Probing the Geometry of the Inner Vestibule of BK Channels with Sugars

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The geometry of the inner vestibule of BK channels was probed by examining the effects of different sugars in the intracellular solution on single-channel current amplitude (unitary current). Glycerol, glucose, and sucrose decreased unitary current through BK channels in a concentration- and size-dependent manner, in the order sucrose > glucose > glycerol, with outward currents being reduced more than inward currents. The fractional decrease of outward current was more directly related to the fractional hydrodynamic volume occupied by the sugars than to changes in osmolality. For concentrations of sugars ≤1 M, the i/V plots for outward currents in the presence and absence of sugar superimposed after scaling, and increasing K^+_i from 150 mM to 2 M increased the magnitudes of the i/V plots with little effect on the shape of the scaled curves. These observations suggest that sugars ≤1 M reduce outward currents mainly by entering the inner vestibule and reducing the movement of K^+ through the vestibule, rather than by limiting diffusion-controlled access of K^+ to the vestibule. With 2 M sucrose, the movement of K^+ into the inner vestibule became diffusion limited for 150 mM K^+_i and voltages >+100 mV. Increasing K^+_i then relieved the diffusion limitation. An estimate of the capture radius based on the 5 pA diffusion-limited current for channels without the ring of negative charge at the entrance to the inner vestibule was 2.2 Å. Adding the radius of a hydrated K^+ (6–8 Å) then gave an effective radius for the entrance to the inner vestibule of 8–10 Å. Such a functionally wide entrance to the inner vestibule together with our observation that even small concentrations of sugar in the inner vestibule reduce unitary current suggest that a wide inner vestibule is required for the large conductance of BK channels.

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INTRODUCTION

K^+ channels play a key role in controlling the excitability of nerve and muscle cells (Hille, 2001). Under physiological conditions, the opening of K^+ channels allows K^+ to flow through the channels down its electrochemical gradient from the intracellular to the extracellular solution, creating an outward current that shifts the intracellular potential more negative, restoring the resting potential. Physiological and structural studies have suggested that the conduction pathways of different K^+ channels have some common features (MacKinnon et al., 1998; Patten et al., 1999; Lu et al., 2001). A typical conduction pathway of a K^+ channel is comprised of a narrow selectivity filter that connects a large inner vestibule with a shallow extracellular vestibule (Hille, 1973; Doyle et al., 1998; Zhou et al., 2001). For outward currents, K^+ would first move from the bulk intracellular solution into the inner vestibule, through the inner vestibule to the selectivity filter, through the selectivity filter to the extracellular vestibule, and then from the extracellular vestibule into the bulk extracellular solution (Andersen, 1983a; Hille, 2001; Jiang et al., 2002; Consiglio et al., 2003). Our present study examines the contribution of the geometry of the inner vestibule to the transport of K^+ through large conductance Ca^{2+}- and voltage-activated K^+ (BK) channels.

BK channels are of special interest as they have the largest single-channel conductance of K^+-selective channels (Latorre and Miller, 1983; Latorre et al., 1989; Hille, 2001; Yellen, 2002). The inner vestibule of BK channels readily allows small cations such as Na^+, Tl^+, K^+, NH_4^+, Rb^+, Mg^{2+}, Ca^{2+}, and Sr^{2+} to enter (Blatz and Magleby, 1984; Yellen, 1984a; Eisenman et al., 1986; Ferguson, 1991; Sugihara, 1998). Of these cations, Tl^+, K^+, Rb^+, and NH_4^+ can proceed through the selectivity filter, whereas the other cations act mainly in the inner vestibule as fast or flickery blockers (Blatz and Magleby, 1984; Yellen, 1984a,b; Eisenman et al., 1986; Ferguson, 1991). The entry of such diverse cations into the inner vestibule of BK channels is not surprising, as the vestibule is large enough to accept quaternary ammonium ions such as TEA^+ (Blatz and Magleby, 1984; Yellen, 1984a; Villarroel et al., 1988) and polyamines (Snetkov et al., 1996; Zhang et al., 2004). Other K^+ channels also have inner vestibules with diameters larger than the selectivity filters (Armstrong, 1971; Armstrong and Hille, 1972; Doyle et al., 1998; Zhou et al., 2001), but the inner vestibules of these other K^+ channels may be smaller than that of BK channels (Li and Aldrich, 2004; Webster et al., 2004). Whereas small

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Abbreviation used in this paper: WT, wild-type.

cations can readily enter the vestibule of BK channels, negative charge at the entrance to the inner vestibule of BK channels (Brelidze et al., 2003; Nimigean et al., 2003) and dipolar negative charge directed into the inner vestibule, by analogy to KcsA channels (Doyle et al., 1998; Roux and MacKinnon, 1999; Zhou and MacKinnon, 2004b), would make it electrostatically unfavorable for anions to enter the vestibule. Thus, it appears that the inner vestibule of BK channels forms a cation-selective pathway to carry ions from the intracellular solution to the selectivity filter.

Our study explores further the geometry of the inner vestibule of BK channels by using sugar molecules as probes. Sugars (or polymers) that are smaller than the entrance to the inner vestibule would be expected to enter the vestibule and decrease the conductance by interfering with the passage of the permeating ions (Bezrukov and Vodyanoy, 1993; Sabirov et al., 1993; Vodyanoy et al., 1993; Parsegian et al., 1995; Oh et al., 1997; Qu and Dahl, 2004). Sugar would also be expected to increase the viscosity of the solutions, decreasing the diffusion coefficient of K^+ (Robinson and Stokes, 1970; Weast, 1976). Sugars (or polymers) larger than the entrance to the inner vestibule would be expected to have little effect on the conductance if the flux of K^+ into the vestibule is not diffusion limited in the presence of the sugar (Parsegian et al., 1995).

We used intracellular sugar to probe the inner vestibule of BK channels in three ways: to place a lower limit on the size of the inner vestibule by determining whether sugars can enter the vestibule and interfere with the passage of K^+; to decrease the diffusion coefficient of K^+ to determine whether the unitary current amplitudes saturate at positive voltages when the diffusion of K^+ from the intracellular solution into the inner vestibule is limited; and to estimate the effective entrance diameter of the inner vestibule using unitary currents when K^+ access to the inner vestibule is diffusion limited.

We find that glycerol, glucose, and sucrose decrease outward unitary currents through BK channels in a concentration- and size-dependent manner, in the order sucrose > glucose > glycerol. The decrease in unitary current was not due to an increase in osmolality, but was directly related to the conductivity of the solution and inversely related to the fractional hydrodynamic volume occupied by sugar.

With 150 mM symmetrical KCl and no sugar, the rate of diffusion of K^+ from the intracellular bulk solution into the inner vestibule did not limit outward unitary currents up to the examined voltage of +300 mV. With 2 M sucrose the outward unitary currents saturated at voltages >+100 mV, becoming diffusion limited. This allowed the capture radius of the entrance to the inner vestibule to be calculated (Lauger, 1976; Andersen, 1983a,b; Kuo and Hess, 1992; Hille, 2001), giving a value of 2.2 Å in 2 M sucrose and 150 mM K^+ for BK channels with the ring of negative charge at the entrance to the inner vestibule neutralized by mutation. The capture radius gives the theoretically required dimensions of a cylindrical pore to capture sufficient ions by diffusion to provide for the diffusion-limited current, assuming that the ions are point charges (without physical dimensions). Structures of crystallized K^+ channels suggest that K^+ is hydrated when it enters the inner vestibule (Doyle et al., 1998; Zhou et al., 2001). To take the hydration into account, the radius of hydrated K^+ (6–8 Å) was added to the capture radius (2.2 Å), giving an effective radius for the entrance to the inner vestibule of 8–10 Å. This wide entrance to the inner vestibule and our observation that even small concentrations of sugar in the inner vestibule reduce unitary current suggest that a functionally wide inner vestibule is required for the large conductance of BK channels. Preliminary results have appeared in abstract form (Brelidze and Magleby, 2004a).

MATERIALS AND METHODS

Channel Expression and Mutagenesis

Constructs encoding wild-type BK channels (mSlo1) were provided by L. Salkoff (Washington University, St. Louis, MO) (mbr5; Schreiber et al., 1999) and Merck research laboratories (Pallanck and Ganetzky, 1994) as modified by Merck (McManus et al., 1995). Wild-type (WT) BK channels expressed from these two clones had equal unitary currents.

The cRNA was transcribed using the mMessage mMachine kit (Ambion) and injected into enzymatically separated *Xenopus laevis* oocytes (Dahl, 1992; Hsiao et al., 2001) at ~0.5–2 ng per oocyte, 2–8 d before recording. After injection, oocytes were kept at 16°C in modified OR2 solution (Brelidze and Magleby, 2004b). The vitelline layer of injected oocytes was removed manually before patch clamp recording.

The mutant cDNA construct for the double mutation E321N and E324N was made from the BK channel provided by Merck using the Quick-Change XL Site-directed Mutagenesis kit (Stratagene) and verified by sequencing (DNA Core Lab Sequencing Facility, University of Miami School of Medicine), as previously described (Brelidze et al., 2003).

Solutions

The extracellular (pipette) and intracellular solutions contained (in mM) 150 KCl, 5 *N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES) to buffer pH (adjusted to pH 7.0), and 1 EGTA and 1 N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid (HEDTA) to bind Ca^{2+} to prevent possible block from contaminating Ca^{2+} (Ferguson, 1991). No Ca^{2+} was added to the solutions with the exception of experiments at negative potentials in which Ca^{2+} was added to the intracellular solution for a free [Ca^{2+}] of ~5 μM to facilitate the opening of the channel. This low level of Ca^{2+} has little effect on the inward current amplitude (Ferguson, 1991; Brelidze et al., 2003). The extracellular solution also typically contained 60 μM $GdCl_3$ to block endogenous mechanosensitive channels (Yang and Sachs, 1989). In some experiments, the concentration of intracellular K^+ (K^+_i)

was varied as indicated. Extracellular K^+ was 150 mM in all experiments. Intracellular solutions, where indicated, contained glycerol, D(+)-glucose, or sucrose. Glucose converts between three isoforms in solution, linear, α-ring, and β-ring structures, with the linear form comprising ~0.1% of the total (Mathews and van Holde, 1990). When solutions contained sugar, the molarity of the KCl was kept constant at either 0.15 or 2 M, as indicated. Consequently, the ratio of the number of K^+ ions to water molecules would increase in the presence of sugar (see DISCUSSION).

The osmolality and conductivity of the solutions were measured using a Wescor vapor pressure osmometer 5520 and Yellow Spring Instrument conductance meter model 35, respectively, calibrated with appropriate standards. EGTA and HEDTA were from Fluka, KCl, glycerol, and sucrose from Fisher, and TES, D(+)-glucose, and $GdCl_3$ from Sigma-Aldrich. Solutions were changed using valve-controlled, gravity-fed perfusion of a microchamber (Barrett et al., 1982). Although the effects of the sugars on unitary current amplitude were completely reversible, typically only one type of sugar at one concentration was examined on each patch of membrane because the time required to completely exchange the solutions with sugar was excessive due to the increased viscosity of the solutions.

Fractional Hydrodynamic Volume of Sugars and Conductivity Ratios

The hydrodynamic radii of glycerol, glucose, and sucrose used in this paper, as determined by viscosity measurements, are 3.1, 4.2, and 5.2 Å (Schultz and Solomon, 1961), and include the associated waters of hydration that do not shear with movement. Slightly smaller hydrodynamic radii for these sugars have also been reported (Sabirov et al., 1993). From the hydrodynamic radii, the fractional hydrodynamic volumes of the solution occupied by glycerol, glucose, and sucrose for 1 M solutions were calculated to be 0.075, 0.19, and 0.36, respectively. As expected, these volumes are greater than the occupied fractional volumes of 0.071, 0.11, and 0.21 (Weast, 1976), which do not include waters of hydration. The ratios of the measured conductivities of the intracellular solutions with and without sugar, χ_s/χ_0, are listed in Table I.

TABLE I

Effect of Sugar on Conductivity, Unitary Currents, and Predicted Unitary Currents

Sugar	Glycerol			Glucose			Sucrose		
	χ_s/χ_0	i_S/i_0	Pred.	χ_s/χ_0	i_S/i_0	Pred.	χ_s/χ_0	i_S/i_0	Pred.
400 mM	0.93	0.99	0.97	0.89	0.92	0.92	0.75	0.86	0.86
1 M	0.86	0.89	0.92	0.70	0.83	0.81	0.47	0.62	0.64
2 M	0.72	0.74	0.85	0.43	0.54	0.62	0.14	0.22	0.28

χ_s/χ_0 gives the ratio of the conductivities of the experimental solutions with 150 mM KCl in the presence and absence of sugar, i_s/i_0 gives the observed ratio of unitary current amplitudes at +200 mV in the presence and absence of the indicated sugars, and Pred. is the predicted value of i_s/i_0 obtained with Eq. 5.

Diffusion Coefficient in 2 M Sucrose

The diffusion coefficient of K^+ in 2 M sucrose was required to estimate a capture radius. Diffusion coefficients depend on the temperature, the ionic species, the concentration of the ions in solution, and the viscosity of the solution, which is changed with sugar (Robinson and Stokes, 1970; Hille, 2001).

To estimate the diffusion coefficient for K^+ in the presence of 2 M sucrose, we took advantage of the observation that the single ion diffusion coefficient in dilute aqueous solution is related to the limiting equivalent conductivity (as the concentration goes to zero) by a constant (Hille, 2001). On this basis,

$$D_s/D_0 = \lambda_s/\lambda_0, \quad (1)$$

where D_s/D_0 and λ_s/λ_0 are the ratios of the single ion diffusion coefficients in dilute aqueous solutions and of the limiting equivalent conductivities, respectively, in the presence and absence of sugar. We estimated the ratio of the limiting equivalent conductivities using a solution of 5×10^{-4} M KCl with and without 2 M sucrose, and found $\lambda_s/\lambda_0 = 0.143 \pm 0.004$ at 23°C. The single ion diffusion coefficient of K^+ for a dilute aqueous solution at 25°C is 1.96×10^{-5} cm^2/s (Hille, 2001). Assuming a 2% decrease in diffusion coefficient per degree centigrade (Lide, 1994), the single ion diffusion coefficient for K^+ in a dilute aqueous solution is 1.88×10^{-5} cm^2/s at 23°C. Using this value for D_0 in Eq. 1 together with the ratio of the limiting equivalent conductivity for K^+ with and without 2 M sucrose (from above) gives a single ion diffusion coefficient of K^+ in a dilute aqueous solution with 2 M sucrose of: $D_s = 1.88 \times 10^{-5}$ cm^2/s $\times$ 0.143 = 0.269×10^{-5} cm^2/s at 23°C. To obtain an estimate of the single ion diffusion coefficient of K^+ in other than a dilute solution, it is necessary to correct for the reduction of the single ion diffusion coefficient by the increased concentration of KCl. This was done by assuming that the fractional decrease in the single ion diffusion coefficient for K^+ with increasing KCl is the same as the fractional decrease in the diffusion coefficient of a solution of KCl. Such an assumption should introduce negligible error, as the single ion diffusion coefficients of K^+ and Cl^- are nearly identical. The ratio of the diffusion coefficient for a solution of 150 mM KCl to that for a limiting solution of KCl is 1.841/1.993 = 0.924 (Robinson and Stokes, 1970). The estimated single ion diffusion coefficient of K^+ in a 150 mM KCl solution with 2 M sucrose then becomes $0.924 \times 0.269 \times 10^{-5}$ cm^2/s = 0.25×10^{-5} cm^2/s at 23°C.

Single-channel Recording and Data Analysis

Single-channel currents were recorded using the inside-out configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 200A amplifier (Axon Instruments, Inc.). Data were sampled every 3 μs using a Digidata 1200A and pClamp7 software, or a Digidata 1322A and pClamp9 software (Axon Instruments, Inc.).

Unitary current amplitudes were measured from all-point histograms of the current records using custom software or Clampfit 9.0 (Axon Instruments, Inc.). Unitary current amplitudes from three or more different patches studied under the same conditions were subsequently averaged. Error bars indicate the SEM. Omission of error bars indicates that the SEM was less than the radius of the symbol.

BK channels were identified by 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). However, endogenous BK channels are observed so infrequently (Brelidze and Magleby, 2004b) that they are not expected to contribute to the results of this study. Unitary currents in symmetrical 150 mM KCl without sugar in the intracellular solution were recorded in every experiment for both WT and mutant channels to verify the control response before switching to solutions with sugars. Solutions with high concentrations of sugar are very viscous, so appropriate precautions were taken to assure complete solution changes.

The effective cutoff frequencies for the low pass filtering ranged from 4 to 33 kHz. Experiments were performed at 22–25°C.

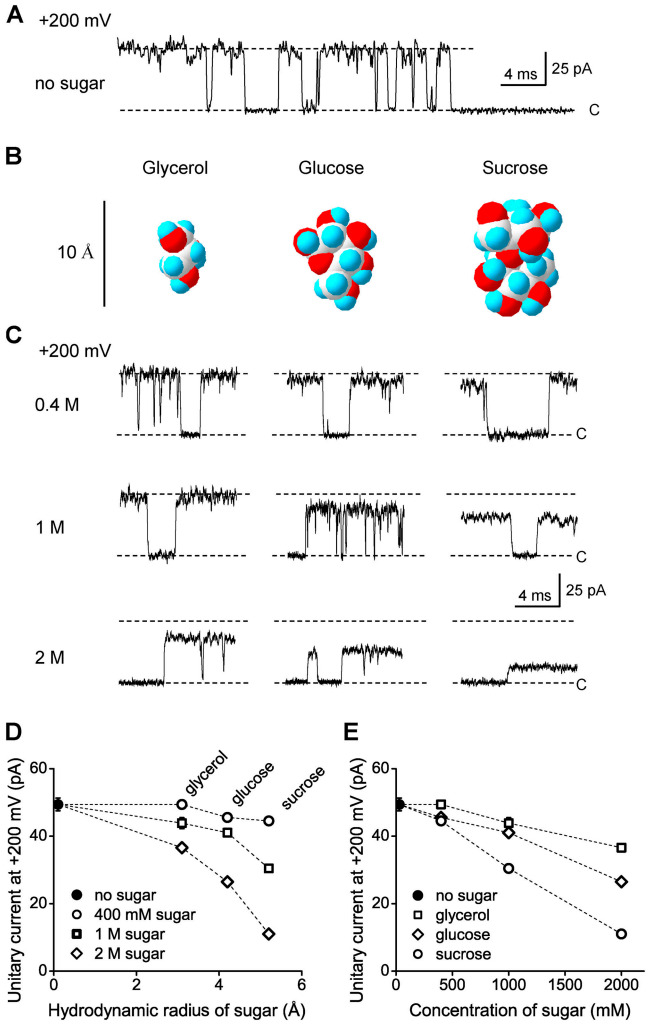

Figure 1. Intracellular sugars decrease outward unitary currents through WT BK channels. (A) Representative outward single-channel currents recorded at +200 mV with no sugar in the intracellular solutions. Closed channel current level is indicated by C. Symmetrical 150 mM KCl and effective low pass filtering of 4 kHz in A and C. (B) Structures of the three sugar molecules used in this study shown as space filling surfaces. The images were obtained with Swiss Protein Viewer using atomic coordinates. The atomic coordinates for glycerol were from ChemFinder structural databases (http://chemfinder.cambridgesoft.com/), and for glucose and sucrose from the library of molecular structures at New York University (http://www.nyu.edu/pages/mathmol/library/library.html). The atoms are identified as: oxygen, red; carbon, white; and hydrogen, cyan. Scaling for the figure was based on dimensions determined from CPK models of the sugars (Harvard Apparatus). (C) Representative outward single-channel currents recorded at +200 mV with sugars in the intracellular solutions at the indicated concentrations. The type of sugar present in the solution for each column of currents in C is shown above the current records in B. (D) Plots of unitary current amplitudes at +200 mV versus the hydrodynamic radii of the different sugars over a range of concentrations, as indicated. The hydrodynamic radii were 3.1 Å, 4.2 Å, and 5.2 Å for glycerol, glucose, and sucrose, respectively, as determined by viscometry (Schultz and Solomon, 1961). (E) Plots of the unitary current amplitudes at +200 mV versus sugar concentration for the indicated sugars. The filled circles in D and E correspond to the unitary current amplitude at +200 mV in the absence of sugar.

RESULTS

Sugars Decrease Unitary Current Amplitudes in BK Channels

This study explores the geometry of the inner vestibule of BK channels using the three sugars, glycerol, glucose, and sucrose, as probes. The scaled structures of these sugars are presented in Fig. 1 B, where it can be seen that molecular size increases as glycerol < glucose < sucrose. Single-channel (unitary) currents recorded at +200 mV with 0.4, 1, and 2 M sugar in the intracellular solution are shown in Fig. 1 C for comparison to current in the absence of sugar in Fig. 1 A. For all three sugars, increasing the concentration reduced the unitary current amplitudes, with glycerol giving the least reduction at each examined concentration, and sucrose the greatest. For example, the least reduction of the unitary currents was by <3% for 0.4 M glycerol, whereas the largest reduction was by 78% for 2 M sucrose. The unitary current amplitudes are plotted against the hydrodynamic radius of the sugars in Fig. 1 D and against the concentrations of the sugars in Fig. 1 E. The hydrodynamic radius of each sugar, as determined by viscosity measurements, gives the radius of a hypothetical sphere whose hydrodynamic behavior is the same as that of the sugar molecule plus the water of hydration, which is too firmly bound to partake in the viscous shearing process (Schultz and Solomon, 1961). Increasing the effective molecular size and/or concentration of the sugar decreased the unitary current amplitude (Fig. 1, D and E). In addition to reducing the unitary current, intracellular sugars also changed the gating kinetics of the channels. The effect on gating will be considered elsewhere.

Sugars do not Produce Discrete Blocking Events

The high concentrations of sugars required to reduce unitary currents (Fig. 1) suggest that either the access rate of sugars to their site of action is extremely slow or that sugars reduce conductance without binding tightly to specific sites. If sugars bound for any appreciable amount of time, then discrete blocking events might be observed. Consistent with the lack of discrete block, the reduction of unitary current amplitudes by sugars was not associated with discrete blocking events or an increase in open channel current noise (Fig. 1, A and C). This is in contrast to the reduction of unitary currents in BK channels by intracellular Na^+, which gives a resolvable flickery block (Yellen, 1984a,b).

Since the examined sugars would be too large to enter the selectivity filter of the channel (see DISCUSSION), their site of action should be confined to a side of the channel intracellular to the selectivity filter. Thus, sugars might be reducing unitary currents by entering the inner vestibule and restricting the passage of K^+ ions to the selectivity filter, and/or by reducing dif-

fusion of K^+ from the bulk intracellular solution into the inner vestibule. Sugars may also be acting in some other manner by changing the structure of the channel to reduce conductance, perhaps through osmotic stress. The following sections will examine these possibilities.

Unitary Current Saturates with 2 M Sucrose and 150 mM K^+ at Voltages >+100 mV

If sugars are decreasing the unitary currents in Fig. 1 by reducing the diffusion of K^+ from the intracellular bulk solution into the inner vestibule of the channel, then the current through the channel should be saturated and not increase with increased voltage because the diffusion of K^+ from the intracellular solution into the inner vestibule will no longer be sufficient to deliver as much K^+ as could be driven through the channel by the high voltages (Lauger, 1976; Andersen, 1983b; Yellen, 1984a; Kuo and Hess, 1992; Hille, 2001). To explore this possibility of diffusion-limited currents (Lauger, 1976; Andersen, 1983b; Yellen, 1984a; Kuo and Hess, 1992; Hille, 2001), plots of unitary current amplitudes were made over a wide range of voltage with 0, 0.4, 1, and 2 M intracellular glycerol, glucose, and sucrose for solutions with 150 mM symmetrical K^+. Sugars reduced unitary current amplitude over the range of examined voltage (Fig. 2, A–C). There was no indication of diffusion-limited currents with 0.4 and 1 M sugar up to the examined +200 mV, as no plateau in unitary current amplitudes with increasing voltage was observed (Fig. 2, A and B). Supporting the lack of diffusion-limited currents for sugars ≤1 M, the unitary current amplitudes in the presence of each sugar could be approximated over the examined voltage range (Fig. 2, A and B, dashed lines) by a direct downscaling of the unitary current versus voltage curve in the absence of sugar (continuous line). If diffusion into the inner vestibule were starting to limit currents, then the observed currents with sugar should have become appreciably less than the scaled currents at the higher voltages because diffusion into the vestibule would not keep pace with the voltage-driven transit of K^+ through the channel.

In contrast, when sugar concentration was increased to 2 M the unitary currents with sugars could no longer be described by simple scaling. For 2 M glycerol and glucose, the unitary currents started to approach diffusion limitation, as indicated by the deviation of the unitary currents from the scaled dashed lines at higher voltages, and with 2 M sucrose the unitary currents appeared to reach a plateau, suggesting saturation (Fig. 2 C). To examine further whether saturation was reached with 2 M sucrose, unitary currents were collected at higher voltages (up to +300 mV), where a clear limit (plateau) of unitary current with increasing voltage was observed (Fig. 2 D). Thus, the saturation in unitary current with increasing voltage suggests that the current is limited by the diffusion of K^+ from the intracellular solution into the inner vestibule for 2 M sucrose, 150 mM symmetrical K^+, and voltages >+100 mV.

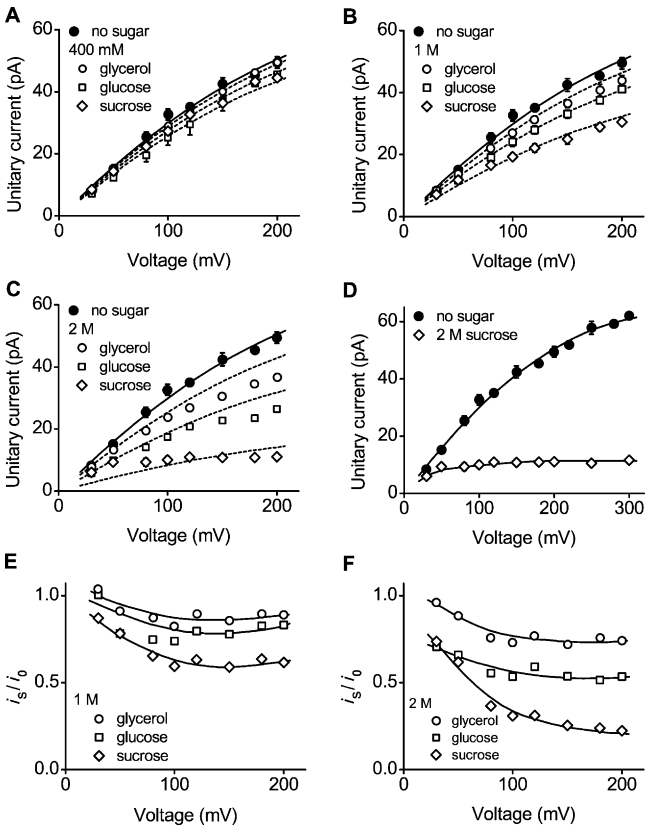

Figure 2. Intracellular sugar decreases outward unitary currents through WT BK channels in a size- and concentration-dependent manner over the examined range of voltages. (A–D) Plots of outward unitary current amplitudes versus voltage without and with the indicated sugar. The continuous lines are polynomial fits to the unitary currents in the absence of sugar, whereas the dashed lines for each sugar are empirical predictions obtained by scaling the continuous lines by a single value obtained from Eq. 5, as listed in Table I in the predicted column. Symmetrical 150 mM KCl. (D) Unitary current amplitudes saturate with 2 M sucrose at voltages >+100 mV with 150 mM K^+_i. (E and F) Outward currents drive sugar into the vestibule, decreasing the currents. Plots of the ratios of the outward unitary currents in the presence and absence of 1 M (E) and 2 M (F) sugar. The lines in D–F have no theoretical meaning.

Fig. 2 E plots the ratio of unitary currents with 1 M sugar to those without sugar versus voltage for data from Fig. 2 B, and Fig. 2 F presents the same type of plots with 2 M sugar for data from Fig. 2 C. For both 1 and 2 M sugar, the ratios initially decreased as the voltage first increased, reaching a plateau that was then maintained (except for 2 M sucrose where the ratios continued to decrease). A decreasing ratio with voltage indicates that the fractional resistance to the outward movement of K^+ increases more in the presence of sugar than in the absence of sugar, whereas a constant

ratio would indicate that any fractional changes in resistance with voltage are the same with and without sugar. The decreasing ratios in Fig. 2 E at the lower voltages are reflected in the small underprediction of the currents by the scaled lines in Fig. 2 B.

Currents with ≤1 M Sugar and 150 mM K^+_i Are Not Diffusion Limited for the Examined Voltages

The observations in the previous section suggested that the unitary currents were not approaching a diffusion limit with 1 M sucrose (Fig. 2 B), whereas with 2 M sucrose the currents became diffusion limited (Fig. 2 D). If this is the case, then increasing intracellular K^+ to 2 M should relieve the apparent diffusion limitation observed with 2 M sucrose by providing additional K^+ to diffuse into the vestibule (Lauger, 1976; Andersen, 1983b; Yellen, 1984a; Hille, 2001). For the same reason, if the currents with 1 M sucrose were not limited by diffusion of K^+ from the intracellular solution into the inner vestibule, then increasing K^+_i should have little effect on the shape of the unitary current amplitude versus voltage (i/V) plots. To test these predictions, unitary currents were collected with 150 mM and 2 M K^+_i for both 1 M and 2 M sucrose and also without sucrose. Examples of unitary currents are presented in Fig. 3 A, and the i/V plots are presented in Fig. 3 (B and D). Increasing K^+_i from 150 mM to 2 M increased unitary current amplitudes in the absence of sucrose (filled circles shifting to filled squares in Fig. 3 B), and also in the presence of both 1 M sucrose (open circles shifting to open squares Fig. 3 B) and 2 M sucrose (open circles shifting to open diamonds in Fig. 3 D), and this was the case over the observed range of voltage.

To determine whether the currents were approaching diffusion limitation with 1 M sucrose, we examined whether increasing K^+_i from 150 mM to 2 M in the presence of 1 M sucrose changed the general shape of the i/V plot. The i/V plot with 1 M sucrose and 2 M K^+_i (open squares in Fig. 3 B) was shifted to the right to compensate for the change in reversal potential and replotted in Fig. 3 C as open squares. The i/V plot with 1 M sucrose and 150 mM K^+_i (Fig. 3 B, open circles) was then upscaled by multiplying by 1.8 over the entire range of voltage, and replotted as open circles in Fig. 3 C. The shapes of the shifted and scaled i/V plots for 150 mM and 2 M K^+_i in the presence of 1 M sucrose approximated each other. For comparison, the unitary currents in the absence of sucrose (Fig. 3 B, filled symbols) were also shifted, scaled, and replotted in Fig. 3 C as filled symbols. The shifted and scaled i/V plots in the absence of sugar approximated each other just as they did in the presence of 1 M sucrose, except that the currents at each K^+ were about twice as large as in the absence of sugar. The absence of saturation in current with sugar at high voltage and the superposition of the shifted and scaled i/V plots at 150 mM and 2 M K^+_i suggests that the currents did not become diffusion limited for sucrose ≤1 M.

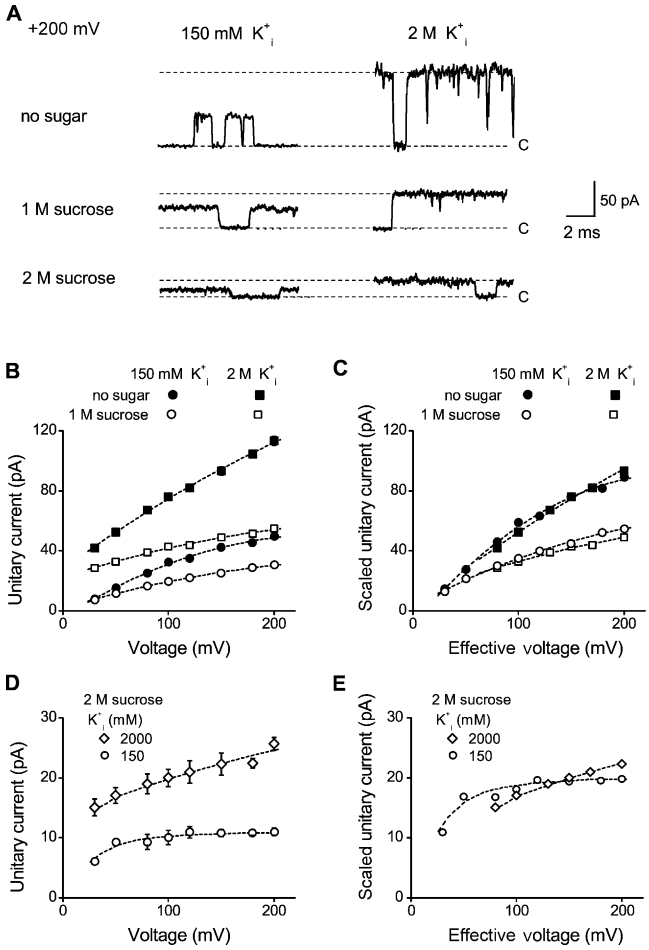

Figure 3. Outward unitary currents are diffusion limited for WT BK channels with 2 M intracellular sucrose and 150 mM K^+_i. (A) Representative outward unitary currents recorded at +200 mV from WT BK channels without and with 1 and 2 M sucrose, for 150 mM and 2 M K^+_i. Effective filtering: 4 kHz. (B) Plots of outward unitary current amplitude versus voltage without and with 1 M sucrose at the indicated K^+_i. (C) Currents with 1 M sucrose and 150 mM K^+_i are not diffusion limited. The data in B are replotted after shifting the 2 M K^+_i data to the right by 50 mV to correct for the shift in reversal potential and scaling the data by 1.8. The data points with 2 M K^+_i for shifted voltages >200 mV are not shown. (D and E) Increasing K^+_i removes the saturation of outward current at high voltage with 2 M sucrose, consistent with diffusion-limited currents in 2 M sucrose with 150 mM K^+_i. The unitary currents with 2 M sucrose and with 150 mM K^+_i or 2 M K^+_i are plotted in D. The data in D are then replotted in E after shifting and scaling the data in the same manner as for C. The lines in B–D have no theoretical meaning.

Currents with 2 M Sucrose are Diffusion Limited for 150 mM K^+_i

In contrast to the above observations with 1 M sucrose and 150 mM K^+_i where the currents did not saturate,

and increasing K^+_i from 150 mM to 2 M had little effect on the scaled shape of the i/V plot (Fig. 3, B and C), with 2 M sucrose the currents did saturate, and increasing K^+_i from 150 mM to 2 M relieved the saturation, changing the shape of the i/V plot. This is shown in Fig. 3 D where the saturation (plateau) in unitary current with 150 mM K^+_i and 2 M sucrose for voltages >+100 mV (open circles) is absent with 2 M K^+_i and 2 M sucrose over the examined voltage range (open diamonds). Replotting the data from Fig. 3 D in Fig. 3 E after shifting the data with 2 M K^+_i to the right to compensate for the shift in reversal potential (open diamonds) and up-scaling the data with 150 mM K^+_i by 1.8 (open circles) illustrates that the saturation of unitary currents with 2 M sucrose and 150 mM K^+_i is relieved when K^+_i is increased to 2 M.

Increasing K^+_i to relieve the saturation of unitary currents at high voltages would also increase the ionic strength, leading to the screening of charges on the proteins and lipids (Hille, 2001). It is unlikely, however, that charge screening rather than increased entry of K^+ into the vestibule is responsible for relieving the saturation of currents by 2 M K^+_i because increasing K^+_i from 150 mM to 2 M with or without 1 M sucrose had little effect on the scaled shape of the i/V plots (Fig. 3 C). Thus, increases in ionic strength per se would not be expected to change the shape of the i/V plots sufficiently to relieve the saturation. Consistent with this possibility, Park et al. (2003) have found that the surface charge on the lipids is not sensed by ions in the inner vestibule of BK channels.

Estimating the Capture Radius of BK Channels in the Presence of 2 M Sugar

The findings in the previous sections indicate that unitary currents with 150 mM K^+_i were not diffusion limited with 1 M sucrose at the examined voltages, but were diffusion limited with 2 M sucrose. When currents are diffusion limited, it is possible to estimate a capture radius that gives a measure of the effective size of the entrance to the pore (Lauger, 1976; Andersen, 1983a,b; Kuo and Hess, 1992; Hille, 2001). The theoretical approach for estimating the capture radius assumes a cylindrical neutral pore (Hille, 1970; Lauger, 1976). BK channels do not have a neutral pore but have a ring of eight fixed negative charges at the entrance to the inner vestibule that doubles the outward unitary current amplitudes when K^+_i is 150 mM (Brelidze et al., 2003; Nimigean et al., 2003). To estimate capture radius we first neutralized the ring of charge by replacing the glutamates with neutral asparagines using the double mutation E321N/E324N on each of the four α subunits (Brelidze et al., 2003).

With the ring of negative charge neutralized, the unitary current amplitudes with 150 mM K^+_i and no sugar

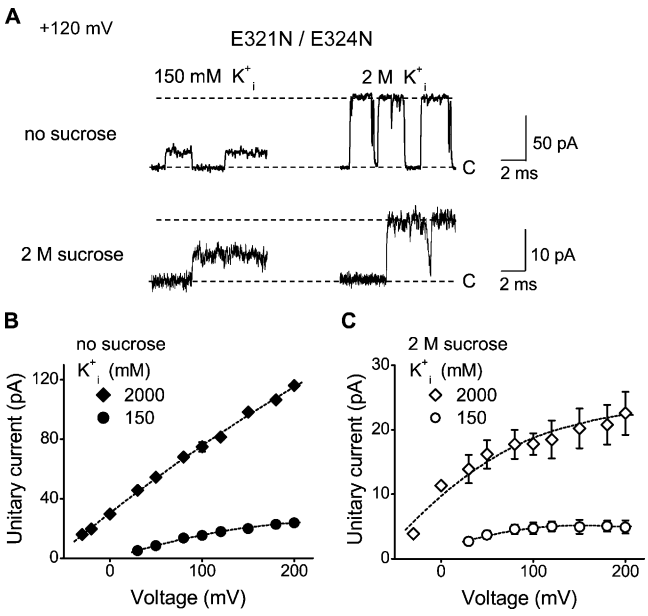

Figure 4. Outward unitary currents with 2 M intracellular sucrose and 150 mM K^+_i are diffusion limited for mutant BK channels without the ring of negative charge. (A) Representative outward single-channel currents recorded at +120 mV from E321N/E324 mutant channels with the indicated K^+_i in the absence and presence of 2 M sucrose. Effective filtering: 4 kHz. (B and C) Plots of outward unitary current amplitudes versus voltage for E321N/E324N mutant channels in the absence (B) and presence (C) of 2 M sucrose at the indicated K^+_i. High K^+_i removes the saturation observed with 2 M sucrose. The lines in B and C have no theoretical meaning.

were reduced to about half, from 35 pA for the WT channel to 18 pA for the mutant channel at +120 mV (compare filled circles in Fig. 3 B with those in Fig. 4 B), because the neutralized ring of charge no longer attracted K^+ into the vestibule (Brelidze et al., 2003; Nimigean et al., 2003). The addition of 2 M sucrose then further reduced the unitary currents in the mutant channel to 5.0 pA at +120 mV (Fig. 4, A and C, note changes in scale). Increasing K^+_i to 2 M then increased the unitary currents with 2 M sucrose to 18 pA at +120 mV (Fig. 4, A and C). As was observed for WT channels with their ring of negative charge, unitary currents for mutant channels without the ring of charge did not saturate with 150 mM K^+_i and no sugar (Fig. 4 B, filled circles) but did saturate with 150 mM K^+_i and 2 M sugar (Fig. 4 C, open circles). Increasing K^+_i to 2 M then relieved the saturation of current for the mutant channels (Fig. 4 C, diamonds), suggesting that the plateau in unitary current in the mutant channel with 150 mM K^+_i and 2 M sucrose was due to diffusion-limited movement of K^+ into the channel. Although 2 M K^+_i relieved the saturation, the observation that the curvature of the i/V curve was more pronounced with 2 M sucrose than without 2 M sucrose, indicates that the increased K^+_i did not entirely remove the effects of the

sugar on limiting the flow of current at higher voltages for the mutant channel (see DISCUSSION).

Having established that the outward current was diffusion limited in mutant BK channels with 150 mM K^+_i and 2 M sucrose, it was then possible to estimate a capture radius for the intracellular entrance to the conduction pathway. The maximal diffusion-limited current with hemispheric access to a cylindrical pore without fixed charge, i_{DL}, is:

$$i_{DL} = 2\pi z e_0 r_c D c, \quad (2)$$

where z is the valence of the permeant ion, e_0 is the value of an elementary charge, r_c is the capture radius, D is the diffusion coefficient, and c is the bulk concentration of the permeant ion expressed as ions/cm^3 (Hille, 1970; Lauger, 1976; Andersen, 1983b; Kuo and Hess, 1992). If the permeant ion were a point charge with no physical size, then r_c would be the required radius of the pore to give the limiting current. However, since the permeant ion itself has physical dimensions, then the effective radius r_E of the entrance to the pore would have to be increased by the radius of the permeant ion to allow entry of all ions whose centers strike the area enclosed by the capture radius, such that

$$r_E = r_c + r_i, \quad (3)$$

where r_i is the radius of the permeant ion (Lauger, 1976; Andersen, 1983a). For the data in Fig. 4 C with i_{DL} of 5.0 pA, 150 mM K^+_i and a diffusion coefficient for K^+ in 2 M sucrose of 0.25×10^{-5} cm^2/s (see MATERIALS AND METHODS), the calculated r_c would be 2.2 Å (Eq. 2). Given an atomic radius for K^+ of 1.33 Å and assuming that no waters of hydration surround K^+, the effective radius of the entrance to the inner vestibule, r_E, would be 3.53 Å. However, K^+ would most likely enter the vestibule of BK channels with its waters of hydration attached, so that r_E would be 8–10 Å (see DISCUSSION).

Sugars Decrease Inward Unitary Current Less than Outward Unitary Current

The previous sections showed that increasing the molecular size and concentration of sugar progressively decreased the outward currents until the currents became diffusion limited with 2 M sucrose at high voltages. The sugar-induced reduction of current that occurred before the diffusion limit was reached would be consistent with sugar entering the inner vestibule and slowing the movement of K^+ through the vestibule to the selectivity filter. Such an additional resistance to the flow of K^+ through the channel might be expected to scale with the concentration and molecular size of the sugars. An alternative explanation is that sugars may act to change the structure of the channel in some manner

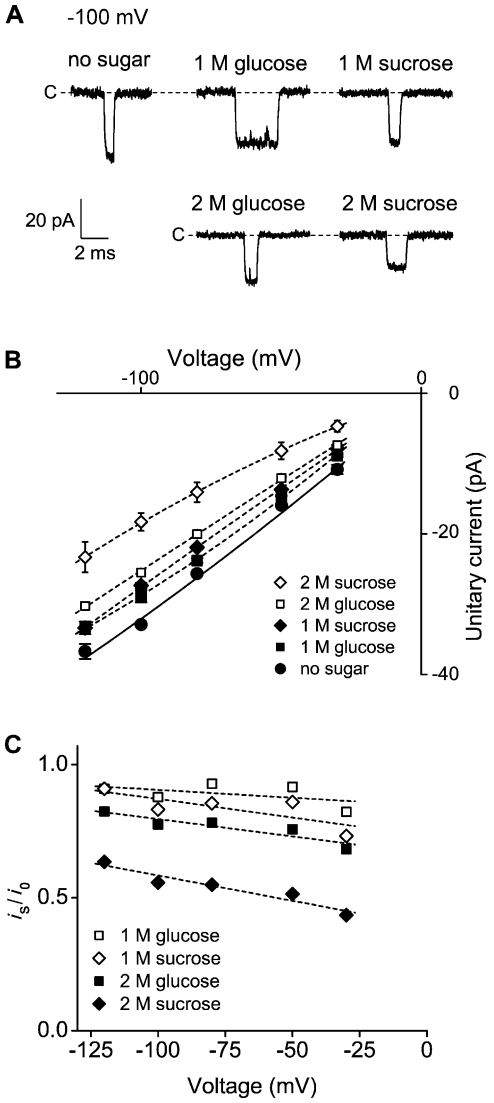

Figure 5. Sugars differentially decrease inward and outward unitary currents in WT BK channels. (A) Representative inward single-channel currents recorded at −100 mV from WT BK channels with and without the indicated sugars. Effective filtering: 4 kHz. (B) Plots of inward unitary current amplitudes versus voltage with and without the indicated sugars. (C) Inward currents drive sugar out of the vestibule. Plots of the ratios of the inward unitary currents in the presence of the indicated sugars (i_s) to the inward unitary currents in the absence of sugars (i_0) versus voltage. Symmetrical 150 mM K^+_i. The lines in B and C have no theoretical meaning.

to decrease the flux of K^+ through the selectivity filter. If such a structural change were the major mechanism of action of sugars, then sugars might be expected to reduce inward currents similar to the reduction of outward currents. To explore this possibility, the action of intracellular sugars on inward unitary current amplitudes was examined. Sugars did decrease inward unitary current amplitudes, but the decrease was less than for outward currents (compare Fig. 5 A and Fig. 5 B to

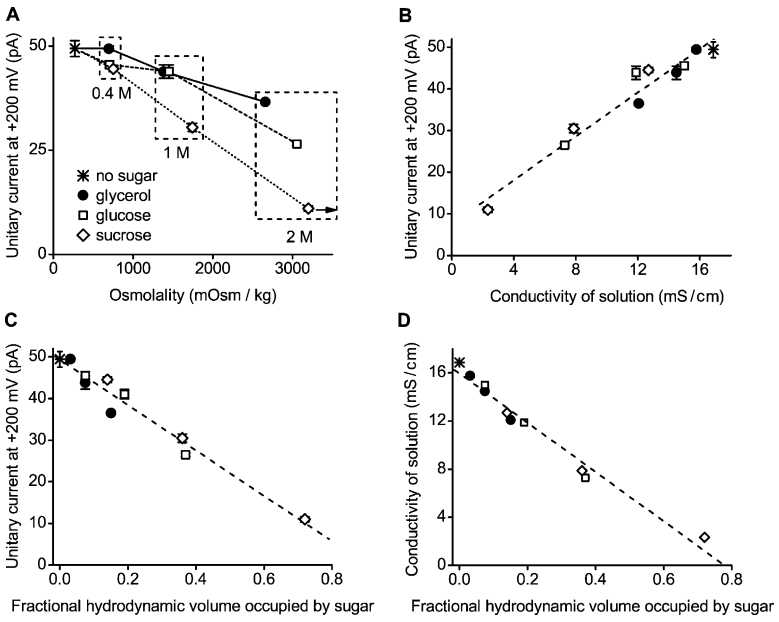

Figure 6. The reduction of outward unitary currents in WT BK channels is strongly correlated with sugar-induced changes in diffusion coefficient, conductivity, and fractional hydrodynamic volume occupied by the sugar, but not osmolality. (A–C) Plots of the outward unitary current at +200 mV versus osmolality (A), conductivity (B), and fractional hydrodynamic volume of the solution occupied by sugar (C). The arrow in A indicates that the osmolality of the solution with 2 M sucrose was greater than the plotted maximum value that the osmometer could read. (D) Plot of conductivity versus fractional hydrodynamic volume. Dashed lines in B, C, and D are linear regression fits to the all the plotted points, with correlation coefficients, R, of 0.97, −0.98, and −0.99 respectively. For B, C, and D, the data for each sugar are presented for concentrations of 0.4, 1, and 2 M. As the concentration of each sugar increases, the conductivity of the solution decreases and the fractional hydrodynamic volume occupied by each sugar increases.

Fig. 1 C and Fig. 2, B and C). For example, at −120 mV, 1 M glucose decreased inward unitary current by 9% compared with a 20% decrease for outward unitary currents at +120 mV, and 2 M sucrose decreased inward unitary current by 37% compared with a 69% decrease for outward unitary current.

In contrast to the saturation of outward unitary current at large driving forces with 2 M sucrose (Fig. 2 D), a saturation of inward currents was not observed (Fig. 5 B). Thus, sugars have a differential effect on inward and outward currents. This differential effect is clearly evident in plots of the ratio of the unitary current amplitudes with sugar to those without versus voltage. For inward currents, these ratios increased as the absolute magnitude of the inward driving force increased (Fig. 5 C), indicating that the resistance to the inward passage of K^+ through the channel decreased in the presence of sugar as the inward flux of K^+ through the channel increased. For outward currents, the ratios of the unitary currents with sugar to those without decreased to a plateau (or continued to decrease in 2 M sucrose) as the outward driving force was increased (Fig. 2, E and F), indicating that the resistance to outward passage of K^+ through the channel in the presence of sugar increased as the outward flux of K^+ through the channel increased.

Do Osmotic Forces Contribute to the Reduction of Unitary Currents by Sugars?

Sugars at the molar concentrations used in the above experiments would have marked effects on the osmolalities of the solutions. If osmolality is a major factor determining conductance, then the reduction of unitary current amplitudes should be similar for solutions of the same osmolality. To examine this possibility, we measured the osmolalities of the various solutions and plotted unitary current amplitude versus osmolality in Fig. 6 A for data at +200 mV, where the boxes enclose data from different sugars at the same concentrations. Although there was a general decrease in unitary current with increasing osmolality, some of the unitary currents were of similar amplitude with markedly different osmolality, such as 1 M sucrose and 2 M glucose. If osmolality were the major factor in determining unitary current amplitudes, then all of the plotted points in Fig. 6 A should fall on a single regression line. The lack of such correlation indicates that the major action of sugars on reducing current is not through changes in osmolality. A similar lack of correlation was found for data obtained at +80 mV (unpublished data).

Nevertheless, there was a general decrease in unitary current amplitude with increasing osmolality (Fig. 6 A). Such a decrease would be expected because increasing the concentration of sugar increases the viscosity of the solutions (Weast, 1976), which decreases the mobilities of ions, giving rise to a decreased conductivity and decreased diffusion coefficients (Robinson and Stokes, 1970; Hille, 2001). To explore this possibility, the unitary currents at +200 mV were plotted against the conductivities of the solutions in Fig. 6 B. There was a strong correlation between unitary current and conductivity (R = 0.97). A similar strong correlation was also found for data obtained at +80 mV, except that the point for 2 M sucrose tended to be a little above the regression line (not depicted) rather than a little below the regression line as observed at +200 mV (leftmost point in Fig. 6 B).

Because the sugar-induced reduction of unitary current would be expected to be related in some manner

to the volume displaced by the sugar molecules with their associated waters of hydration (Robinson and Stokes, 1970), we also plotted unitary current versus the fractional hydrodynamic volume of the solution occupied by the sugar and its waters of hydration (see MATERIALS AND METHODS), and found a strong negative correlation ($R = -0.98$), indicating that the unitary currents decreased as the fractional hydrodynamic volume of the sugars increased (Fig. 6 C). The strong correlations in Fig. 6 (B and C) also suggest that the conductivity of the solutions would be related to the fractional hydrodynamic volume displaced by sugar. This was found to be the case (Fig. 6 D), with a strong negative correlation coefficient ($R = -0.99$).

Empirical Description of the Reduction of Unitary Current by Sugars

The relationship between fractional hydrodynamic volume of the sugar and the unitary currents was approximated by the dashed line in Fig. 6 C, calculated with the following equation

$$i_S/i_0 = 1 - C_S V_F B, \quad (4)$$

where i_S/i_0 is the ratio of the unitary current with and without sugar, V_F is the fractional hydrodynamic volume occupied by the specified sugar for a 1 M concentration, C_S is the molar concentration of the sugar, and B is a constant with a value of 1.11. Whereas Eq. 4 is an empirical description, it is consistent with a simple physical model for the action of sugar. The observations in the previous sections suggest that sugar molecules enter the inner vestibule. Once in the vestibule, the sugar molecules with their waters of hydration would reduce the effective volume of the vestibule available for diffusion of K^+ to the selectivity filter. For a vestibule of fixed length, the average cross-sectional area of the vestibule available for diffusion would be proportional to the volume of the vestibule. If sugars are at the same concentration in the vestibule as in the bulk solution, then the fractional reduction in volume of the vestibule would be approximated by the fractional volume of the bulk solution occupied by sugar. If the unitary current is proportional to the volume of the vestibule not occupied by sugar, then

$$i_S/i_0 = 1 - C_S V_F, \quad (5)$$

which is of the same form as Eq. 5, except for the constant B. Eq. 5 indicates that the currents would go to zero when the vestibule is 100% filled with sugar. Because K^+ itself has physical dimensions and would require space to diffuse, the current would go to zero before the vestibule were fully filled with sugar, giving a possible physical basis for the constant B in Eq. 4. $B = 1.11$ would then suggest that to reduce currents to near zero, sugar with associated waters of hydration would need to occupy only ~90% of the volume of the vestibule.

The dashed lines in Fig. 2 (A–C) show that Eq. 5 approximated the reduction in outward current for concentrations of glycerol, glucose, and sucrose ≤1 M and for 2 M glycerol and glucose up to +100 mV. For sugar of 1 M and voltages ≤+50 mV, Eq. 5 underpredicted the currents somewhat (Fig. 2 B), as would be expected from the changing ratios in Fig. 2 E. Eq. 5 overpredicted the currents for 2 M glycerol and glucose for voltages >+100 mV, and Eq. 5 did not describe the currents with 2 M sucrose, suggesting, as would be expected, that factors in addition to those accounted by the Eq. 5 are involved in the reduction of current by intracellular sugar. Such factors would include an increased length of the effective diffusion pathway through the vestibule in the presence of sugar, the possible concentration of sugar in the vestibule by the outward flux of K^+ through the channel (Fig. 2 E, see DISCUSSION), a diffusion-controlled limit on the rate of entry of K^+ into the vestibule at high voltages and 2 M sucrose, the fact that K^+ itself occupies volume, requiring a minimal space in order to diffuse (as discussed above), and that Eq. 5 ignores the resistance of the selectivity filter (see DISCUSSION). Nevertheless, Eq. 5 provides a description of the reduction of outward unitary currents by sugar over limited ranges of experimental conditions without the use of free parameters: the values of C_S and V_F used in Eq. 5 are set by the hydrodynamic volumes of the specific sugars and their concentrations.

DISCUSSION

This study probed the geometry of the inner vestibule of BK channels by applying sugars of different sizes and concentrations to the intracellular side of the channel. All experiments were performed with single-channel recording so that the effects of sugars on the unitary current amplitudes could be clearly separated from any effects on gating. In addition, unitary currents obtained at positive potentials were recorded in the presence of Ca^{2+} chelators to prevent block of currents by contaminant Ca^{2+} (Marty, 1981; Oberhauser et al., 1988; Ferguson, 1991; Cox et al., 1997). We found that high concentrations of intracellular sugars (hundreds of mM to M) decreased both outward and inward unitary current amplitudes in a size- and concentration-dependent manner. The effectiveness of current reduction was sucrose > glucose > glycerol, and the block of outward currents was greater than of inward currents. In the presence of 2 M sucrose, outward unitary currents saturated at voltages >+100 mV, revealing diffu-

sion-limited currents of 10.9 pA for WT BK channels and 5.0 pA for BK channels in which the ring of negative charge at the entrance to the inner vestibule was removed by substitution of uncharged amino acids (E321N/E324N). The diffusion-limited current for BK channels without the ring of charge was used to calculate a capture radius of 2.2 Å in the presence of 2 M sucrose.

Sugars Do Not Reduce Current through Specific Binding

The lack of increased open channel noise and/or flickery block when currents were reduced by sugars (Fig. 1) suggests that sugars are not acting by binding tightly to specific sites that block K^+ ion permeation through the pore. The high concentrations of sugars required to reduce the current also argue against tight binding. Sugars are also unlikely to enter or bind within the selectivity filter, as the expected diameter of the selectivity filter for K^+-selective channels of ~3.3 Å (Hille, 1973; Doyle et al., 1998; Hille, 2001; Zhou et al., 2001) is less than the size of the sugars used in our study. Thus, a more general action of sugars than tight binding to specific blocking sites is required to reduce current.

Sugars do not Reduce Current through Structural Changes or Osmotic Effects

Sugars in concentrations similar to those used in our study can stabilize protein structure (Gekko and Timasheff, 1981; Arakawa and Timasheff, 1982; Hall et al., 1995; Davis-Searles et al., 2001) by extracting the lubricant water that maintains conformational flexibility (Priev et al., 1996) and also through van der Waals contacts and hydrogen bonds between the sugars and the protein (Kullman et al., 2002). In addition, glycerol/water mixtures can change the properties of lipids (Johansson et al., 1993). If these potential actions of sugar resulted in changes in the structure of the conduction pathway of the channel, especially of the selectivity filter, then this could lead to a decrease in unitary current. Arguing against such a mechanism as the major action of sugars is the asymmetric effect of sugars on inward and outward currents. The observation of saturation of unitary currents with 2 M sucrose at high positive voltages and the absence of saturation at high negative voltages is consistent with sugar acting to reduce the diffusion of K^+ from the bulk solution to the selectivity filter for the outward currents (see below), but would be difficult to account for by sugar-induced changes in the structure of the selectivity filter, which might be expected to have similar effects on both inward and outward currents. Thus, although we cannot rule out that sugar-induced structural changes may contribute to the reduction of unitary currents, our observations suggest that the major action of sugars on reducing currents is through other mechanisms.

The intracellular application of sugars in our experiments creates an osmotic gradient across the channel, and hence, a small streaming potential that would shift the reversal potential to the right along the voltage axis. The largest shift expected in our experiments (for 2 M sugar) would be ~2 mV (Alcayaga et al., 1989). This small shift in the reversal potential due to osmotic forces would have little effect on the measured magnitudes of the unitary currents in our study.

Further support that the sugar-induced change in osmolality is not a major cause of the reduced currents with sugars is our observation that the reduction of unitary current was strongly correlated with two interrelated factors, the fractional hydrodynamic volume of the solution occupied by the sugars and the conductivity of the solutions, but only weakly correlated with the osmolality of the solutions (Fig. 6).

Sequence of Outward Movement of K^+ through BK Channels

The transition of K^+ ions through BK channels to generate outward currents involves (1) movement of K^+ from the bulk intracellular solution into the inner vestibule; (2) movement through the vestibule to the selectivity filter; (3) entering the selectivity filter; (4) movement from the intracellular to the extracellular side of the selectivity filter; and (5) movement from the extracellular side of the selectivity filter into the bulk extracellular solution (Andersen, 1983a; Berneche and Roux, 2001; Hille, 2001; Morais-Cabral et al., 2001). Discussion in the previous sections suggested that the major action of sugars was not through changes in osmolality or through specific binding at the entrance to or within the selectivity filter. Consequently, sugars could be reducing currents by slowing the movement of K^+ from the bulk solution to the selectivity filter (steps 1 and 2 above). Slowing the movement of K^+ would reduce the concentration of K^+ at the entrance to the selectivity filter, decreasing step 3 above. Since entry of K^+ into the intracellular side of the selectivity filter is required for the transit through and exit of K^+ from the extracellular side of the selectivity filter, then slowing the movement of K^+ from the bulk solution to the selectivity filter would also slow steps 4 and 5 above.

Diffusion-limited Currents and the Capture Radius

Aqueous solutions containing sugar have greater viscosity than pure water, decreasing the diffusion coefficient for added ions (Robinson and Stokes, 1970; Weast, 1976). If the diffusion of K^+ were slowed sufficiently, then at very high positive voltages, the diffusion of K^+ from the intracellular bulk solution into the inner vestibule would be insufficient to replace the outward flux of K^+ driven through the channel by the high electric

field. Consequently, the current would become diffusion limited, reaching a maximum (saturating) current that does not increase with further increases in voltage (Lauger, 1976; Andersen, 1983a,b; Kuo and Hess, 1992; Hille, 2001). Saturating outward unitary currents were observed with 2 M intracellular sucrose for voltages >+100 mV (Fig. 2 D). The saturating current was then relieved by providing more K^+ to diffuse from the bulk intracellular solution into the inner vestibule by increasing K^+_i from 150 mM to 2 M (Fig. 3 D and Fig. 4 C), indicating diffusion-limited outward currents with 2 M sucrose.

When currents through an uncharged cylindrical pore are diffusion limited, it is possible to estimate a theoretical capture radius for the entrance to the pore (Lauger, 1976; Andersen, 1983a,b; Kuo and Hess, 1992; Hille, 2001), where the capture radius specifies the size of a pore that would be required to capture sufficient ions by diffusion to provide the limiting current (Eq. 2). The calculation requires the absence of fixed charge at the entrance to the pore. Consequently, we replaced the ring of charge at the inner vestibule of BK channels with neutral amino acids (Brelidze et al., 2003) and remeasured the saturating current. The observed saturating current of 5.0 pA (Fig. 4 C) gave an estimated capture radius of 2.2 Å (Eq. 2).

If BK channels have four pore helices that direct their COOH-terminal end charge toward the center of the inner vestibule, as KcsA channels have (Doyle et al., 1998; Roux and MacKinnon, 1999; Zhou and MacKinnon, 2004b), or other structures that increase the electrostatic negativity of the inner vestibule, then this negativity could attract K^+ ions into the vestibule, leading to larger currents and an overestimate of the capture radius. Electrical gradients in the convergence region to the entrance of the intracellular vestibule arising from the imposed electrical potential could also increase flux of K^+ into the vestibule, leading to an overestimation of the capture radius. On the other hand, unless the potential drop immediately within the inner vestibule is sufficiently strong to counter back diffusion, some K^+ may diffuse back out of the vestibule to the intracellular bulk solution, so that all ions that diffuse into the vestibule are not captured, leading to an underestimation of the capture radius. Also, sugars in the vestibule would increase the resistance for diffusion in the vestibule. The relative contributions of these various factors to errors in the estimate of the capture radius are not clear, but our observation that the saturating current remains relatively constant over a wide voltage range (Fig. 2 D and Fig. 4 C) suggests that errors from these possible factors, all of which would be voltage dependent, are either small or they cancel out.

Why the Entrance to the Inner Vestibule of BK Channels Is so Big

Eq. 2 for the capture radius assumes that the ions are point charges. To take the physical size of the permeant ion into account, the capture radius has to be increased by the radius of the permeant ion to give the effective radius (r_E in Eq. 3) for the entrance to the inner vestibule. If K^+ enters the vestibule without its waters of hydration, then the capture radius would be increased by the radius of K^+ (1.33 Å) to give an r_E of 3.53 Å for a diameter of 7.1 Å. It is most likely, however, that K^+ enters the vestibule of BK channels with its waters of hydration intact, as the crystallized structure of MthK, a large conductance Ca-modulated K^+ channel, shows a wide entrance of ~20 Å connecting the water in the bulk intracellular solution with the inner vestibule (Jiang et al., 2002), and eight waters of hydration can be observed surrounding K^+ in the inner vestibule of Fab-coordinated KcsA K^+ channels (Zhou et al., 2001; MacKinnon, 2003).

The observed eight waters of hydration are those in a consistent position to be captured by the x-ray crystallography. The effective number of waters of hydration surrounding each K^+ may approach 12 (Hille, 2001). Thus, the hydrated radius of K^+ may approach 6–8 Å (Zhou et al., 2001; Fig. 10.4 in Hille, 2001), in which case the effective radius r_E would be in the range of 8–10 Å for the calculated capture radius of 2.2 Å (Eq. 3). An r_E of 8–10 Å would give an estimated diameter to the entrance of the inner vestibule in BK channels of ~16–20 Å, in general agreement with the structure of MthK. A wide entrance into the inner vestibule of BK channels is also consistent with the observations that quaternary ammonium compounds readily enter the vestibule (Blatz and Magleby, 1984; Villarroel et al., 1988), including those as large as decyltriethylammoniuim (Li and Aldrich, 2004).

The above observations suggest why the diameter of the inner vestibule of BK channels is so large compared with the diameter of the selectivity filter. Since the entrance to the inner vestibule from the bulk intracellular solution is not associated with a special coordination structure to replace the waters of hydration, as at the entrance to the selectivity filter (Doyle et al., 1998; Roux and MacKinnon, 1999; Morais-Cabral et al., 2001), K^+ must enter the vestibule with its waters of hydration intact, as the energetic costs to physically strip the waters of hydration without such coordination sites would be prohibitively high (Hille, 2001). Consequently, the entrance to the inner vestibule must be sufficiently wide so that sufficient hydrated K^+ can diffuse into the vestibule to supply the needed current. Furthermore, once the hydrated K^+ enters the vestibule it must then diffuse to the selectivity filter. When K^+ diffuses, its waters of hydration are readily replaced with those in the surrounding water, facilitating diffusion (Hille, 2001).

Thus, a wide inner vestibule is required to first capture the large diameter hydrated K^+ from the bulk solution and then to facilitate its diffusion through the water-filled vestibule to the selectivity filter. For MthK, the ~20-Å-wide entrance to the inner vestibule narrows to ~12 Å halfway through the vestibule, and maintains this diameter until the entrance to the selectivity filter (Jiang et al., 2002). Considering that the hydrated diameter of a single K^+ is in the range of 12–16 Å, the waters of hydration would act to center the K^+ in the middle of the vestibule lined up with the entrance to the selectivity filter, as previously observed (Zhou et al., 2001). A BK channel can carry 170 pA of outward unitary current at +250 mV with 3.4 M K^+_i, or ~1 ion per nanosecond (Brelidze and Magleby, 2004b). Consequently, the diffusion of K^+ from the bulk solution into and through the inner vestibule to the selectivity filter, the removal of waters of hydration as K^+ enters the selectivity filter, the transit through the selectivity filter, and the exit of K^+ to the extracellular solution can all occur in ~1 nS.

The saturating unitary current in the WT BK channel, at 10.9 pA, was more than twice the 5.0 pA observed when the ring of negative charge was neutralized (Figs. 2 and 4). Thus, the ring of negative charge approximately doubles the diffusion-limited outward current in the presence of 2 M sucrose, similar to the doubling of the nondiffusion limited outward currents in the absence of sucrose (Brelidze et al., 2003; Nimigean et al., 2003). From Eq. 2 it can be seen that to double the diffusion-limited current in the absence of the ring of negative charge would require a doubling of the capture radius. Thus, the ring of negative charge allows the capture radius of the inner vestibule to be half the size that it would need to be in the absence of the ring of charge to carry the same current.

The observation that the negative ring of charge doubles the unitary current suggests that the diameter of the entrance to the inner vestibule of BK channels would limit current in the absence of charge. For KcsA channels without the ring of charge, the diameter of the entrance into the inner vestibule may be a major factor controlling the currents (Chung et al., 2002; Nimigean et al., 2003), as it is for BK channels (see below). In contrast, the rate of ion exit from the selectivity filter in Kv2.1 may be a major rate limiting step for Kv2.1 channels (Consiglio et al., 2003).

Sugar in the Inner Vestibule Reduces Outward and Inward Unitary Current

The above discussion suggests that the inner vestibule would be large enough for sugars to enter. On this basis, the presence of sugars in the vestibule would be expected to interfere with the passage of K^+ from the intracellular bulk solution through the vestibule to the selectivity filter. Our data support this conclusion. For sucrose ≤1 M, glycerol and glucose ≤2 M, and for voltages ≤200 mV, diffusion-limited currents were not observed, yet sugars still reduced the unitary currents (Figs. 1 and 2). Because the diffusion of K^+ from the bulk solution into the vestibule was not rate limiting for these experiments, then these observations suggest that sugars are entering the vestibule and slowing the transit of K^+ through the vestibule to the selectivity filter.

Sugars within the vestibule would reduce the effective area available for diffusion through the vestibule. An empirical model that assumed that the fractional reduction of outward current was proportional to the fractional volume of the vestibule occupied by sugar and associated waters of hydration approximated the reduction in outward current by glycerol, glucose, and sucrose for sugar concentrations ≤1 M for examined voltages up to +200 mV and for 2 M glycerol and glucose for voltages up to +100 mV. For other experimental conditions and for 2 M sucrose, the simple model no longer approximated the currents, as might be expected, since it did not take into account a number of factors such as the resistance of the selectivity filter and that currents would become diffusion limited at high enough voltage. For outward currents that are not diffusion limited, the reduction in current by sugar most likely includes a reduction in both steps 2 and 3 in the transit process (see above), as the decreased movement of K^+ through the vestibule (step 2) would also decrease the effective concentration of K^+ at the entrance to the selectivity filter, which would decrease the rate of entry into the selectivity filter (step 3).

Our observation that decreasing the fractional volume of the inner vestibule by even small amounts (<10%) by adding sugar led to decreases in the outward unitary currents (Fig. 2 A; Table I) suggests that the passage of K^+ through the inner vestibule is a major determinant of the outward unitary current (step 2 in the transit process in the above discussion). If this were not the case, then small increases in the resistance of the inner vestibule to the passage of K^+ should have had negligible effects on the unitary currents. If the general geometry of the conduction pathway in BK channels is similar to that of MthK channels, then the majority of the voltage drop across the conduction pathway of BK channels in the absence of sugar (or for small amounts of sugar in the inner vestibule) would be across the selectivity filter rather than the inner vestibule (Jiang et al., 2002). On this basis, it might be speculated that the voltage drop across the selectivity filter is so large for moderate to high voltages that K^+ that reaches the selectivity filter rapidly passes through, so that a major limitation on conductance becomes the passage of K^+ through the inner vestibule (step 2) where the voltage drop and driving force are much less.

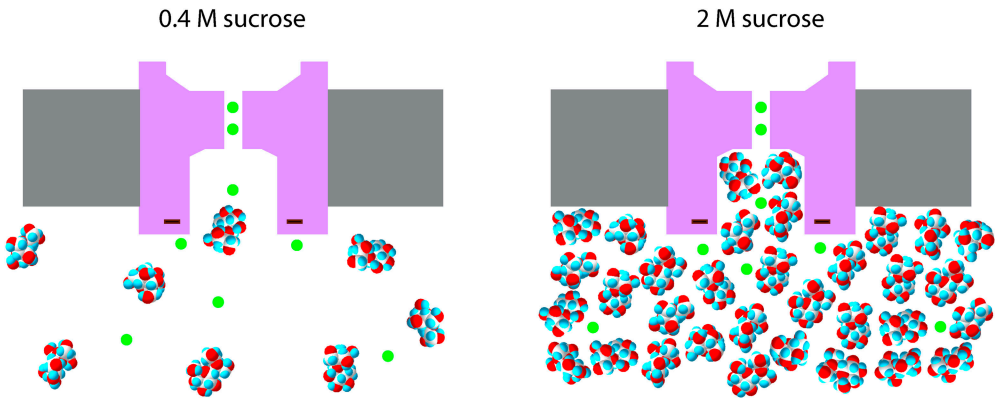

Figure 7. Cartoon of a BK channel with 150 mM K^+ and 0.4 and 2 M sucrose. The inner vestibule is drawn with a depth of 20 Å and a diameter of 18.5 Å. The actual physical dimensions and shape are likely to be different. The number of ions visible is the average number that would be contained in an 8-Å slice in the plane of the figure centered on the centerline of the channel. It is assumed that the concentration of sucrose in the channel is the same as in the bulk intracellular solution. The negative charge at the entrance to the inner vestibule attracts additional K^+ ions (Brelidze et al., 2003). The selectivity filter is drawn with two K^+ ions (Morais-Cabral et al., 2001), and the pore helices are likely to provide a favorable electrostatic environment for a K^+ ion in the vestibule (Roux and MacKinnon, 1999).

Step 1, the diffusion of K^+ from the bulk intracellular solution into the inner vestibule was not a major rate-limiting step in our experiments in the absence of sugar with 150 mM K^+_i for examined voltages (driving forces) up to +300 mV. Step 1 did become the rate-limiting step for voltages >+100 mV when 2 M sucrose was used to slow diffusion of K^+ into the inner vestibule. Given sufficiently high voltage, step 1 would be expected to become the rate-limiting step, even in the absence of sugar, because the high voltage should drive K^+ through the channel faster than the diffusion-limited entry of K^+ into the inner vestibule.

The empirical model described by Eq. 5 would underpredict the inward currents (unpublished data), because inward currents were reduced less than outward currents by sugar. Nevertheless, for inward currents, sugars in the vestibule would slow the movement of K^+ from the selectivity filter to the intracellular bulk solution, leading to an increase in the concentration of K^+ deep in the vestibule. Such increased K^+ would increase the occupancy of the K^+ site at the inner side of the selectivity filter, repelling the inward movement of K^+ from deeper sites in the selectivity filter (Morais-Cabral et al., 2001; Zhou and MacKinnon, 2004a), reducing the inward movement of K^+ through the selectivity filter.

Sugar reduced outward currents more than inward currents. For outward currents, the fractional reduction by sugar increased with increasing driving force (Fig. 2, E and F), and for inward currents, the fractional reduction decreased with increasing driving force (Fig. 5). The mechanism for this differential effect is not clear, but several factors may contribute. Movement of K^+ ions in the inner vestibule would be expected to drag sugar molecules along with them, both due to direct collisions and because the waters of hydration of K^+ and sugar would overlap to some degree (Robinson and Stokes, 1970). Such a drag effect would act to increase the concentration of sugar in the vestibule for outward currents and decrease the concentration for inward currents, giving a differential effect on the current magnitudes as the driving force increases. For inward currents, the sugar in the vestibule would slow the efflux of K^+ from the vestibule, resulting in accumulation of K^+ near the selectivity filter, which could then give rise to a counter electrostatic force on the K^+, reducing the current. Sugar in the entrance to the vestibule would reduce the entrance area available for diffusion, but at the same time, because of the constant molarity of K^+ as the concentration of sugar is increased, the microscopic concentration of K^+ in the solution between the sugar molecules would be greater, so that, on average, the same numbers of K^+ would be available to diffuse into the vestibule per macroscopic unit area. In any case, the speculative mechanisms considered in this paper are mainly useful to point out questions that will require quantitative approaches to answer.

Physical Obstruction of the Inner Vestibule by Sugar

If it is assumed that the inner vestibule of BK channels is 18.5 Å in diameter and 20 Å deep, then a volume of bulk solution equivalent to the volume of the vestibule would contain, on average, 0.49 molecules of K^+ for a 150 mM solution, and 1.3, 3.2, and 6.5 molecules of sugar for the 0.4, 1, and 2 M solutions of sugar used in our experiments. Fig. 7 presents a cartoon showing a longitudinal 8-Å-thick section through the center of the assumed BK channel with 0.4 and 2 M sucrose. This 8-Å section would contain 53% of the assumed volume of the vestibule and hence, assuming equal partition between vestibule and bulk intracellular solution, would also contain 53% of the molecules in the vestibule. The large fractional volume of the slice occupied by sucrose is readily apparent in Fig. 7, especially at the higher concentration of 2 M. This large volume of sugar mole-

cules would increase the viscosity of the solution, decreasing the diffusion coefficient for K^+ (Weast, 1976). The ring of negative charge at the entrance to the inner vestibule increases the effective concentration of K^+ in the vestibule ~3.3-fold (Brelidze et al. 2003). On this basis, the volume of the slice in the vestibule would contain ~0.9 K^+. The physical obstruction to the passage of K^+ by the sugar molecules is readily apparent. It is the physical presence of sugar, both in the vestibule and in the bulk solution (for diffusion limited currents), that leads to the reduction of currents by sugars.

Differential Action of Sugars in the Bulk Solution and in the Inner Vestibule

Our observations indicate that 0.4 M glycerol (the smallest sugar examined) can reduce unitary current (Fig. 2 A), whereas a very high (2 M) concentration of sucrose (the largest sugar examined) was required to induce diffusion-limited outward current. Why is this the case? In simplified terms, the inner vestibule can be viewed as a resistor in series with the selectivity filter. On this basis, even small amounts of sugar in the vestibule would increase the resistance of the vestibule, and hence, the total resistance to passage of K^+ through the channel, reducing unitary currents.

In contrast, because the diffusion of K^+ from the bulk solution up to the entrance of the inner vestibule can occur from a larger area than the cross-sectional area of the entrance, the diffusion coefficient for K^+ must be decreased by a large amount (using 2 M sucrose) before diffusion into the vestibule becomes a limiting factor on unitary current at high voltages. Thus, sugar in the vestibule acts in a graded manner on unitary currents, whereas sugar in the bulk solution has little effect until the flux of K^+ driven through the channel by the high voltage approaches the rate of diffusion of K^+ from the bulk solution into the vestibule. At this point, the rate of diffusion into the vestibule from the bulk solution sets the magnitude of the diffusion-limited current.

The Large Inner Vestibule of BK Channels Is Required for their Large Unitary Conductance

K^+-selective channels differ markedly in their single channel conductance even though the signature sequence TVGYG in their selectivity filters is typically conserved among K^+ channels (Heginbotham et al., 1994; Doyle et al., 1998). BK channels, which have the largest conductance of all K^+-selective channels (Hille, 2001), have fixed negative charge at the entrance to the inner vestibule that doubles the magnitude of the outward unitary currents (Brelidze et al., 2003; Nimigean et al., 2003). However, even after neutralization of these fixed negative charges, the conductance of BK channels is reduced only from ~300 to ~150 pS (Brelidze et al., 2003; Nimigean et al., 2003), which still gives a conductance much larger than for most of the other K^+-selective channels. Thus, there must be other factors, in addition to the fixed negative charge, that contribute to the large conductance of the BK channels.

Differences in the physical size of the inner vestibules of K^+ channels could be one of the factors (Li and Aldrich, 2004). Consistent with this proposal, the conductance of Shaker K^+ channels is 5–10 times less than the 300 pS conductance of BK channels, and estimates of the diameter of the inner vestibule of Shaker channels made using a Cd^{2+} bridge to lock the channel in the open state suggest a narrow vestibule of ~8–9 Å to accommodate the Cd^{2+} bridge (Webster et al., 2004). Blocking studies with decyltriethylammonium ions also suggest smaller dimensions for the inner vestibule of Shaker channels than for BK channels (Li and Aldrich, 2004). Further support that large inner vestibules are associated with large conductances are the findings that the MthK channel, with a large conductance of ~200 pS, has a larger inner vestibule (Jiang et al., 2002) than estimated values for the inner vestibule of Shaker (Webster et al., 2004). Theoretical support that larger inner vestibules give rise to larger conductances are molecular dynamics simulations showing that increasing the diameter of the inner vestibule in a modeled KcsA type channel from 3.0 to 10 Å leads to steady increases in conductance (Chung et al., 2002).

Consistent with the above observations, our study suggests that BK channels with their large conductance also have a large effective diameter of 16–20 Å for the entrance to the inner vestibule, as estimated from the capture radius (2.2 Å) and the radius of hydrated K^+ (6–8 Å). Decreasing the effective volume of the inner vestibule of BK channels with sugar decreases unitary currents in a graded manner (Figs. 1, 2, and 6 C). Thus, the large inner vestibule for BK channels both contributes to and is essential for their large conductance by allowing hydrated K^+ to readily enter and pass through the vestibule to the selectivity filter.

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