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Ca^{2+}-Activated K^+ Conductance of the Human Red Cell Membrane: Voltage-Dependent Na^+ Block of Outward-Going Currents

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Summary. Human red cells were prepared with various cellular Na^+ and K^+ concentrations at a constant sum of 156 mM. At maximal activation of the K^+ conductance, $g_K(Ca)$, the net efflux of K^+ was determined as a function of the cellular Na^+ and K^+ concentrations and the membrane potential, V_m, at a fixed $[K^+]_{ex}$ of ~ 3.5 mM. V_m was only varied from $(V_m - E_K) \approx 25$ mV and upwards, that is, outside the range of potentials with a steep inward rectifying voltage dependence (Stampe & Vestergaard-Bogind, 1988). $g_K(Ca)$ as a function of cellular Na^+ and K^+ concentrations at $V_m = -40$, 0 and 40 mV indicated a competitive, voltage-dependent block of the outward current conductance by cellular Na^+. Since the present Ca^{2+}-activated K^+ channels have been shown to be of the multi-ion type, the experimental data from each set of Na^+ and K^+ concentrations were fitted separately to a Boltzmann-type equation, assuming that the outward current conductance in the absence of cellular Na^+ is independent of voltage. The equivalent valence determined in this way was a function of the cellular Na^+ concentration increasing from 0.5 to 1.5 as this concentration increased from 11 to 101 mM. Data from a previous study of voltage dependence as a function of the degree of Ca^{2+} activation of the channel could be accounted for in this way as well. It is therefore suggested that the voltage dependence of $g_K(Ca)$ for outward currents at $(V_m - E_K) > 25$ mV reflects a voltage-dependent Na^+ block of the Ca^{2+}-activated K^+ channels.

Key Words Ca^{2+}-activated K^+ conductance · human red cell membrane · voltage-dependent block · cellular Na^+

Introduction

The presence of Ca^{2+}-activated K^+-selective ion channels have been demonstrated in the membrane of many cell types, excitable as well as nonexcitable (for reviews *see* Latorre & Miller, 1983; Schwarz & Passow, 1983; Hille, 1984). That an increase in the intracellular concentration of ionized Ca elicits an increase in K^+ net efflux was first discovered in experiments with human red cells incubated in the presence of extracellular Ca^{2+} and poisons inhibiting the glycolytic processes (Gardos, 1958).

In an extensive study of the K^+ fluxes across the membranes of resealed human red cell ghosts containing arsenazo III, Yingst and Hoffman (1984) examined transmembrane interactions of cellular Na^+ and extracellular K^+ on the Ca^{2+}-activated K^+ efflux at given, constant levels of cellular ionized calcium. At $[K^+]_{ex} = 2$ mM and constant $[K^+]_c$ values of 25 and 100 mM, respectively, the efflux of ^{42}K was found to be substantially lower at a cellular Na^+ concentration of 50 mM than in the absence of cellular Na^+. This inhibition of ^{42}K efflux by cellular Na ions was not seen at $[K^+]_{ex} = 20$ mM, indicating the existence of a transmembrane interaction between cellular Na and extracellular K ions.

In the meantime, it has been shown that the Ca^{2+}-activated conductance, $g_K(Ca)$, is based on an ionic channel (Hamill, 1981; Grygorczyk & Schwarz, 1983) and that this channel is of the multi-ion type exhibiting single-filing (Vestergaard-Bogind, Stampe & Christophersen, 1985). In addition, it has been shown that the channel is an inward rectifier, that is, the conductance for outward-going currents at a constant $[K^+]_c$ increases sharply with decreasing $(V_m - E_K)$ within the range of 20 to 0 mV (Vestergaard-Bogind, Stampe & Christophersen, 1987). In the above cited experiments of Yingst and Hoffman (1984), $(V_m - E_K)$ values within this range of steep voltage dependence might well occur at the extracellular K^+ concentration of 20 mM. Further, the suggested transmembrane interaction was based on determined flux values. In the present report, we describe the effect on $g_K(Ca)$ of variations in $[Na^+]_c$ and $[K^+]_c$ at a fixed $[K^+]_{ex}$ of ≈3.5 mM.

Materials and Methods

Cells

Cells from freshly drawn blood from healthy human donors were depleted of ATP and 2,3-diphosphoglycerate as previously described (Vestergaard-Bogind & Stampe, 1984). In the experi-

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ments all chloride was replaced by nitrate, and the cells were therefore, after the adjustment of cellular Na and K concentrations, washed and Ca-loaded in nitrate salt solutions. The cells were loaded with Ca to a given concentration of ionized Ca of ≈30 μM using ≈1 μmol of ionophore A23187 per liter cells (for details *see* Vestergaard-Bogind et al., 1987).

Adjustment of Cellular Na and K Concentrations

The procedure applied here is based on the observation made by Wieth (1970) that in cells in which all Cl ions had been replaced by SCN ions, the cell membranes became highly permeable to both Na and K ions at 0°C. After ATP depletion, the cells were washed three times in 10 volumes of salt solution (90 mM (KSCN + NaSCN), 112 mM sucrose, 0.5 mM EGTA[1]). The cells were then incubated in this salt solution at 0°C and a cytocrit of ≈5% for 12 to 18 hr. Varying the ratio between KSCN and NaSCN, it was in this way possible to produce cells in which a given fraction of the cellular K ions had been replaced by Na ions in a 1 : 1 exchange, that is, with a constant sum of Na and K ions of 156 mM.

The addition of 112 mM sucrose assured that the cellular concentration of permeable anions was kept at the normal value of ~95 mM, thereby ensuring the constant cell volume. Suspending the cells in 90 mM KSCN, 112 mM sucrose solution, the resulting cells had normal Na and K concentrations and could be used as control cells with respect to possible effects of the SCN treatment. In experiments where an increased $[K^+]_c$ and a correspondingly decreased $[Na^+]_c$ was aimed at, some of the sucrose was replaced by K-gluconate, in this way attaining an exchange of cellular Na^+ for extracellular K^+, still maintaining a normal concentration of permeable anions. After the SCN treatment, the cells were washed three times in 10 volumes of salt solution containing 90 mM KNO_3 and 66 mM $NaNO_3$.

Phthalate Method

Cellular contents of K^+, Na^+, and ^{45}Ca and extracellular concentrations of K^+ were determined by the phthalate method as previously described (Vestergaard-Bogind et al., 1985).

Membrane Potential

Changes in membrane potential (V_m) were determined according to the method of Macey, Adorante and Orme (1978). The experiments were carried out with cells suspended in buffer-free salt solution at a hematocrit of 3.1% in the presence of 20 μM of the protonophore CCCP, which mediates a fast electrochemical equilibration of protons across the cell membranes. In our experience, the presence of 20 μM CCCP does not affect g_K(Ca). The shifts in extracellular pH mediated by the ionophore in response to hyper- or depolarizations do not change the magnitude of the net efflux of K^+ within a pH_{ex} range of 6.0 to 8.0 (Stampe & Vestergaard-Bogind, 1988). At $V_m = E_K = E_{Cl}$, that is under zero current condition, the size of the unidirectional fluxes were the same in the presence and absence of CCCP. In experiments where large net effluxes of K^+ took place, a reduction in net efflux (of up to 30%) was observed in parallel experiments without CCCP added. Estimating in these experiments the unidirectional influx with ^{42}K, the V_m could be calculated from the flux ratio inserting the single file exponent 2.65. It was found that the degree of hyperpolarization was correspondingly increased so that the g_K(Ca) value was the same as with CCCP present, whereas the anion conductance was reduced.

Experimental Procedure

In all experiments, packed cells with a cellular pH of ≈7.1 and loaded with Ca ($[Ca^{2+}]_c \approx 30$ μM) were transferred into buffer-free salt solution [3 mM KCl, 0 to 153 mM NaCl, isotonicity maintained by addition of sucrose (264 mM sucrose taken as isotonic)], containing 20 μM CCCP, thermostatted at 37°C and vigorously stirred. Since the cells were transferred into the salt solutions with open K^+ channels, the efflux started instantaneously (for further details, *see* Vestergaard-Bogind et al., 1987).

Calculations

The net efflux of K^+ in mmol per liter cells per hr was calculated from the initial, constant decrease in cellular K^+ content by linear regression. The correlation coefficient, $r \geq 0.97$. The value of the Nernst potential for K ions at the peak of hyper- or depolarization was calculated from the extracellular K^+ concentration at that time and the initially determined intracellular concentrations. The membrane potential (V_m) was calculated from the CCCP-mediated electrochemical equilibrium of protons across the cell membranes. A pH difference ($pH_{ex} - pH_c$) of one unit was taken to be equivalent to a membrane potential of 61.5 mV (inside-negative).

The Ca^{2+}-activated K^+ conductance, g_K (ca) was calculated from the equation of Hodgkin & Huxley (1952).

$$I_K = J_K \cdot F = (V_m - E_K) \cdot g_K(\mathrm{Ca}). \quad (1)$$

Here I_K is the current of K ions across the membrane, that is $J_K \cdot F$, where J_K is the next flux of K ions in μmol/(cm² · sec) and F is the Faraday constant. In the calculation of J_K per cm² of membrane, it was assumed that the area of 1 liter of cells equals $1.75 \cdot 10^7$ cm². The conductance is accordingly obtained in μS/cm².

Results

At decreased cellular K^+ concentration and a correspondingly increased cellular Na^+ concentration, a marked decrease in g_K(Ca) was found. This decrease was not an effect of submaximal Ca^{2+}-activation, since at a $[Na^+]_c$ value of 75 mM the K_m(Ca) was found to be within the normal range of 1–3 μM. In order to obtain reasonably large net effluxes, the experiments were conducted with NO_3 ions as the only permeable anion, taking advantage of the fact that g_{NO_3} is about 2.5 times larger than g_{Cl} (Vestergaard-Bogind et al., 1985; Bennekou & Stampe,

[1] *Abbreviations:* CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol *bis* (β-aminoethyl ether)-N,N'-tetraacetic acid.

1988). If not otherwise mentioned, $[K^+]_{ex}$ was 3 mM in the salt solutions, achieving in this way the previously observed maximal activation by extracellular K^+ of $g_K(Ca)$ at normal $[K^+]_c$ (Vestegaard-Bogind et al., 1987).

Besides the Ca^{2+}-activated K^+ conductance, the only important conductance is the anion conductance. At zero current across the cell membranes, that is at the peak of hyper- or depolarization, V_m is therefore given by the equation

$$V_m = \frac{E_{An} \cdot g_{An} + E_K \cdot g_K(Ca)}{g_{An} + g_K(Ca)} \quad (2)$$

where E_{An} is the Nernst equilibrium potential and g_{An} the corresponding conductance of the anion. Positive values of V_m were obtained by shifting E_{An} to positive values by substituting sucrose for extracellular salt, the concentration of the latter always kept higher than 15 mM to avoid the net efflux observed by Jones and Knauf (1985) at lower ionic strength.

In Fig. 1*A*, the net effluxes of K^+, J_K, from cells with different K^+, and accordingly, Na^+ concentrations, are shown as a function of V_m. The net efflux at $[K^+]_c$ = 135 mM, $[Na^+]_c$ = 21 mM were obtained in experiments from two types of ATP-depleted, Ca-loaded NO_3 cells. The values indicated by (○) were obtained with cells, which had been suspended overnight in 90 mM KSCN and 112 mM sucrose, a treatment which resulted in cells with unchanged cellular K^+ and Na^+ concentrations. The flux values indicated by (●) were obtained in experiments with ATP-depleted NO_3 cells, which had been stored overnight in a high K^+ salt solution, that is, cells which had not been treated with thiocyanate solution.

A possible influence of the thiocyanate treatment on $g_K(Ca)$ could thus be examined. Compared to the control experiment, the net effluxes of K^+ from the thiocyanate-treated NO_3 cells were a little lower and the membrane potentials were a little more negative. The calculated $g_K(Ca)$ values were, however, identical in the two experiments. Thus, the K^+ conductance was not significantly affected by the thiocyanate treatment, whereas the higher degree of hyperpolarization indicates that the NO_3 conductance was diminished slightly.

In Fig. 1*B*, $g_K(Ca)$ at −40, 0 and 40 mV are plotted *versus* $[K^+]_c$ and $[Na^+]_c$, the sums of these concentrations, as mentioned, kept constant at 156 mM. The $g_K(Ca)$ values were calculated from the net flux curves and the corresponding V_m and E_K values shown in Fig. 1*A*. The curves in Fig. 1*B* were drawn by eye, the dashed lines representing extrap-

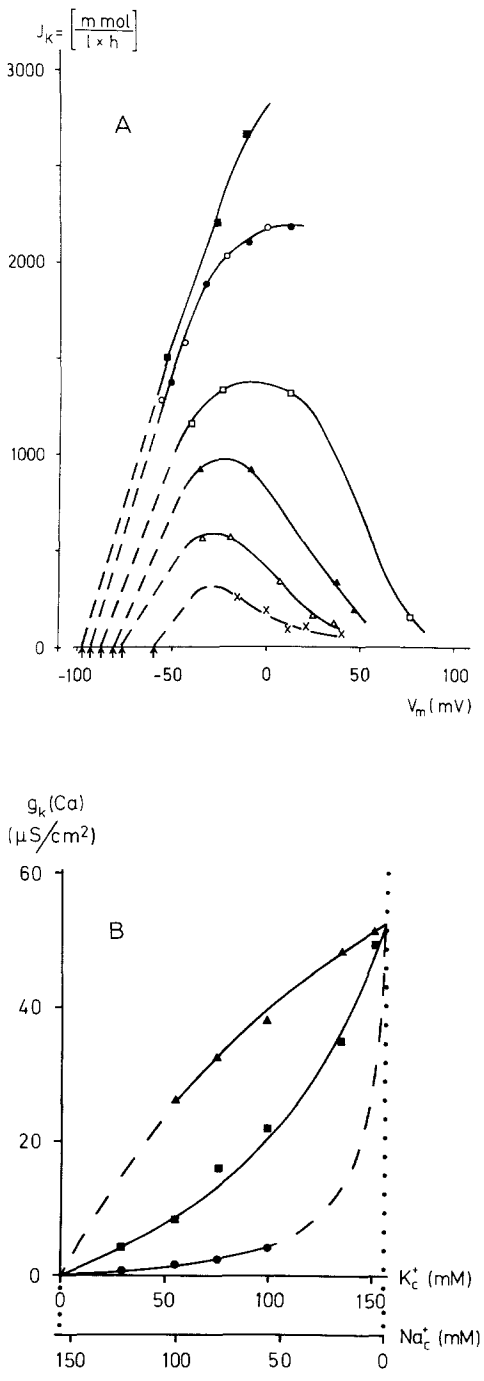

Fig. 1. (*A*) K^+ net efflux (J_K in mmol per hr per liter of cells) from cells, preloaded with Ca to a concentration of ionized Ca of ≈30 μM as functions of membrane potential (V_m in mV) at six different ($[K^+]_c$ + $[Na^+]_c$) values: (150 + 6) mM (■), (135 + 21) mM (○) (thiocyanate-treated cells) and (●) (control cells), (99 + 57) mM (□), (75 + 81) mM (▲), (55 + 101) (△) mM and (29 + 127) mM (×). In all experiments, $[K^+]_{ex}$ was about 3.5 mM. The calculated E_K values at the peak of hyper- or depolarizations are shown as (↑) on the abscissa axis. (*B*) Conductances ($g_K(Ca)$ in μS/cm²) at three different V_m values (−40 (▲), 0 (■) and 40 mV (●)) as a function of $[K^+]_c$ and $[Na^+]_c$ in mM. The data are calculated from the flux values and the corresponding V_m and E_K values shown in *A*. The curves are drawn by eye

olations into the ranges where experimental data could not be obtained.

In a separate series of experiments, it was found that at a $[Na^+]_c$ of ≈45 mM an increase in $[K^+]_{ex}$ from the standard concentration of ≈3.5 to ≈7 mM resulted in an increase in $g_K(Ca)$ of about 20%, a further increase in $[K^+]_{ex}$ being without effect. In connection with the present results, the effect of a higher $[K^+]_{ex}$ in the experiments with the highest $[Na^+]_c$ values imply minor change in the g_0 and K_B values, whereas the value of the equivalent valence hardly would change (*see* Discussion).

Discussion

$g_K(Ca)$ as a function of cellular Na^+ and K^+ concentrations at $V_m = 0$ mV, as shown in Fig. 1*B*, indicates that the K^+ channels are blocked by Na ions in a competitive way. A comparison of the function at 0 mV with the corresponding functions at −40 and 40 mV, respectively, further indicates a strong voltage dependence of the block.

If a block of a one-ion channel is not influenced by other ions, then the fraction of blocked, R_B, to not blocked, $(1 - R_B)$, channels is given by a Boltzmann distribution at equilibrium

$$\frac{R_B}{1 - R_B} = \frac{[B]}{K_B} \cdot \exp\left[\frac{z'FV_m}{RT}\right] \tag{3}$$

where $[B]$ is the activity of the blocking ion, K_B the dissociation constant of the complex at $V_m = 0$ mV and z' the effective valence of the blocking reaction (Hille & Schwarz, 1978). For a one-ion channel with competition between blocking and permeant ions, Eq. (3) may not be satisfied with a constant z' or K_B, and in case of a multi-ion channel, this is much more pronounced (Hille & Schwarz, 1978). Since the conductance in the present case is based on a multi-ion channel with at least three sites and four potential barriers, the blocking Na ions may bind to one or more sites. In the following, we assume that Na^+ can enter the channel from the cellular side of the membrane and may cross all potential energy barriers but the last one (on the extracellular side), whereas Na ions do not enter the channel from the extracellular side. This leads to 17 different blocked states (Hille & Schwarz, 1978) and a mathematical expression for the competition between K and Na ions on the channel sites becomes extremely complicated.

In the absence of cellular Na^+ and at physiological concentrations of K^+ (130 mM intracellularly and ≈5 mM extracellularly), the single-channel *I-V* curve in patch-clamp experiments was linear within the voltage of −100 to 80 mV (P. Christophersen, *personal communication*). Also, French and Wells (1977) noted that the delayed rectifier showed a linear *I-V* relation for outward currents in the absence of axonal Na^+. These results indicate that at a single-channel level, no voltage dependence of the K^+ conductance exists at low $[K^+]_{ex}$ values. It seems reasonable, therefore, to correct the Na^+ blockade of the channel for voltage while the conductance of the unblocked channels are assumed to be voltage independent and Eq. (3) can be reformulated as

$$g = g_0 \frac{1}{1 + \frac{[B]}{K_B} \cdot \exp\left[\frac{z'FV_m}{RT}\right]} \tag{4}$$

where g_0 is the conductance in the absence of Na^+ at the given K^+ concentration and K_B, formally, is the activity of Na^+ required to block half of the channels at $V_m = 0$ mV. A linearized form of Eq. (4) is

$$\ln\left[\frac{g_0}{g} - 1\right] = \ln\frac{[B]}{K_B} + \frac{z'FV_m}{RT}. \tag{5}$$

For each set of ionic concentrations, values of g_0, K_B and z' were calculated by fitting the data to Eq. (4) using the Gauss-Newton method. In Fig. 2A the fit of the experimental data at 57 mM Na^+ is shown, whereas in Fig. 2*B* the fits of data at 11 and 101 mM Na^+ to the linearized form (Eq. (5)) are shown. All data supplemented with some previously published are presented in the Table.

As seen from the Table, the equivalent valence, z', increases with increasing substitution of cellular K for Na ions from 0.5 at 11 mM Na^+ to 1.5 at 101 mM Na^+. An increase in equivalent valence with the concentration of the blocking ion has previously been reported by Adelman and French (1978). In a study of block of the delayed rectifier by external Cs ions, they found an increase in z' from 0.6 to 1.3 as the concentration of Cs was increased from 5 to 200 mM. In their treatment of multi-ion channels in a potential-barrier theory, Hille and Schwarz (1978) have shown that an equivalent valence >1 for a monovalent blocker should be anticipated in a multi-ion channel. Assuming that the present channel has four energy barriers and three energy wells and a high degree of occupancy (Vestergaard-Bogind et al., 1985) an equivalent valence of up to 1.5 of the blocking cellular Na^+ is not surprisingly high, but gives further support to the notion that the Ca^{2+}-activated K^+ channel of the human red cell membrane is a multi-ion channel. An equivalent valence >1 is best explained by a model where Na^+ can occupy at least two of the sites in the channel, and

Table. The parameters g_0, K_B and z' (and their calculated standard deviations) determined by fitting to Eq. (4) the conductances calculated from the flux values and the corresponding V_m and E_K values shown in Fig. 1*A*

	$[Na^+]_c$ (mM)	$[K^+]_c$ (mM)	$[Ca^{2+}]_c$ (μM)	g_0 (μS/cm^2)	K_B (mM)	z'
A	6	150	30	—	—	—
B	11	145	1.6	27 ± 2	16 ± 3	0.55 ± 0.06
C	11	145	3.0	38 ± 4	18 ± 6	0.54 ± 0.11
D	21	135	30	56 ± 4	37 ± 8	0.79 ± 0.19
E	57	99	30	42 ± 5	75 ± 29	1.11 ± 0.29
F	81	75	30	39 ± 3	53 ± 10	1.27 ± 0.12
G	101	55	30	32 ± 1	35 ± 2	1.47 ± 0.04
H	127	29	30	—	—	—

In experiments *A* and *H*, the range of conductance was too narrow to yield the fitting parameters. The results in rows *B* and *C* are from previously published experiments (Stampe & Vestergaard-Bogind, 1988).

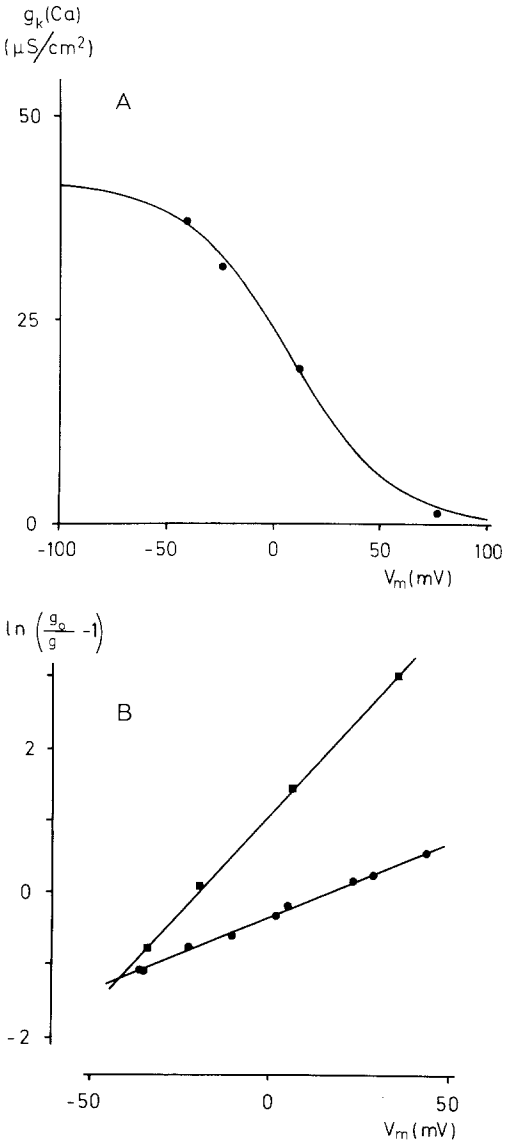

Fig. 2. (*A*) g_K(Ca) in μS/cm^2 as a function of V_m in mV. Data (●) are plotted together with the values calculated from Eq. (4) (full line). The fitted parameters are shown in the Table, row *E*. (*B*) Data from two different experiments (row *B* (●) (11 mM Na^+) and row *G* (■) (101 mM Na^+) in the Table) plotted according to Eq. (5). The slope of each line determining the equivalent valence (z' = 0.54 and 1.47, respectively) of the blocking Na ion. For further details, see the Table.

accordingly some channels sometimes contain two Na ions simultaneously (*cf.* Hille, 1984 (p. 296)). In this model, the selectivity filter will be close to the extracellular side of the channel.

Previously, voltage-dependent blocking of outward currents from the delayed rectifier by axonal Na^+ has been reported (Bezanilla & Armstrong, 1972; Hille, 1975; French & Wells, 1977). The delayed rectifier was the first ion channel, which was shown to be a multi-ion channel, characterized by a flux ratio exponent of ≈2.5 (Hodgkin & Keynes, 1955). All the same, French and Shoukimas (1985) estimated an equivalent valence of Na^+ in the voltage dependence of the blocking of this channel of only 0.5.

Turning to the highly K^+-specific Ca^{2+}-activated K^+ channels, Marty (1983) and Yellen (1984) in patch-clamp studies of the maxi-K^+ channel of bovine chromaffin cells found that internal Na^+ blocked the outward current through the channel in a voltage-dependent way. Yellen (1984) described the blockade as a flickery block where the rate of block is very voltage dependent ($z' \approx 0.9$), whereas the unblock rate is voltage independent. An equivalent valence of 1.38 of a putative cellular ion blockade of a Ca^{2+}-activated K^+ channel from myotubes has been reported by Suarez-Isla and Rapoport (1986). In the maxi-channel of T-tubules, the conductance is blocked by Na^+ on the *cis* side with an equivalent valence of 0.7 (Latorre, 1986).

We have previously described fractional decreases in g_K(Ca) with voltage at different degrees of Ca^{2+}-activation of the conductance (Stampe & Vestergaard-Bogind, 1988). With $[K^+]_c$ and $[Na^+]_c$ values of 145 and 11 mM, respectively, the slopes of the apparently linear relationships between g_K(Ca) and V_m decreased with decreasing $[Ca^{2+}]_c$. The data could be fitted to a two-state model with an equivalent valence of the gating particle of 0.4. but alternatively they were suggested to reflect a voltage-dependent block of outward currents by an intracellular ion. This alternative, with Na^+ as the cellular ion, is supported by the fact that the previously published experimental data could be fitted to Eq. (4), resulting in z' values (*see* rows *B* and *C* in

the Table), which arranges well according to the sequence of Na concentrations. Using the linear transform, Eq. (5), the data are plotted in Fig. 2*B*. We conclude, therefore, that voltage dependence of the basic conductance at various ($[K^+]_c$ + $[Na^+]_c$) values, as well as at various $[Ca^{2+}]_c$ values (Stampe & Vestergaard-Bogind, 1988), can be described by a model in which Na^+ competes in a voltage-dependent way with K^+ on at least two channel sites.

We have previously reported that outward currents increased sharply towards a zero current value of ≈160 $\mu S/cm^2$ as ($V_m - E_K$) decreased from ≈20 to 0 mV (Vestergaard-Bogind et al., 1987). With a flux ratio exponent of 2.65 (Vestergaard-Bogind et al., 1985), the ratio between the unidirectional influx and the net efflux will increase sharply within this range of ($V_m - E_K$) and a possible knock off (Armstrong, 1975) of Na^+ from the inner site might increase equally strongly. Thus, voltage-dependent block of outward current by cellular Na ions might constitute a part of the previously reported inward rectification.

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