

Protons Block BK Channels by Competitive Inhibition with K^+ and Contribute to the Limits of Unitary Currents at High Voltages

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ABSTRACT Proton block of unitary currents through BK channels was investigated with single-channel recording. Increasing intracellular proton concentration decreased unitary current amplitudes with an apparent pK_a of 5.1 without discrete blocking events, indicating fast proton block. Unitary currents recorded at pH_i 8.0 and 9.0 had the same amplitudes, indicating that 10^{-8} M H^+ had little blocking effect. Increasing H^+ by recording at pH_i 7.0, 6.0, and 5.0 then reduced the unitary currents by 13%, 25%, and 53%, respectively, at +200 mV. Increasing K^+_i relieved the proton block in a manner consistent with competitive inhibition of K^+_i action by H^+_i . Proton block was voltage dependent, increasing with depolarization, indicating that block was coupled to the electric field of the membrane. Proton block was not described by the Woodhull equation for noncompetitive voltage-dependent block, but was described by an equation for cooperative competitive inhibition that included voltage-dependent block from the Woodhull equation. Proton block was still present after replacing the eight negative charges in the ring of charge at the entrance to the intracellular vestibule by uncharged amino acids. Thus, the ring of charge is not the site of proton block or of competitive inhibition of K^+_i action by H^+_i . With 150 mM symmetrical KCl, unitary current amplitudes increased with depolarization, reaching 66 pA at +350 mV (pH_i 7.0). The increase in amplitude with voltage became sublinear for voltages >100 mV. The sublinearity was unaffected by removing from the intracellular solutions Ca^{2+} and Ba^{2+} ions, the Ca^{2+} buffers EGTA and HEDTA, the pH buffer TES, or by replacing Cl^- with $MeSO_3^-$. Proton block accounted for ~40% of the sublinearity at +200 mV and pH 7.0, indicating that factors in addition to proton block contribute to the sublinearity of the unitary currents through BK channels.

KEY WORDS: Ca^{2+} -activated K^+ channels • ring of charge • conductance • fast block • proton block

INTRODUCTION

Protons decrease single-channel (unitary) current amplitudes through a variety of ion channels, including Na^+ (Daumas and Andersen, 1993; Benitah et al., 1997), Ca^{2+} (Prod'hom et al., 1987; Tytgat et al., 1990; Klockner and Isenberg, 1994; Chen et al., 1996), CNG (Root and MacKinnon, 1993; Gordon et al., 1996; Morrill and MacKinnon, 1999), and K^+ channels (Davies et al., 1992; Coulter et al., 1995; Lopes et al., 2000; Xu et al., 2000; Geiger et al., 2002; Nimigeon et al., 2003). For Na^+ channels, proton block increases with depolarization, suggesting that the site of proton block is within the transmembrane electric field (Woodhull, 1973; Daumas and Andersen, 1993; Hille, 2001; Geiger et al., 2002). For L-type Ca^{2+} channels, CNG channels, and Kir1.1 potassium channels the sites of proton action or binding are formed by negatively charged glutamate residues (Chen et al., 1996; Morrill and MacKinnon, 1999; Xu et al., 2000). BK channels also have their unitary conductance reduced by protons (Laurido et al.,

1991; Habartova et al., 1994), and have glutamate residues that control the magnitude of K^+ current (Brelidze et al., 2003b; Nimigeon et al., 2003), which might form a site for proton block. Here we investigate in detail the effect of protons on the amplitudes of unitary currents through BK channels and examine whether the glutamate residues that form a ring of negative charge at the intracellular vestibule are the site of proton block.

Some of this material has appeared in abstract form (Brelidze et al., 2003a).

MATERIALS AND METHODS

Expression of BK Channels in Oocytes and Mutagenesis

The constructs encoding wild-type BK channels (mSlo1) used in these experiments were gifts from Dr. Lawrence Salkoff (mbr5; Schreiber et al., 1999) and Merck research laboratories (Pallanck and Ganetzky, 1994) as modified by Merck (McManus et al., 1995). BK channels expressed from these two clones had the same unitary currents.

Xenopus laevis oocytes were separated by enzymatic treatment as described (Dahl, 1992; Hsiao et al., 2001). The cRNA was transcribed using mMessage mMachine kit (Ambion) and injected in *Xenopus laevis* oocytes at ~0.5–2 ng per oocyte, 2–8 d before recording. After injection, oocytes were kept at 14–16°C in modified OR2 solution: (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1

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mM MgCl₂, 1 mM Na₂HPO₄, 50 mg/l penicillin/streptomycin (Sigma-Aldrich), 50 mg/l gentamicin (Gibco), 4.8 mM HEPES, pH 7.5). The vitelline layer of injected oocytes was manually removed before patch clamp recording.

The mutant cDNA construct with the double mutation E321N and E324N was made using the Quickchange XL site-directed mutagenesis kit (Stratagene) and checked by sequencing (DNA Core Lab Sequencing Facility, University of Miami School of Medicine), as described previously (Brelidze et al., 2003b).

Solutions

Unless indicated, the extracellular (pipette) and intracellular solutions contained (mM): 150 KCl, 5 N-tris[Hydroxymethyl]-methyl-2-aminoethane-sulfonic acid (TES) to buffer pH (adjusted to pH 7.0), and 1 ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetra-acetic acid (EGTA) and 1 N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid (HEDTA) to bind Ca²⁺ to prevent possible Ca²⁺ block from contaminating Ca²⁺ (Ferguson, 1991). The intracellular solution also typically contained 50 μM (+)-(18-crown-6)-2,3,11,12-tetra-carboxylic acid (crown ether) to bind Ba²⁺ to prevent Ba²⁺ block of the channel (Vergara and Latorre, 1983; Diaz et al., 1996). The extracellular (pipette) solution also typically contained 60 μM GdCl₃ to block endogenous mechano-sensitive channels (Yang and Sachs, 1989). The concentration of intracellular K⁺ ([K⁺]_i) was varied as indicated by changing the concentration of KCl. Extracellular [K⁺] was kept constant at 150 mM in all experiments unless indicated. TES was used as a pH buffer. The optimal pH range for buffering by TES is from 6.8 to 8.2, but weaker buffering extends the range from ~6.0 to 9.0. In the experiments where the intracellular pH (pH_i) was changed from 5.0 to 9.0, the pH_i was found to remain sufficiently stable to carry out the experiments. To further test for proper buffering of pH, in a few experiments where indicated 5 mM TES was increased to 10 mM, and 10 mM propionic acid, 10 mM 2-[N-Morpholino]ethanesulfonic acid (MES), and 10 mM N-tris[Hydroxymethyl]methyl-4-aminobutane-sulfonic acid (TABS) were also added. The pH of the solutions was adjusted by adding KOH and HCl to avoid adding Na⁺. In a few experiments where indicated 5 mM 1,2-bis(σ-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), which is less sensitive to pH than EGTA and HEDTA (Tsien, 1980), was added to act as a Ca²⁺ chelator. At pH 5.0, 5 mM BAPTA plus the 1 mM EGTA and 1 mM HEDTA would reduce the expected contaminant Ca²⁺ of 5–10 μM in the solution to a free Ca²⁺ of 0.02–0.04 μM (calculated with MAXC, www.stanford.edu/~cpatton/maxc.html; Bers et al., 1994). EGTA and HEDTA were from Fluka, propionic acid, KCl, HCl, and KOH from Fisher; TES, MES, TABS, GdCl₃, and crown ether from Sigma-Aldrich; and BAPTA from Molecular Probes. Solu-

tions were changed using valve-controlled, gravity-fed perfusion of a microchamber (Barrett et al., 1982).

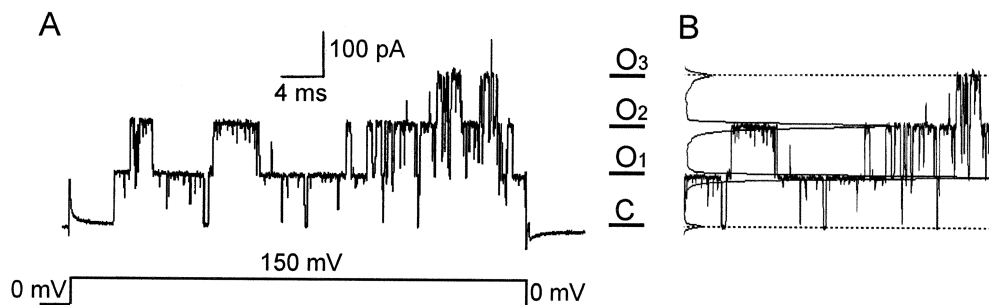
Single-channel Recording and Data Analysis

Single-channel currents were recorded from BK channels expressed in oocytes using the inside-out configuration of the patch-clamp technique (Hamill et al., 1981). Patch pipettes were prepared from borosilicate glass tubing (Warner Instruments). Pipettes were heat polished to obtain resistances of 10–20 MΩ when filled with 150 mM KCl solution. Data were acquired with an Axopatch 200A amplifier, sampled every 3 μs using a Digidata 1200A and pClamp7 software (Axon Instruments, Inc.), and stored and analyzed on a Pentium III computer.

Membrane potentials from –200 to +200 mV were generated by computer through the application of voltages to the external command input on the Axopatch 200A. Voltages less than –200 mV and greater than +200 mV were obtained by application of the computer applied voltages together with a steady-state holding potential preset on the front panel of the Axopatch 200A. For instance to achieve +250 mV, +50 mV was applied through the holding potential on the Axopatch 200A and then a brief (40–100 ms) +200-mV pulse was applied through the computer-controlled external command. For voltages from –50 to +50 mV gap-free acquisition mode was used. For voltages less than –50 mV and greater than +50 mV, the episodic acquisition mode of pClamp 7 was used. The recording system was tested with a model cell and found to give linear i/V curves from –400 to +400 mV.

Unitary currents were measured from all-point histograms of the current records. Fig. 1 A shows a representative single-channel current record as it appears on the screen of the program interface. The corresponding voltage jump is indicated below the record. Fig. 1 B shows the same record as in Fig. 1 A, but with the data at the ends of the recording removed for the histogram analysis. The peaks of the current histograms correspond to the closed (C) and open current levels when one (O₁), two (O₂), and three (O₃) BK channels were open at the same time. The distance between dashed lines placed at the peaks of the all-point histograms divided by the number of current steps between the dashed lines gave the average unitary current of open BK channels present in the patch. Currents recorded from –50 to +50 mV were typically measured by manually setting cursors on the closed and open current amplitudes because the histograms were less likely to show clear peaks due to the low activity. Unitary current amplitudes obtained by these methods were subsequently averaged for estimates obtained from three or more different patches studied under the same conditions, with the error bars in figures indicating the SEM. The absence of visible error bars indicates that the SEM was less than the size of the symbol.

FIGURE 1. Unitary currents were measured with a custom program using histogram analysis. (A) A representative single-channel current is shown as it appears on the screen of the program interface. The membrane potential was jumped from the holding potential of 0 to +150 mV for 42 ms and then jumped back to 0 mV. (B) The same recording as in A but with the data at the ends of the recording removed. The peaks of the current histograms correspond to the closed (C) and open (O₁–O₃) for one to three open channels) current levels. [K⁺]_i was 3.4 M. Effective filtering was 33 kHz.



BK channels were identified from their characteristic Ca^{2+} sensitivity and voltage dependence (Barrett et al., 1982). *Xenopus* oocytes express endogenous BK channels at very low levels (Krause et al., 1996). Endogenous BK channels were so few for our conditions that we did not see any from 20 patches excised from uninjected oocytes harvested from three different frogs. Due to the low levels of expression of the endogenous BK channels and the averaging of results from three or more patches with each patch typically containing 2–3 channels, the results in this study should not be affected by the possible presence of the endogenous BK channels.

The effective filtering ranged from 4 to 33 kHz, and had no effect on the measured unitary conductance over this range because the open and closed current levels were well defined. Experiments were performed at room temperature (21–23°C), unless otherwise indicated.

Fitting the Experimental Data

To analyze the titration curves for the inhibition of unitary current by protons, the data were fitted with Eq. 1

$$i_b/i_0 = k_i^n / (k_i^n + [B]^n), \quad (1)$$

where i_0 and i_b are unitary current amplitudes in the absence and presence of the blocking ion, k_i is the inhibitory constant of the blocker, given by the concentration of the blocker required to achieve 50% reduction of i_0 , n is a pseudo Hill coefficient, and $[B]$ is the concentration of the blocking ion (Daumas and Andersen, 1993; Schild et al., 1997; Lopes et al., 2000; Park et al., 2003). The apparent dissociation constant, pK_a , is defined as $-\log k_i$.

To examine whether incorporating competitive inhibition of H^+ on the action of K^+ into the Woodhull equation would account for the i/V curves at different pH_i , we incorporated the voltage dependence from the Woodhull equation (Eq. 5 in RESULTS) into equations from enzyme kinetics, as described below. The unitary current i_0 in the presence of substrate K^+ can be described by the Hill equation

$$i_0/i_{\max} = [\text{K}^+]^n / ([\text{K}^+]^n + k_{\text{dK}}^n), \quad (2)$$

where $[\text{K}^+]$ is the concentration of the K^+ ions at the site of action, i_{\max} is the maximal current at saturating $[\text{K}^+]$, k_{dK} is the dissociation constant for K^+ from the binding site, and the Hill coefficient n is a measure of cooperativity (Stryer, 1981). In the presence of competitive inhibition of H^+ on the action of K^+ , the apparent k_{d} will be given by $k_{\text{dK}}(1 + [\text{H}^+]/k_{\text{dH}})$, where $[\text{H}^+]$ is the concentration of the blocking ion at the site of action and k_{dH} is the dissociation constant for H^+ from the blocking site (Stryer, 1981). With substitution for the apparent k_{d} , Eq. 2 becomes

$$i_b/i_{\max} = [\text{K}^+]^n / \{[\text{K}^+]^n + (k_{\text{dK}}(1 + [\text{H}^+]/k_{\text{dH}}))^n\}. \quad (3)$$

The ratio of current in the presence (i_b) and absence (i_0) of competitive blocker is then given by the ratio of Eq. 2 to Eq. 3

$$i_b/i_0 = ([\text{K}^+]^n + k_{\text{dK}}^n) / \{[\text{K}^+]^n + (k_{\text{dK}}(1 + [\text{H}^+]/k_{\text{dH}}))^n\}. \quad (4)$$

To account for voltage-dependent concentration of the ions within the electric field of the membrane (Woodhull, 1973), $[\text{H}^+]$ and $[\text{K}^+]$ in Eq. 4 are given by

$$[\text{K}^+] = [\text{K}^+]_i \exp(zdVF/RT) \quad (4a)$$

$$[\text{H}^+] = [\text{H}^+]_i \exp(zdVF/RT), \quad (4b)$$

where $[\text{K}^+]_i$ and $[\text{H}^+]_i$ are the concentrations in the bath, z is the valence of the ion, V is the voltage across the membrane, d is the fraction of the membrane voltage at the binding site (the electrical distance) measured from the inside of the membrane, and $F/RT = 25.5 \text{ mV}$ at 23°C.

The fitting of equations to the experimental data was performed in Origin (Microcal Software, Inc.) using the standard least squares fitting method.

RESULTS

i/V Plots of Unitary Currents Are Sublinear at High Voltages

This paper explores block of BK channels by intracellular protons, but before testing for such block, we examined whether other ions in the solutions might also be blocking the channel. Data were collected at high voltages, as channel block by intracellular ions is typically more pronounced at greater depolarization (Armstrong and Hille, 1972; Woodhull, 1973; Ferguson,

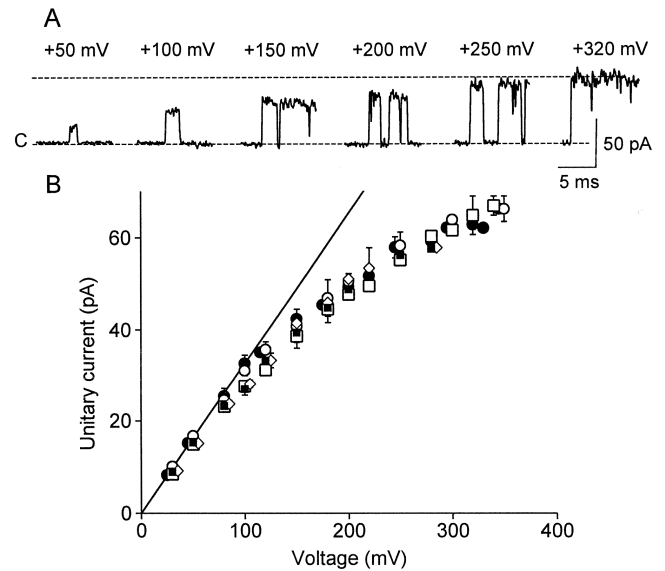


FIGURE 2. Current/voltage plots of unitary currents are sublinear at high voltages, and the Ca^{2+} and pH_i buffers used in the solutions are not the cause of the sublinearity. (A) Representative single-channel currents recorded from BK channels expressed in oocytes with symmetrical 150 mM KCl. Effective filtering: 4 kHz. (B) Plot of the outward unitary currents versus voltage obtained with different solutions. Pipette solution (mM): filled circles, filled squares, open circles, open diamonds, and for the rest of the figures in the paper, 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, 60 μM GdCl_3 , pH 7.0; open squares, 140 KMeSO_3 , 20 HEPES, 2 KCl, 2 MgCl_2 , pH 7.2. Intracellular solution (mM): filled squares, 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, pH 7.0; filled circles, 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, 50 μM crown ether, pH 7.0; open circles, only 150 KCl; open diamonds, 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, 100 μM added Ba^{2+} , pH 7.0; open squares, 140 KMeSO_3 , 20 HEPES, 2 KCl, 1 HEDTA, 0.84 μM free Ca^{2+} , pH 7.2. Some of the symbols are shifted to the left or right by 5 mV so they can be seen. The slope of the straight line corresponds to the average chord conductance of 326 pS at +100 mV for the filled circles.

1991; Dumas and Andersen, 1993; Hille, 2001; Geiger et al., 2002). Single-channel currents were recorded from BK channels expressed in *Xenopus* oocytes using the inside-out configuration of the patch-clamp technique. Representative outward single-channel currents recorded over a range of voltage are shown in Fig. 2 A. The amplitudes of these unitary currents are plotted versus membrane voltage in Fig. 2 B (filled circles). Unitary currents increased with the increase in membrane depolarization. This increase was linear to about +100 mV and then became sublinear with further increases in voltage. In spite of this tendency to saturate, the unitary currents reached the very large value of 66 pA at +350 mV. In the linear range, the average chord conductance at +100 mV was 326 pS (straight line). BK channels are characterized by large conductances (Marty, 1981; Barrett et al., 1982; Yellen, 1984; Blatz and Magleby, 1984; Ferguson, 1991).

The Ca²⁺ and Ba²⁺ Buffers and the pH Buffer TES Used in this Study Do Not Affect Unitary Current Amplitudes

The sublinearity observed in Fig. 2 B could arise from intrinsic properties of the channel or from channel block by ions in the intracellular solutions. To determine whether the Ca²⁺ buffers EGTA and HEDTA, the pH buffer TES, the Ba²⁺ chelator (crown ether), or possible impurities in these agents induced the voltage-dependent sub-linearity in outward currents, we recorded single-channel currents using an intracellular solution that contained only 150 mM KCl and no EGTA, HEDTA, crown ether, or TES. Due to the absence of a pH buffer, the pH of the solution was somewhat unstable over time, ranging from 6.7 to 7.3, as water is only a weak pH buffer. (It will be shown later that changes in pH of this magnitude would have little effect on the unitary current.) As can be seen from Fig. 2 B, the unitary currents recorded in the absence (open circles) and presence (filled circles) of the chelators and buffers were essentially identical. Thus, the chelators and buffers used in our study do not affect the unitary current amplitudes.

Contaminating Ba²⁺ in the Solution Does Not Affect the Unitary Current Amplitude

Studies recording currents from patches of membrane containing large numbers of BK channels showed that contaminating Ba²⁺ in the intracellular solution can significantly reduce macro currents recorded from BK channels (Diaz et al., 1996). Single-channel studies have shown that the reduced currents result from a dual effect of Ba²⁺ on currents through BK channels: Ba²⁺ decreases the effective open probability of the channel by producing long lasting blocks (seconds) of the channel (Vergara and Latorre, 1983; Miller et al., 1987; Bello and Magleby, 1998), and Ba²⁺ can decrease the unitary cur-

rent by a fast block (Vergara and Latorre, 1983; Bello and Magleby, 1998). To test if contaminating Ba²⁺ is producing the sublinearity of currents that we observed at high voltage, we examined unitary currents with and without the Ba²⁺ chelator crown ether added to the intracellular solution. From Fig. 2 B (filled squares for no Ba²⁺ chelator and filled circles with Ba²⁺ chelator) it can be seen that removing the contaminating Ba²⁺ with the chelator had no effect on the unitary current. Thus, contaminating Ba²⁺ in the solution does not contribute to the sublinearity of unitary currents.

As a further test to explore whether contaminating Ba²⁺ might be reducing the unitary currents, we added 100 μ M Ba²⁺ to the intracellular solution containing 1 mM EGTA and 1 mM HEDTA in the absence of crown ether. For these conditions Ba²⁺ produced occasional long blocks of the channel that became more pronounced with voltage, reducing the open probability, as expected (Vergara and Latorre, 1983; Miller et al., 1987; Bello and Magleby, 1998), but there was little change in the peak unitary currents (Fig. 2 B, open diamonds). For voltages >200 mV with this large amount of added Ba²⁺, a slow flickery block developed that reduced the average current, but not the peak unitary currents. Considering that the total contaminant Ba²⁺ is typically <0.1 μ M (Diaz et al., 1996; Bello and Magleby, 1998), a thousand times less than the 100 μ M Ba²⁺ added in this experiment, contaminant Ba²⁺ would not be expected to alter measurements of unitary current amplitudes. Nevertheless, 50 μ M crown ether was added to the intracellular recording solutions to assure that Ba²⁺ was not a factor.

MeSO₃⁻ and HEPES Do Not Reduce Unitary Current Amplitudes

Although we did not use MeSO₃⁻ and HEPES in our experiments, with these ions in the intra- and extracellular solutions Cox et al. (1997) observed that the unitary currents through BK channels reached a maximum value of 32 pA at +120 mV with no further increase with increasing depolarization. To explore why Cox et al. (1997) saw such pronounced saturation compared with our observations (Fig. 2), we examined the possibility that some ion present in their solutions that was not in our solutions may have blocked the channel in a voltage-dependent manner. For example, Guo and Lu (2000) found that HEPES, a commonly used pH buffer, or some accompanying impurities could cause rectification in IRK1 K⁺ channels. We recorded single-channel currents using intracellular and extracellular solutions prepared as described by Cox et al. (1997), with the intracellular solution containing (mM): 140 KMeSO₃, 20 HEPES, 2 KCl, 1 EGTA, and 0.84 μ M free Ca²⁺, pH 7.2; and the pipette (extracellular) solution containing (mM): 140 KMeSO₃, 20 HEPES, 2 KCl, 2 MgCl₂, pH 7.2. Unitary currents recorded with these so-

lutions (Fig. 2 B, open squares) were essentially unchanged from those observed in our solutions (Fig. 2 B, filled circles). Thus, some other factor, perhaps an impurity in the stock chemicals, is responsible for the difference in results between our observations and those of Cox et al. (1997). Consistent with our observations, Talukder and Aldrich (2000) observed unitary currents of ~ 58 pA at +260 mV, similar to the value we observed at this potential.

Intracellular Protons Decrease Unitary Currents of BK Channels in a Concentration-dependent Manner

Protons block ion channels from both the extracellular (Woodhull, 1973; Prod'homme et al., 1987; Tytgat et al., 1990; Daumas and Andersen, 1993; Klockner and Isenberg, 1994; Coulter et al., 1995; Chen et al., 1996; Benitah et al., 1997; Morrill and MacKinnon, 1999; Lopes et al., 2000; Geiger et al., 2002) and intracellular (Davies et al., 1992; Daumas and Andersen, 1993; Habartova et al., 1994; Xu et al., 2000; Nimigeon et al., 2003) surfaces. To investigate whether block by intracellular protons contributes to the sublinearity of the i/V curves in Fig. 2, single-channel currents were recorded over a range of intracellular pH (pH_i). Fig. 3 A shows representative single-channel records at +250 mV for pH_i ranging from 5.0 to 9.0. The unitary currents at pH_i of 7.0 and 5.0 were decreased by $\sim 15\%$ and $\sim 55\%$, respectively, from that at pH_i 9.0. The unitary currents at pH_i 8.0 and 9.0 were essentially the same, indicating that H^+ at 10^{-8} M has little effect on the unitary current. Plots of unitary currents recorded at +250 mV versus pH_i , as well as for currents recorded at +200 and +100 mV, are shown in Fig. 3 B. At pH_i 4.0 the channel still functioned, but clear open levels were difficult to define due to the high levels of block. Thus, currents at $\text{pH}_i < 5.0$ were not studied. Habartova et al. (1994) have previously examined the effect of pH_i from 5.5 to 8.0 on unitary current amplitudes for voltages up to +40 mV, with observations similar to ours over this range.

The unitary current amplitude vs. pH data in Fig. 3 B obtained at different voltages were simultaneously fitted with Eq. 1 in MATERIALS AND METHODS (continuous lines) to estimate the concentration of blocker that is necessary to reduce the currents to 50% of the value in the absence of blocker. The inhibition constant was $8.7 \mu\text{M}$, giving an apparent pK_a of 5.1, with a pseudo Hill coefficient of 0.48. Fixing the Hill coefficient to 1.0 gave worse fits of the data (unpublished data) with the Chi squared value 6.8 times larger. The apparent pK_a falls between the pK_a (as free amino acids) for glutamate (4.5) and histidine (6.4). Even though the pK_a s of these amino acids could be different in the protein (Fersht, 1985; Morrill and MacKinnon, 1999), we examined the region of the channel expected to form the intracellular vestibule (Jiang et al., 2002a) for these

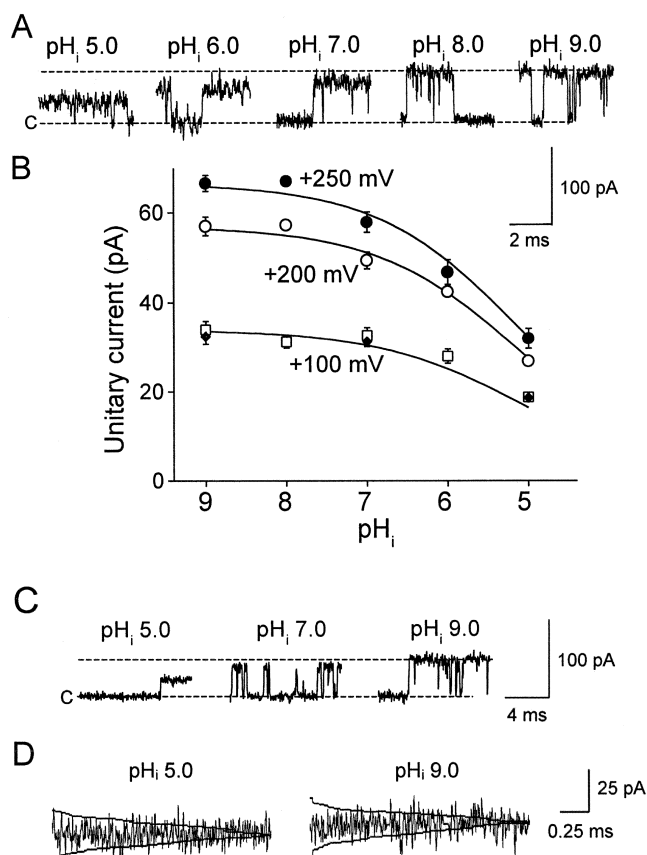
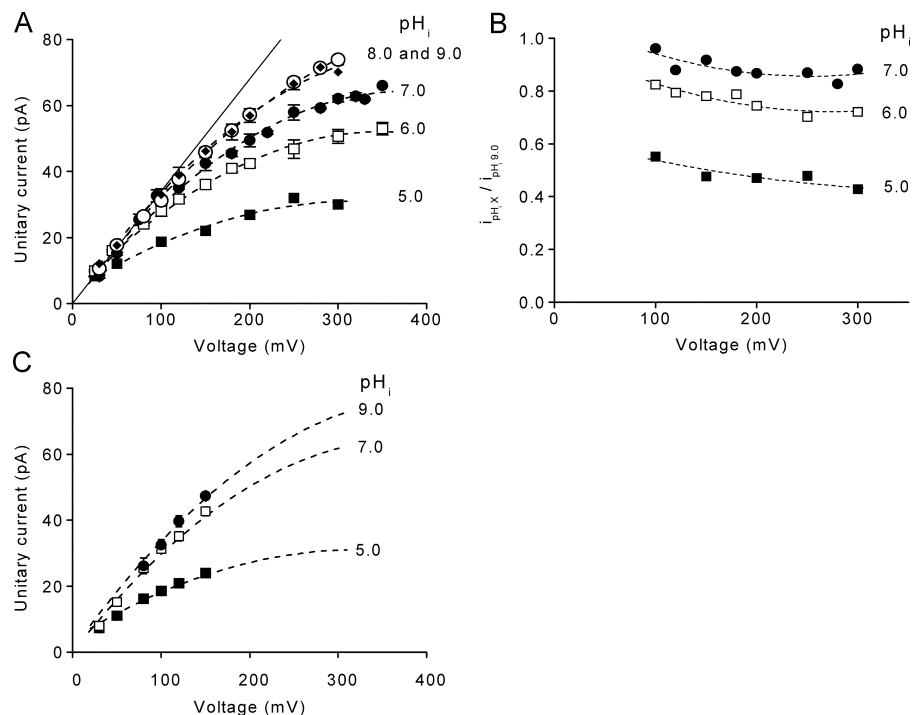


FIGURE 3. Intracellular protons decrease unitary current amplitudes of BK channels. (A) Representative single-channel currents from BK channels with symmetrical 150 mM KCl at the indicated pH_i . Intracellular solution (mM): 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, and 50 μM crown ether. Membrane voltage: +250 mV. Effective filtering: 4 kHz. (B) Semilogarithmic plot of the dose response curves for unitary current amplitudes obtained at +100, +200, and +250 mV versus pH_i . The lines represent simultaneous fits of all the data with Eq. 1, with $k_i = 8.7 \mu\text{M}$ ($\text{pK}_a = 5.1$) and $n = 0.48$. Intracellular solution (mM): open squares, open circles, and filled circles, 150 KCl, 5 TES, 1 EGTA, 1 HEDTA, and 50 μM crown ether; filled diamonds, 150 KCl, 10 propionic acid, 10 MES, 10 TES, 10 TABS, 1 EGTA, 1 HEDTA, and 50 μM crown ether. (C) Single-channel currents from a BK channel at the indicated pH_i . Intracellular solution (mM): 150 KCl, 10 propionic acid, 10 MES, 10 TES, 10 TABS, 1 EGTA, 1 HEDTA, and 50 μM crown ether. Membrane voltage: +150 mV. Effective filtering: 4 kHz. (D) Open-channel current traces at pH_i 5.0 and pH_i 9.0 with all points histograms from the same patch as in C. For the open-channel current trace at pH_i 9.0, two brief closings were replaced by a horizontal line to exclude kinetics from the noise histogram. Effective filtering: 33 kHz.

amino acids. Two glutamate residues per subunit at the entrance to the intracellular vestibule would be candidates for the site of proton action. This possibility is tested in a later section by replacing the glutamates with uncharged amino acids.

The data in Fig. 3, A and B, were obtained with a single pH buffer (TES, pK_a of 7.4). This was done to pre-

FIGURE 4. Proton block of BK channels is concentration and voltage dependent. (A) Plots of unitary current amplitudes versus voltage at the indicated pH_i . The slope of the straight line corresponds to the average chord conductance of 340 pS at +100 mV for the data at pH_i 9.0. (B) Plots of the ratio of the unitary current amplitudes at the indicated pH_i to the amplitudes at pH_i 9.0 versus voltage, where X can be 5.0, 6.0, or 7.0, and is indicated for each curve. The dashed lines are polynomial fits. (C) Plots of unitary current amplitudes versus voltage at the indicated pH_i with four pH buffers in the intracellular solutions. Symmetrical 150 mM $[K^+]$ for A–C. The dashed lines in A and C are polynomial fits to the data in A.



vent the possibility of channel block from additional buffers, but has the disadvantage of weak buffering at the extremes of the pH examined. To test whether the single buffer controlled the pH adequately for our experiments, unitary currents were recorded in the presence of four buffers (10 mM each) to span the pH range: propionic acid (pK_a 4.9), MES (pK_a 6.1), TES (pK_a 7.4), and TABS (pK_a 8.9) and compared with data with 5 mM TES alone.

Representative single-channel records at pH_i 5.0, 7.0, and 9.0 recorded at +150 mV are shown in Fig. 3 C, where increasing the concentration of protons decreases unitary current amplitudes with the four pH buffers, as observed with one buffer. Comparison in Fig. 3 B of data obtained with the four pH buffers (filled diamonds) to that obtained with one pH buffer (open squares), and comparison of Fig. 4 C to Fig. 4 A, showed a similar proton block over a range of voltages, indicating that the pH of the solutions was adequately buffered with the single buffer. Although there was no difference in the unitary current amplitudes when using one pH buffer or four pH buffers for voltages ≤ 150 mV, an apparent discrete block developed with the four buffer solution that became so prominent with depolarizations > 150 mV that the openings became too brief to measure (unpublished data). Hence, Fig. 4 C presents data for ≤ 150 mV. The discrete block in the four buffer solution is not proton block, as it was observed at pH 9.0 as well. Because of the apparent discrete block with the four buffer solutions at large depolarizations, one pH buffer was used for all subsequent experiments except for a few experiments in the next section.

Proton Block is a Fast Block

To investigate whether the proton block is consistent with fast block, we examined the open-channel noise at pH_i 5.0 and 9.0 for data recorded with a single pH buffer and also with four pH buffers for voltages ≤ 150 mV. For data obtained from the same single-channel patch with four pH buffers, the noise (filtering of 33 kHz) was the same at pH_i 5.0 and 9.0, as indicated by the similar width of the all-points histograms superimposed on the currents (Fig. 3 D). This lack of effect of pH on the open-channel noise can also be seen in Fig. 3 C for data obtained from the same patch filtered at 4 kHz. Similar conclusions were obtained from comparison of the standard deviation of the noise of the open-channel current record recorded with a single pH buffer for three patches at pH_i 9.0 and four patches at pH_i 5.0, where the SD of the noise was 15% greater at pH_i 9.0 than pH_i 5.0 rather than less, as might be expected for discrete block ($P = 0.83$, t test). Thus, proton block is a fast block, as the pH-induced reduction in unitary currents is not associated with increased open-channel noise at 4–33 KHz filtering. Association and dissociation rates of protons with proteins can be so fast (Fersht, 1985) that such binding events could go undetected at the filtering used in our experiments, consistent with a fast block.

Proton Block Is Voltage Dependent

If protons reduce unitary currents by acting inside the conduction pathway of the channel, then the reduction of currents by protons should be voltage de-

pendent. To explore this possibility, unitary currents were obtained at pH_i 5.0–9.0 and plotted against voltage in Fig. 4 A. As indicated previously, the superposition of the unitary currents at pH_i 8.0 and 9.0 shows that concentrations of protons $\leq 10^{-8}$ M have little effect on the unitary conductance. The reduction of the unitary currents for the increasing concentrations of protons from pH_i 7.0 to 5.0 is readily apparent. To examine to what extent the proton block is voltage dependent, the ratios of the unitary currents at pH_i 7.0, 6.0, and 5.0 to the unitary currents at pH_i 9.0, where there is no block, are plotted in Fig. 4 B. For each pH_i the ratio decreases with voltage, suggesting that the site of action of protons could be within the electrical field of the membrane. While protons could reduce unitary current amplitudes by $\sim 60\%$ at pH_i 5.0 and +300 mV, the effect of protons was negligible at pH_i 7.0 for voltages ≤ 100 mV. The average chord conductance of BK channels at +100 mV and pH_i 9.0 (in the essential absence of proton block) was 340 pS (straight line, Fig. 4 A), the average chord conductance at +100 mV and pH_i 7.0 was 326 pS (straight line, Fig. 2 B). A paired t test of the chord conductances at pH_i 9.0 and 5.0 at +30, +50, and +100 mV indicated that this small difference in chord conductance was not significant ($P > 0.05$).

The Ca^{2+} buffering ability of the EGTA and HEDTA depends on pH (Miller and Smith, 1984). Even though we used solutions with zero added Ca^{2+} , a small amount of contaminating Ca^{2+} would be expected in the recording solutions due in large part to the Ca^{2+} in the KCl salt, so that some Ca^{2+} would be displaced from the Ca^{2+} buffers at the lower pH_i s. To test whether this displaced Ca^{2+} was blocking the channel, some experiments were also performed with 5 mM BAPTA added to the solutions to chelate Ca^{2+} (see MATERIALS AND METHODS). The reduction of currents by protons was the same in the absence and presence of BAPTA (unpublished data). Thus, the greater decrease in unitary currents at lower pH_i was not due to block by contaminating Ca^{2+} .

Proton Block Contributes to the Sublinearity of Unitary Currents at High Voltages

To determine to what extent proton block contributed to the sublinearity of the i/V plots obtained at pH 7.0 (Fig. 2), we compared i/V plots obtained at pH_i 9.0, where proton block was negligible, to the i/V plots at pH 7.0. From Fig. 4 A it can be seen that only $\sim 40\%$ of the sublinearity with increasing voltage at pH_i 7.0 arises from proton block. The remaining sublinearity, still observed at pH_i 9.0 in the absence of proton block, suggests that factors in addition to proton block contribute to the sublinearity.

The Woodhull Model can Approximate the Proton Block at a Fixed pH_i , but Cannot Describe the Proton Block Over a Range of pH_i

Fig. 4 shows that protons block BK channels in a concentration- and voltage-dependent manner. To determine if this block is consistent with the assumption that protons bind at a site within the electric field to block the channel, we examined whether the Woodhull model (Woodhull, 1973) could describe the data. This model is given by:

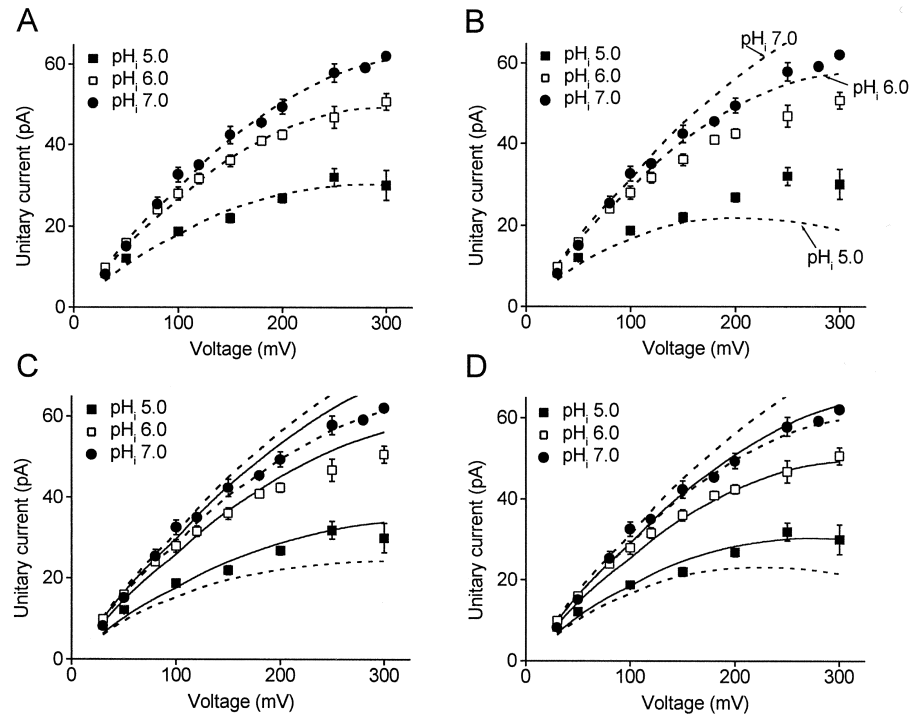
$$i_0/i_b = 1 + ([B]/k_d(0))\exp(zdVF/RT), \quad (5)$$

where i_0 and i_b are currents in the absence and presence of the blocking ion, respectively, $[B]$ is the concentration of the blocking ion, $k_d(0)$ is the dissociation constant of the blocking ion at 0 mV, z is the valence of the blocking ion, V is the voltage drop across the membrane, d is the fraction of the membrane voltage at the binding site (the electrical distance) measured from the inside of the membrane, and RT/F is 25.5 mV at 23°C. Fig. 5 A shows the results when data obtained at each intracellular pH were fitted separately (dashed lines). Under these conditions the Woodhull model could describe the experimental data for each pH_i . The values of the k_d s were 20, 8.7, and 2.1 μM for pH_i s of 5.0, 6.0, and 7.0, respectively, and the values of d were 0.08, 0.12, and 0.13 for pH_i s of 5.0, 6.0, and 7.0, respectively. The values for both the k_d and d parameters changed with pH, suggesting that the block is more complex than described by the Woodhull model. To test this possibility the data at the three different pH_i s were simultaneously fitted to obtain the best global estimates for k_d and d . As can be seen in Fig. 5 B, the Woodhull model did not adequately describe the data at voltages +100 mV when the fitting was over a range of pH_i with a single set of parameter values, suggesting that the mechanism of H^+ block of BK channels is more complicated, as will be described in a later section. Nevertheless, the Woodhull model can give a rough indication of the effective distance within the electric field where the block occurs (parameter d), which ranged from 0.08 to 0.13 from the inner membrane surface in these fits.

Proton Block of BK Channels is K^+ Dependent

Inward currents in the cardiac leak channel Kcnk3 are blocked by external protons, and the block is relieved by external K^+ (Lopes et al., 2000). Outward currents in BK channels are blocked by intracellular Mg^{2+} and the block is relieved by intracellular K^+ in a competitive manner (Ferguson, 1991). In both of these studies, the permeating ion can relieve the block. To investigate whether the proton block of BK channels is also relieved by K^+ , single-

FIGURE 5. Comparison of the Woodhull model and competitive inhibition models for proton block of BK channels. (A) Predictions of the Woodhull model (Eq. 5, dashed lines) fitting data for each pH_i separately. Different k_d and d parameters were found for each pH_i . The values of the k_d s were 20, 8.7, and 2.1 μM for pH_i s of 5.0, 6.0, and 7.0, respectively, and the values of d were 0.08, 0.12, and 0.13 for pH_i s of 5.0, 6.0, and 7.0, respectively. (B) Predictions of the Woodhull model (dashed lines) for simultaneously fitting with Eq. 5 for data at different pH_i with single values for K_d and d of 20 μM and 0.15. (C) Predictions of the competitive inhibition model for simultaneously fitting the data at different pH_i with Eq. 4 when d is the same for both K^+ and H^+ : $d = 0.2$, $k_{dK} = 500 \text{ mM}$, $k_{dH} = 10 \mu\text{M}$, $n = 1$ for the dashed lines, and $n = 0.57$ for the continuous lines. (D) Same as C except that d can be different for K^+ and H^+ . Dashed lines: $d_K = 0$, $d_H = 0.13$, $k_{dK} = 500 \text{ mM}$, $k_{dH} = 10 \mu\text{M}$, and $n = 1$. Continuous lines: $d_K = 0$, $d_H = 0.2$, $k_{dK} = 203 \text{ mM}$, $k_{dH} = 10 \mu\text{M}$, and $n = 0.46$. The parameters were poorly defined in the fits for C and D, but to describe the data it was necessary for n to be ~ 0.5 and for the electrical distance from the inside for d_H to be ~ 0.2 greater than for d_K . The single-channel current at $\text{pH}_i 8.0$ was used as the i_0 current in the absence of protons.



channel currents were recorded over a range of $[\text{K}^+]_i$ and pH_i . Results are presented in Fig. 6 A for data obtained at $\text{pH}_i 5.0$ and 9.0 for $[\text{K}^+]_i$ of 150 mM and 3.4 M . With a $[\text{K}^+]_i$ of 150 mM , increasing the proton concentration by decreasing pH_i from $\text{pH}_i 9.0$ to 5.0 reduced the unitary currents at 200 mV by 53% , whereas with a $[\text{K}^+]_i$ of 3.4 M , increasing the proton concentration the same amount had little effect on the unitary currents. Results are summarized in Fig. 6 B for the data obtained at $+200 \text{ mV}$ with $60, 150, \text{ and } 3,400 \text{ mM}$ $[\text{K}^+]_i$, where it can be seen that increasing $[\text{K}^+]_i$ decreases the proton block.

In the experiments presented in Fig. 6 B, $[\text{K}^+]_i$ was varied from 60 mM to $3,400 \text{ mM}$, giving rise to significant changes in the ionic strength. To test for the effects of ionic strength control experiments would be needed with an “inert” cation that neither blocks the channel nor passes through. Previous studies that have examined large numbers of cations have not found such an ion (Blatz and Magleby, 1984; Eisenman et al., 1986; Latorre et al., 1989; Bello and Magleby, 1998). We tried intracellular choline⁺ and NMDG⁺, and found that they also block BK channels. Our observation that intracellular NMDG⁺ blocks BK channels is consistent with previous findings (Lippiat et al., 1998). Thus, we could not separate the effects of the increase in $[\text{K}^+]_i$ from the increase in ionic strength on the unitary currents through BK channels.

Proton Block of BK Channels Is Consistent with Competitive Inhibition of K^+ by H^+

To investigate if the proton block of BK channels is consistent with competitive inhibition of K^+_i by H^+_i , double reciprocal plots of unitary current versus $[\text{K}^+]_i$ were analyzed, similar to studies of enzyme kinetics (Stryer, 1981). Fig. 7 A shows theoretical double reciprocal plots with no blocker, and with blocker present for competitive and noncompetitive inhibition. For noncompetitive inhibition the plots with and without blocker intersect on the x-axis, and for competitive inhibition they intersect on the y-axis at an x value of zero, where $[\text{K}^+]_i$ is infinite ($1/[\text{K}^+]_i = 0$), because the infinitely high $[\text{K}^+]_i$ fully relieves the block by the competitive inhibitor.

To examine whether proton block is competitive or noncompetitive, Fig. 7 B presents double reciprocal plots of unitary currents obtained in the presence of protons (open circles, $\text{pH}_i 5.0$) and in the absence of protons (filled circles, $\text{pH}_i 9.0$). The intersection of the lines on the y-axis is consistent with competitive block. Thus, H^+ competes with K^+ , either directly at the same site or indirectly through an allosteric mechanism, with high K^+ reducing the action of H^+ .

To determine whether incorporating the voltage-dependent blocking mechanism from the Woodhull equation into a competitive inhibition model would ac-

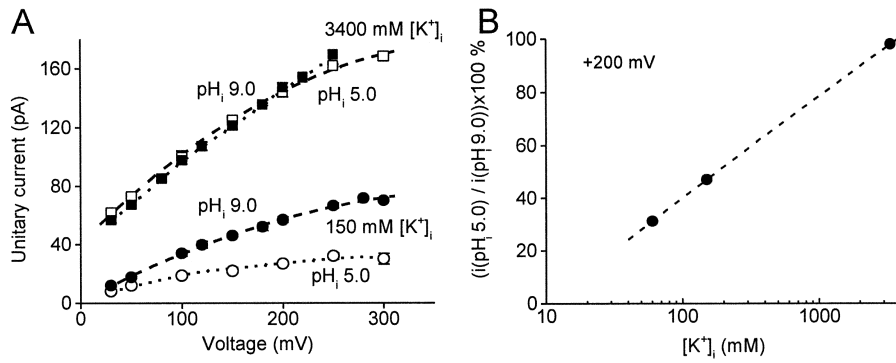


FIGURE 6. Proton block of BK channels is relieved by K^+ . (A) Plots of the unitary current amplitudes versus voltage for the indicated $[K^+]_i$ and pH_i . Dotted lines are predictions of the competitive inhibition model for simultaneously fitting the data at different pH_i and $[K^+]_i$ with Eq. 4 when $d_K = 0.2$, $d_H = 0.21$, $k_{dK} = 200$ mM, $k_{dH} = 10$ μ M, and $n = 1.6$. The parameters were poorly defined, but to describe the data it was necessary for n to be ≥ 1.0 and < 1.7 and for the electrical distance from the inside for d_H to be equal to 1 to 1.1 times d_K . The single-channel current at pH_i 9.0 was used as the i_0 current in the absence of protons. (B) Semilogarithmic plot of the ratio (in %) of the unitary current amplitudes at pH_i 5.0 to the unitary current amplitudes at pH_i 9.0 versus $[K^+]_i$. Dashed lines in A and B are polynomial fits.

count for the i/V curves at different pH_i , we simultaneously fitted the data in Fig. 5 A obtained at three different pHs with Eq. 4 (see MATERIALS AND METHODS). The dashed lines in Fig. 5 C indicate that Eq. 4 does not describe proton block with $n = 1.0$ (Hill coefficient), and the continuous lines indicate that Eq. 4 provides a better description of the data when $n = 0.57$, although there are still differences between the observed and predicted i/V curves at the higher voltages and pH_i s. Thus, the simple competitive inhibition model represented by Eq. 4 does not adequately describe proton block. For this test of competitive inhibition, the fraction d of the electrical distance from the inside for the site of action of K^+ and H^+ was constrained to be the same, as would be the case if they acted at the same site. The value of d for the plots in Fig. 5 C was 0.2, but values of d between 0.2 and 0.5 produced essentially the same fits because of compensating changes in the k_d for protons.

When d was allowed to differ for H^+ and K^+ , which could be the case for allosteric action of proton block, then the data were still not described by Eq. 4 when $n = 1.0$ (Fig. 5 D, dashed lines), but allowing n to be < 1.0 gave an excellent description of the data with: $n = 0.46$, $d = 0$ for K^+ , and $d = 0.2$ for H^+ (continuous lines, Fig. 5 D). Equally good fits could be obtained over a range of d for K^+ as long as there was a well defined distance between the d for H^+ and the d for K^+ , given by $d_H = 0.2 + 0.84d_K$. For example, a fit visually indistinguishable from the continuous lines in Fig. 5 D was obtained with $n = 0.46$, $d = 0.2$ for K^+ , and $d = 0.36$ for H^+ .

Eq. 4 could also describe the effect of high K^+ in relieving the proton block (dotted lines in Fig. 6 A). For this fit $n = 1.6$, the d for $K^+ = 0.2$, and the d for $H^+ =$

0.21. Once again, similar fits could be obtained over a range of d 's. (The implications of the description of the data by Eq. 4 will be considered in the DISCUSSION.)

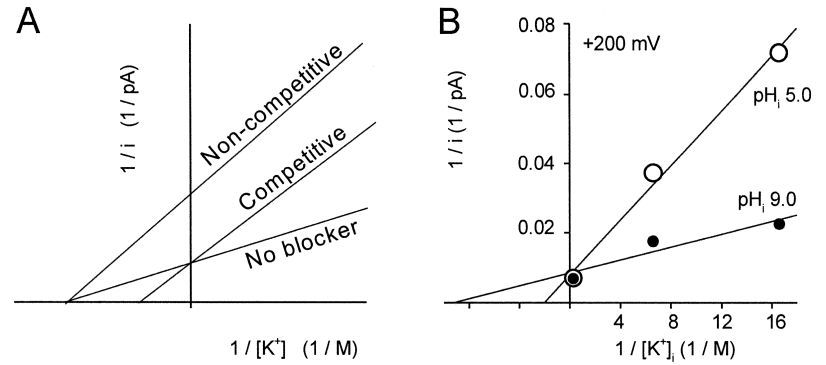
The competitive interaction between K^+ and H^+ and the high concentration of K^+ (150 mM) compared with H^+ (10^{-7} M) would contribute to the lack of significant proton block under physiological conditions. Projecting the line in Fig. 6 B suggests that proton block could be highly significant for lower concentrations of permeant ion. Consistent with this possibility for a different channel, competitive extracellular proton block of L-type Ca^{2+} channels for physiological concentrations of the permeant Ca^{2+} (2 mM) may be sufficiently pronounced to have physiological implications (Chen et al., 1996).

Is the Competitive Inhibition of K^+ by H^+ at the Ring of Charge?

BK channels have a ring of eight negative charges at the entrance to the intracellular vestibule that doubles the unitary conductance for outward currents (Brelidze et al., 2003b; Nimigean et al., 2003). H^+ might reduce (block) unitary currents by protonating or screening the negative charges forming the ring of charge so that less K^+ is concentrated in the vestibule. With less K^+ in the vestibule, less K^+ would be available to transit the channel.

To examine to what extent proton block requires the ring of charge, we neutralized the negative charge in the ring of charge with the double mutation E321N/E324N, and compared proton block for mutated and wild-type (WT) channels. Fig. 8 B plots unitary current amplitudes for mutated channels recorded at pH_i 9.0, 7.0, and 5.0 against voltage, and Fig. 8 A replots data from Fig. 4 A from WT channels. Two observations are

FIGURE 7. Proton block of BK channels is consistent with competitive inhibition of H^+_i on K^+_i . (A) Double reciprocal plots of theoretical unitary current amplitudes versus $[K^+]_i$ to illustrate competitive and noncompetitive block. (B) Double reciprocal plots of unitary current amplitudes versus $[K^+]_i$ at pH_i 5.0 (high concentration of the H^+_i blocker) and at pH_i 9.0 (no blocker).



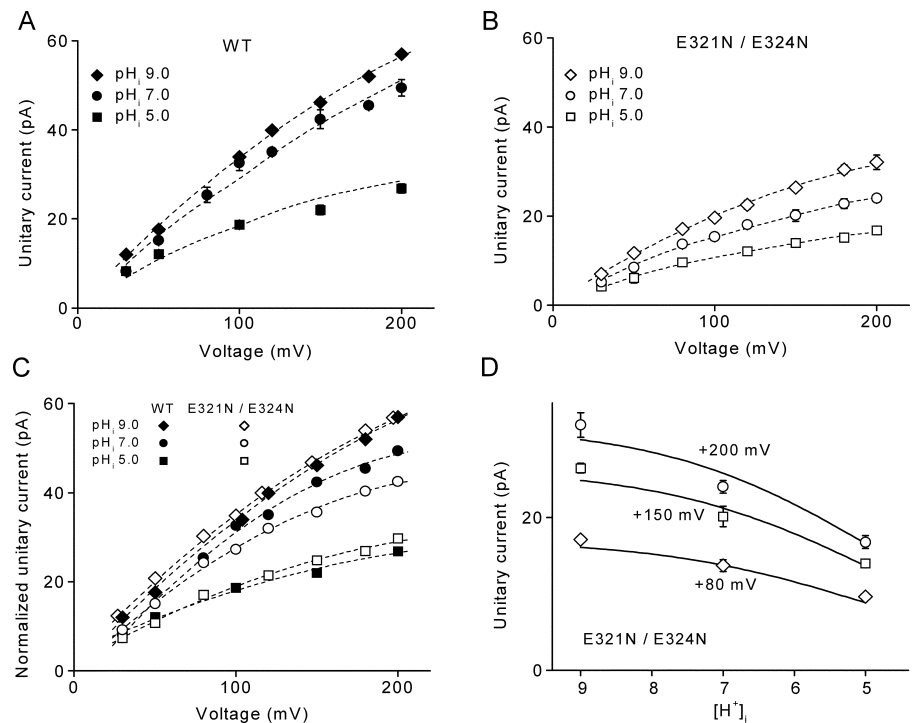
immediately apparent. The unitary currents of the mutant channels for all pH_i s are reduced when compared with WT channels, and protons still block the mutant channel. The reduction in currents would be expected from previous findings that the ring of charge doubles the unitary currents through BK channels by concentrating K^+ in the intracellular vestibule by an electrostatic mechanism (Brelidze et al., 2003b). The further reduction of the unitary currents in the mutant channel at pH_i 7.0 and 5.0, when compared with pH_i 9.0, indicates that protons still block in the absence of the ring of charge.

To determine whether the fractional degree of block was altered by the ring of charge, we multiplied all the data points in Fig. 8 B by 1.77, so that the currents at

pH_i 9.0 for the mutant channels superimposed those for the WT channels. The normalized data in Fig. 8 C show that the fractional block by protons at pH_i 5.0 was similar for mutant and WT channels, and at pH_i 7.0 was somewhat greater for mutant than for WT channels. Thus, the ring of charge is not required for proton block, suggesting a different site of competitive interaction between H^+ and K^+ .

The data for the mutant channel in Fig. 8 B were re-plotted in Fig. 8 D to obtain the pH titration curves. The titration curves obtained at the three different voltages were then simultaneously fitted with Eq. 1, giving a k_i of 12.6 μ M (apparent pK_a of 4.9) and n of 0.29. These values can be compared with the apparent pK_a of 5.1 and n of 0.48 for WT channels (Fig. 3 B).

FIGURE 8. Proton block is still present after the removal of the ring of charge. (A) Plots of unitary current amplitudes versus voltage for WT channels at the indicated pH_i (from Fig. 4). The dashed lines for pH_i 5.0 and 7.0 are the same as the continuous lines in Fig. 5 D. The dashed line for pH_i 9.0 is a polynomial fit. (B) Plots of unitary current amplitudes versus voltage for E321N/E324N mutant channels at the indicated pH_i . The dashed lines for pH_i 5.0 and 7.0 were drawn with Eq. 4 with $d_K = 0.0$, $d_H = 0.15$, $k_{dK} = 200$ mM, $k_{dH} = 2.2$ μ M, and $n = 0.23$, using the data at pH 9.0 for i_0 . The dashed line for pH_i 9.0 is a polynomial fit. (C) The data for WT channels from A, and the data for E321N/E324N channels from B, normalized by multiplying the data in (B) by 1.77. Some of the symbols at pH_i 9.0 are shifted by 4 mV so they can be seen. (D) Semilogarithmic plot of the dose response curves for unitary current amplitudes for E321N/E324N mutant channels at +80, +150, and +200 mV versus pH_i . The solid lines are fits of the data with Eq. 1 with $k_i = 12.6$ μ M ($pK_a = 4.90$) and $n = 0.29$. Symmetrical 150 mM $[K^+]_i$ for A–D.



This study shows that intracellular protons block BK channels in a concentration and voltage-dependent manner with an apparent pK_a of 5.1. All experiments were performed with single-channel (unitary) currents so that the effects of protons on the conductance could be clearly separated from effects on gating. Increasing $[K^+]_i$ progressively relieved the proton block, consistent with competitive inhibition of K^+ by H^+ . We also show that the ring of negatively charged glutamate residues located at the entrance to the intracellular vestibule of BK channels, is not the major site for proton action.

Proton Block of BK Channels Is Voltage Dependent

Protons can decrease the unitary conductance of ion channels by binding to one or multiple sites within the pore of the channels (Daumas and Andersen, 1993; Chen et al., 1996; Benitah et al., 1997; Morrill and MacKinnon, 1999). A characteristic of the block of BK channels by intracellular protons is that the block is voltage dependent, increasing with depolarization. Woodhull (1973) developed a model to describe voltage-dependent proton block of Na^+ currents by H^+ by assuming that the site of block was within the electric field of the membrane so that voltage concentrated H^+ at the blocking site. The Woodhull model could simultaneously describe proton block of BK channels over a range of pH_i s for voltages <100 mV, but could not describe the block at higher voltages. The inability of the Woodhull model to describe the data for BK channels would be expected since we found a competitive inhibition of H^+ on unitary K^+ current amplitudes (Fig. 7), whereas the Woodhull model assumes that the blocking and conducting ions do not interact.

By incorporating the mechanism of voltage-dependent concentration of ions from the Woodhull model into a model for competitive inhibition that also allowed for cooperativity (Eq. 4), we were able to simultaneously describe the voltage and pH dependence of proton block for pH_i from 5.0 to 9.0 and for voltages up to $+300$ mV with a single set of parameters (Fig. 5 D). For 150 mM $[K^+]_i$, the best fits were obtained when the electrical distance through the membrane field measured from the inside of the membrane was $\sim 20\%$ greater for H^+ than for K^+ , suggesting the site of proton block may be different from the site of action for K^+ . However, although Eq. 4 could also account for data obtained at two different $[K^+]_i$ as well (Fig. 6 A), it did not adequately describe the experimental observations when simultaneously fitting data for 60 mM, 150 mM, and 3.4 M $[K^+]_i$ over multiple pH_i s and voltages (unpublished data). Thus, although Eq. 4 can be used to provide a quantitative description of competitive proton block of BK channels over a range of voltage

and pH_i for a narrow range of $[K^+]_i$, this equation is best viewed as a semiempirical equation because it does not fully account for the effect of $[K^+]_i$ because it does not take into account such factors as changes in the electric field introduced by the blocking and conducting ions themselves and the effect of the ring of charge on concentrating K^+ .

Since the Woodhull model and Eq. 4 could approximate the voltage dependence of the proton block, it is of interest to relate the electrical distance for the block determined with these equations to the known structures of K^+ channels. The fractional electrical distance of proton block from the inside of the membrane for the Woodhull model ranged from 0.08 to 0.15, and for Eq. 4 the electrical distance for the site of proton action was ~ 0.2 greater than for the site of action of K^+ . These numbers would be consistent with the blocking site for protons being located within the conduction pathway of the channel, ~ 10 – 20% of the electrical distance through the electric field of the membrane. Whether this places the blocking site deep in the vestibule at the entrance to the selectivity filter or within the selectivity filter itself is not clear. Electrostatic calculations indicate that there is little voltage drop in the intracellular vestibule of the bacterial MthK channels (Jiang et al., 2002b). If this is also the case for BK channels, then the site of proton block may be just within the selectivity filter.

However, estimates of the electrical distance for intracellular block of BK channels by TEA place the fractional blocking site for TEA 0.26 from the intracellular side (Blatz and Magleby, 1984). This electrical distance for block by TEA is greater than for block by protons, even though the large size of TEA would be expected to exclude TEA from the selectivity filter (Hille, 2001; Zhou et al., 2001). Thus, it is difficult to relate electrical distance to physical structure, especially since the size and number of the ions themselves could alter the apparent electrical distance. If the proposed intracellular RCK domains of the BK channel (Jiang et al., 2002a) extend the intracellular conduction pathway for BK channels, as proposed for the intracellular NH_2 and $COOH$ terminus for GIRK1 and KirBac1.1 channels (Nishida and MacKinnon, 2002; Kuo et al., 2003), then the extended conduction pathway might also contribute to the electrical distance.

Our observation that proton block of BK channels is a fast block with no detectable increase in the open-channel noise does not rule out direct channel block. As pointed by Woodhull (1973), proton binding to proteins can be so fast (Fersht, 1985) that single binding (blocking) events would not necessarily be detected for the frequency response of our experiments. Thus, direct channel block could occur without detectable increases in open-channel noise.

An observation of voltage-dependent block, however, does not require that the block occurs within the electric field of the membrane. Voltage-dependent block could also arise from the coupling of a blocker outside of the electric field to the movement of permeant ions inside the electric field. Such coupling is consistent with the voltage-dependent block of K^+ channels by extracellular TEA and Ca^{2+}_i (Armstrong, 1971; Gomez-Lagunas et al., 2003; Thompson and Begenisich, 2003). The coupling works as follows. When the average position of K^+ in the selectivity filter is near the extracellular side of the channel, the positive charge of K^+ electrostatically repels the blocker from the extracellular blocking site. Shifting the membrane potential more negative then moves the average position of K^+ toward the inner side of the selectivity filter away from the blocking site so that the blocker can now bind. Since the extracellular vestibule is so shallow (Jiang et al., 2002a,b), the extracellular blocker can be close to the entrance of the selectivity filter while remaining outside of the electric field. Thus, the voltage-dependent movement of K^+ within the selectivity filter gives voltage dependence to a blocker outside of the electric field.

On this basis, the possibility arises that some or all of the voltage dependence of proton block from the intracellular side of the membrane may arise from a coupling between K^+ in the selectivity filter and H^+ in the intracellular vestibule. To what extent such a mechanism contributes to the voltage dependence of proton block will require further experimentation to resolve.

Does Channel Block Arise through the Screening of Surface Charge?

Proton binding to surface charges on lipid membranes can decrease the conductance of some channels by neutralizing (screening) negative surface charges that might normally increase the conductance of the channel by concentrating K^+ at the entrance to the pore (Klockner and Isenberg, 1994; Chen et al., 1996; Benitah et al., 1997; Morrill and MacKinnon, 1999). A lipid-screening mechanism would also be consistent with a fast block because screening should not increase the open-channel noise. A lipid-screening mechanism for block of BK channels by protons appears unlikely, however. A recent study by Park et al. (2003) showed through experiments measuring the kinetics of Ba^{2+} block of BK channels incorporated into membranes comprised of either neutral or charged lipids that the surface charge on the lipids was too far away from the pore of the channel (due to the large size of the BK channel) to concentrate cations at the entrance to the pore of the channel. Theoretical calculations in the same study supported the experimental conclusion. Thus, if the lipid surface charge is too far away to concentrate K^+ at the entrance to the pore, then removing lipid surface charge with protons

would have little effect on K^+ at the entrance, so the lipid surface charge is not the site of proton block.

The next question is whether surface charges on the protein of the channel itself might be the site of proton block. Glutamate residues frequently are sites of proton block in other channels (Chen et al., 1996; Morrill and MacKinnon, 1999; Xu et al., 2000). As a possible candidate for the site of proton block in BK channels, we considered the ring of fixed negative charges formed by eight glutamate residues at the entrance to the intracellular vestibule (Brelidze et al., 2003b; Nimigean et al., 2003). This ring of charge doubles the conductance of BK channels by concentrating K^+ in the vestibule by an electrostatic mechanism (Brelidze et al., 2003b). Protonation or screening of the ring of charge by protons might decrease the concentration of K^+ at the entrance to the vestibule, decreasing the unitary currents. Support suggesting that protons might act at the ring of charge is the observation in KcsA channels that the introduction of an aspartate residue in the region corresponding to the ring of charge in BK channels significantly increased proton block of unitary KcsA currents (Nimigean et al., 2003).

In contrast, we found in BK channels that the percentage reduction of unitary currents by proton block at pH_i 5.0 was the same in the presence and absence of the ring of charge (Fig. 8 C), indicating that the ring of charge is not the major site of action of proton block for BK channels. This relative lack of effect of the ring of charge on proton block may reflect that the ring of charge would increase the concentrations of both K^+ and H^+ at the entrance to the intracellular vestibule, with the increased K^+ compensating for the effects of the increased H^+ . The absence of a ring of charge would then decrease the concentrations of both K^+ and H^+ at the entrance to the intracellular vestibule so that the percentage reduction in currents by protons would be the same with or without the ring of charge at pH 5.0. If protons also bind to the carboxyl residues, in addition to screening, the binding would reduce the effective number of charges in the ring of charge. However, since removing the ring of charge has little effect on proton block (Fig. 8), then partially removing charge from the ring of charge by the binding of protons would also be expected to have little effect on proton block. At pH_i 7.0 the percentage reduction of currents due to proton block was slightly less in the presence of the ring of charge than in its absence, suggesting that, if anything, the ring of charge may reduce proton block of BK channels. Thus, the ring of charge is not the site of proton block.

The apparent dissociation constants for proton block of WT BK channels and for mutant BK channels without the ring of charge were 5.1 and 4.9 respectively. Except for the E321 and E324 residues in each of the four

subunits that form the ring of charge, there are no apparent candidate residues with a pK_a close to 5.0 in the intracellular vestibule of the BK channel pore. It is possible that the site of the proton block is a composite site (oxygen atoms in the selectivity filter?), rather than a site provided by a single residue, or that the surrounding structures change the pK_a (Fersht, 1985). Apparent Hill coefficients of 0.29–0.48 when describing the data with a competitive inhibitory model with a cooperativity factor (Eq. 4) suggests a lack of positive cooperativity in the proton block of the BK channel.

Do Protons Block through an Allosteric Mechanism?

In the previous section it was indicated that the surface charge of the membrane lipids is too far away from the pore of the channel to increase the concentration of K^+ or H^+ at the entrance to the vestibule. This conclusion does not rule out that the surface charge of the lipids may play some role in proton block. Park et al. (2003) have proposed that the observed decrease in the conductance of BK channels that occurs when negatively charged lipids are replaced with neutral lipids arises from lipid-induced conformational changes of the selectivity filter that depend on the charge of the lipid. On this basis, protons might reduce conductance through a similar allosteric mechanism by changing the properties of the lipids by binding to the negative charges on the lipid head groups. Arguing against such an allosteric mechanism for proton block is that it is difficult to envision how such a mechanism could give rise to the voltage dependence of the block. Furthermore, such an allosteric mechanism for proton block might be expected to reduce unitary current amplitudes for inward, as well as outward, currents, which is not observed (Habartova et al., 1994). Although if the allosteric mechanism were coupled to depolarization in some manner, then it might not be observed at negative potentials. Protons might also decrease conductance in a different type of allosteric manner by binding to a site within the electric field of the membrane but outside of the pore of the channel. Such an allosteric mechanism could provide voltage-dependent proton block and also competitive inhibition. Whereas direct block of the pore of the channel by protons is a simpler mechanism for proton block, and therefore is to be preferred, our data are not sufficient to exclude more complex allosteric mechanisms.

The Proton Block of BK Channels Contributes to the Sublinearity of Unitary Currents at High Voltages at pH 7.0

We observed that the unitary currents through BK channels increased sublinearly with voltage for depolarizations >100 mV. Our findings indicated that $\sim 40\%$ of this sublinearity arose from proton block for data collected at pH 7.0, and became progressively greater

for more acidic pH_i . Even in the effective absence of protons at pH_i 9.0, the i/V plot still deviated from linearity at high voltages, indicating that some factors besides proton block contribute to the sublinearity of currents at the higher voltages. Sublinearity was still observed in solutions that contained no added buffers, or in solutions with chelators to remove trace amounts of Ca^{2+} or Ba^{2+} , suggesting that some intrinsic property associated with the channel contributes to the sublinearity. This sublinear increase might result from a limitation on the diffusion of K^+ from the bulk solution to the intracellular vestibule of the channel (Lauger, 1976; Andersen, 1983; Kuo and Hess, 1992; Hille, 2001), and/or intrinsic properties of the channel (Yellen, 1984). These points will be dealt with in greater detail elsewhere.

Although proton block could be appreciable under extreme conditions of low pH_i and high voltages, it would be expected that intracellular protons would have little blocking effect on BK channels for physiological voltages and pH. Nevertheless, the marked effect of proton block on unitary currents at the large positive voltages routinely used to investigate mechanisms of selectivity and conductance indicates that proton block needs to be taken into account unless the pH is ≥ 8.0 .

Limits of K^+ Permeation through BK Channels

BK channels have the largest single-channel conductance (250–350 pS) of all known K^+ channels (Marty, 1981; Pallotta et al., 1981; Yellen, 1984; Latorre et al., 1989; Hille, 2001). In spite of their large conductance, BK channels are highly selective for K^+ over Na^+ (Blatz and Magleby, 1984; Yellen, 1984; Eisenman et al., 1986; Latorre et al., 1989; Hille, 2001). The largest unitary currents that we observed in this study were ~ 170 pA with 3.4 M $[K^+]_i$, 150 mM $[K^+]_o$ outside, a voltage of +250 mV, and a pH_i of 9.0. The cord conductance for these conditions was ~ 536 pS at +100 mV. A current of 170 pA requires an average net transfer of ~ 1.1 K^+ ions through the channel per nanosecond. The water molecules of the inner sphere surrounding a K^+ ion are replaced with a time constant of ~ 1 ns (Diebler et al., 1969; Hille, 2001). To enter the selectivity filter most of the hydration shell would have to be removed (Bezannilla and Armstrong, 1972; Hille, 1973) by substitution of the carbonyl oxygen atoms within the selectivity filter for the waters of hydration (Zhou et al., 2001; Hille, 2001; MacKinnon, 2003). Thus, the dehydration rate of K^+ as it enters the selectivity filter for the largest unitary currents that we observed is similar to the time required for substitution of waters in the inner hydration shell of K^+ while K^+ is in the bulk solution.

The maximum conductance for the outward currents that we observed, 536 pS, was essentially the same as

the maximum theoretical conductance of 550 pS calculated by Berneche and Roux (2003) from the structure of the KcsA channel using a transmembrane potential profile from the open MthK channel (Jiang et al., 2002b). Yet, for experimental conditions with 100–150 mM $[K^+]_i$, the unitary currents in BK channels are 3–4 times higher than those in KcsA channels (Blatz and Magleby, 1984; LeMasurier et al., 2001). Why then is the maximum theoretical conductance for KcsA channels the same as the maximum experimentally observed conductance for BK channels? BK channels have a ring of negative charge in the intracellular vestibule that is absent in KcsA channels (Brelidze et al., 2003b; Nimigean et al., 2003). This ring of charge doubles the conductance of BK channels for outward currents (Brelidze et al., 2003b; Nimigean et al., 2003). The effect of the ring of charge on increasing conductance decreases as the $[K^+]_i$ is increased, becoming negligible at very high $[K^+]_i$ (3.4 M), suggesting that the action of the ring of charge is through electrostatic attraction of K^+ to the vestibule (Brelidze et al., 2003b). Because the maximum theoretical conductance for KcsA channels (Berneche and Roux, 2003) and the maximum experimentally observed conductance for BK channels (Fig. 6) were both obtained with high $[K^+]_i$ where the effects of the ring of charge would be negligible, then it would be expected that these maximal estimates would be similar for BK and KcsA if these two ion channels have relatively similar conduction pathways except for the ring of charge.

We thank Dr. Wolfgang Nonner and Dr. Xiaowei Niu for helpful discussions.

This work was supported in part by grants to K.L. Magleby from the Florida Department of Health Biomedical Research Program, B029, and from the National Institutes of Health, AR32805.

Olaf S. Andersen served as editor.

Submitted: 22 September 2003

Accepted: 2 February 2004

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