselective cationic currents (Voets et al, 1996).

For the candidate of Ba^{2+}-sensitive IRK channel in BAEC, we observed the expression of Kir2.1 channel using RT-PCR analysis. As shown in Fig. 2, PCR product (388 bp) of Kir2.1 channel gene was amplified only with preceding reverse transcription.

Effects of intracellular proton on Ba^{2+}-sensitive IRK currents

In order to investigate the action of cytosolic proton

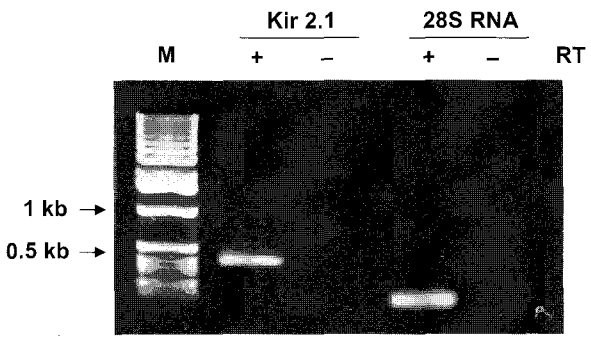

Fig. 2. RT-PCR analysis of Kir2.1 expression in bovine aortic endothelial cell (BAEC). Total RNAs isolated from BAEC were amplified by PCR with (+) or without (–) preceding reverse transcription (RT). Used primer pairs were sequence-specific to bovine Kir2.1 channel and 28S RNA. The resultant PCP products were separated and visualized on a 1.1% agarose gel containing ethidium bromide. M; DNA size marker.

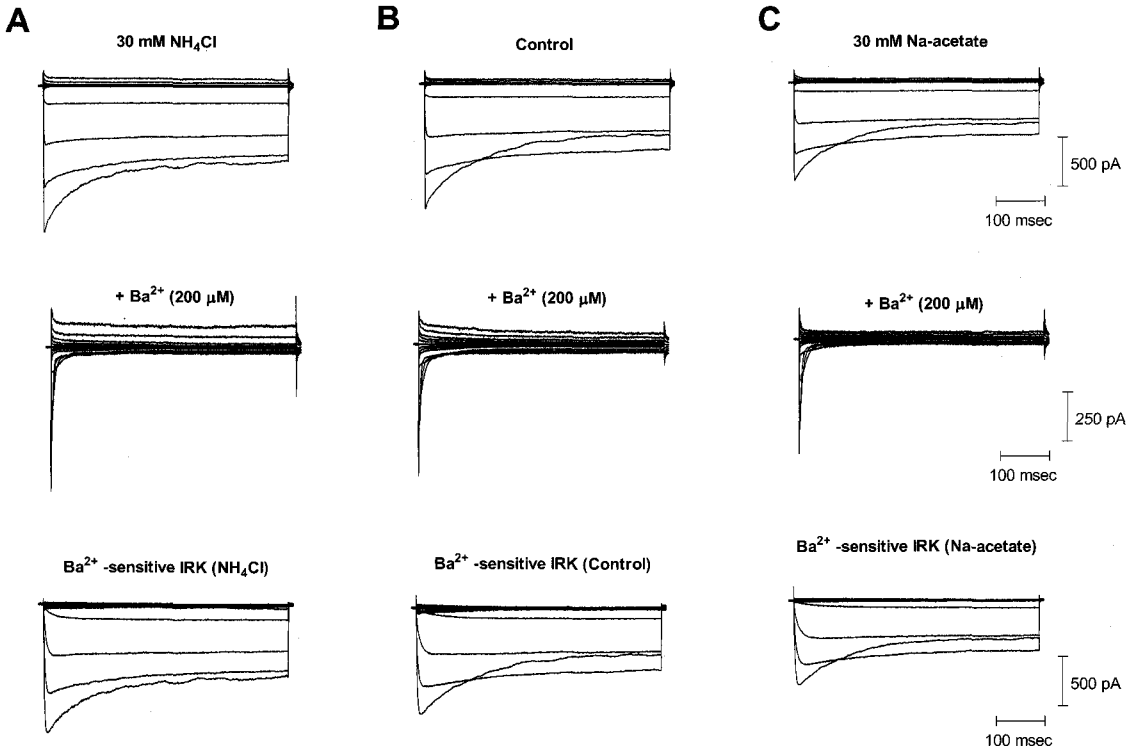

Fig. 3. Alterations in intracellular pH modulate the Ba^{2+}-sensitive currents in bovine aortic endothelial cell (BAEC). A Representative current traces of modulating Ba^{2+}-sensitive currents in BAEC by the changes in intracellular pH recorded during voltage step pulses from −140 mV to +100 mV in 20 mV interval held at 0 mV. Compared to the control (B), intracellular alkalinization with 30 mM NH_4Cl (A) augmented and intracellular acidification with 30 mM sodium acetate (C) inhibited the Ba^{2+}-sensitive inwardly rectifying K^+ current.

concentration on IRK currents we changed the intracellular pH by the extracellular application of ammonium chloride or sodium acetate. Ammonium chloride can absorb intracellular protons to form NH_4^+ after entering the cytosol as NH_3, which increase intracellular pH from 7.07±0.07 to 7.61±0.09 (Fig. 4B). On the other hand, sodium acetate can go into the cytosol as COOH form and release H^+, which will decrease intracellular pH (to 6.53±0.04). Compared to the control, reduced intracellular proton concentration by ammonium chloride augmented the amplitude of Ba^{2+}-sensitive IRK currents (Fig. 3A). Conversely, intracellular acidification by sodium acetate attenuated the amplitude of IRK currents (Fig. 3C). At the ramp pulse protocol, ammonium chloride increased the Ba^{2+}-sensitive IRK currents by 21.3±2.2% (calculated at −130 mV), while sodium acetate decreased the IRK current by 21.6±0.5% (Fig. 4D).

Effects of extracellular proton on Ba^{2+}-sensitive IRK currents

We also observed whether proton in extracellular side could affect Ba^{2+}-sensitive IRK currents in BAECs. As shown in Fig. 5A, IRK currents were inhibited by the perfusion with acidic extracellular solution in a pH-dependent manner. The amplitude of IRK currents at −130 mV was decreased to 40.2±1.3% of control upon switching the extracellular pH to 4.0 from 7.4. The pH value for half-maximal inhibition (pK) of IRK current was 5.11. However, the perfusion with alkaline extracellular solution slightly increase the amplitude of IRK current, which was smaller than the results of intracellular alkalinization.

DISCUSSION

In the present study, we investigated the regulatory actions of intracellular and extracellular proton on Ba^{2+}-sensitive IRK currents as a major determinant of the resting membrane potential in BAECs. Our results prove that the activity of IRK channel, probably Kir2.1, was inhibited by proton at both sides of plasma membrane.

Like other second messengers, proton is an important regulator of cellular functions, such as ion channel activities. A number of ion channels are modulated by protons; these include N-methyl-D-aspartate receptors (Traynelis & Cull-Candy, 1990), large conductance Ca^{2+} activated K^+ channels (Wellner-Kientz et al, 1998) and IRK channels. Among the various subtypes of IRK channels, Kir1.1 (Doi et al, 1996) and Kir2.3 (Coulter et al, 1995; Zhu et al, 1999) have been well known to be inhibited by intra- and extracellular proton. Especially in Kir2.3 channels, molecular structures enabling pH sensitivity have been already identified using the chimeric and mutant channels (Coulter et al, 1995; Qu et al, 1999).

In contrast to Kir2.3, Kir2.1 known as dominant IRK in endothelium has been reported as pH insensitive channel (Doi et al, 1996; Qu et al, 1999). Instead, this channel was

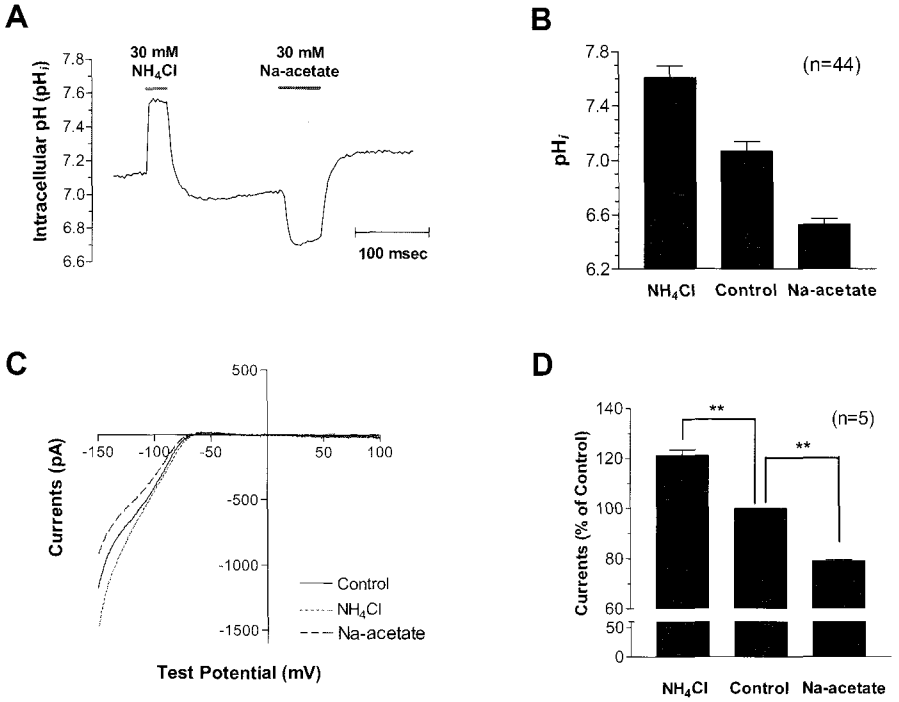

Fig. 4. The amplitude of Ba^{2+}-sensitive currents are dependent on the intracellular pH in bovine aortic endothelial cell (BAEC). The substitution with NH_4Cl or sodium acetate in external solution induces intracellular alkalinization or acidification (A and B), which was measured using intracellular pH-sensitive fluorescent probe, BCECF/AM. The amplitude of Ba^{2+}-sensitive currents recorded in response to the linear ramp pulses were increased by 30 mM ammonium chloride and decreased by 30 mM sodium acetate (C). The degree of increase or decrease was calculated from the current amplitude at −130 mV (D). **; $P<0.01$.

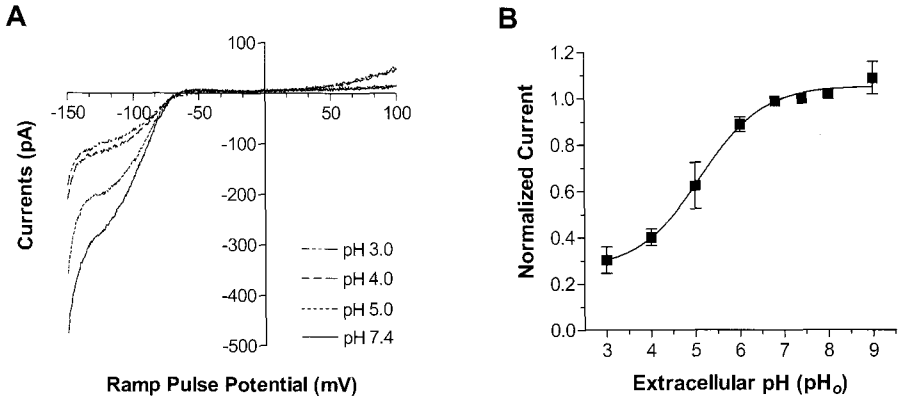

Fig. 5. Alterations in extracellular pH also modulate the inwardly rectifying K^+ (IRK) currentsin BAECs. (A) The amplitude of IRK currents recorded using linear ramp pulse protocol were reduced by the perfusion with acidic extracellular solution. (B) The concentration-response relationship for current inhibition in response to the changes in extracellular pH is shown. The pH value for half-maximal inhibition (pK) was 5.11.

suggested to be modulated by endogenous G-protein-linked phosphatase (Kamouchi et al, 1997). However, according to the results of this study, changes in intra- and extracellular pH affected the amplitude of Ba^{2+}-sensitive IRK currents, even though the sensitivity to extracellular pH changes was lower than Kir2.3 channels. In expressed Kir2.3 channels, the half-maximal inhibition was achieved at extracellular pH of 6.7 (Zhu et al, 1999), which is less acidic value than that obtained from this study (pKa 5.11).

We identified the expression of Kir2.1 channels in BAECs using RT-PCR analysis. Since nucleotide sequence data of bovine IRK channels was not available except Kir2.1, we did not exclude the possibility about the existence of other type of IRK channels in BAEC. For confirming the domi-

nance of the Kir2.1 channel in BAEC, it will be helpful to analyze the conductance values using single channel experiments. If the measured currents in this study were mainly Kir2.1 currents, it was an interesting and unpredicted data that the channel activity was regulated by the relatively small changes in intracellular pH. Especially, increase in intracellular pH augmented the amplitude of IRK current, which means that channel was finely regulated by not only intracellular acidification but also alkalinization. Unfortunately, when we measured the alterations of IRK currents by the application of ammonium chloride or sodium acetate using whole cell voltage clamp experiments, the real changes in intracellular pH would be different in some degree from the fluorometrically measured values due to the buffering effects of pipette solution. To clarify the precise values of intracellular pH for half-maximal inhibition, data from single channel recording with inside-out mode will be needed.

Membrane potential has been recognized as a major determinant of Ca^{2+} entry and consequently release of various vasoactive substances (Wakabayashi & Groschner, 1997). Ca^{2+} influx activated by store depletion after bradykinin stimulation was found to be inhibited by the depolarization with high extracellular K^+ solution (Schilling et al, 1989; Laskey et al, 1990). Therefore, the modulation of IRK current by intracellular pH can be an influencing factor for the Ca^{2+} influx and cellular functions in BAECs. Further studies are required to identify the extensive effects of intra- or extracellular pH changes on biological functions and proliferation of endothelial cells in relation with cellular signaling.

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