

Apparent Change in Ion Selectivity Caused by Changes in Intracellular K^+ during Whole-Cell Recording

Charles J. Frazier, Eric G. George, and Stephen W. Jones

Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106 USA

ABSTRACT In whole-cell recordings from HEK293 cells stably transfected with the delayed rectifier K^+ channel Kv2.1, long depolarizations produce current-dependent changes in $[K^+]_i$ that mimic inactivation and changes in ion selectivity. With 10 mM K_o^+ or K_i^+ , and 140–160 mM Na_o^+ , long depolarizations shifted the reversal potential (V_R) toward E_{Na} . However, similar shifts in V_R were observed when Na_o^+ was replaced with *N*-methyl-D-glucamine (Na_o^+). In that condition, $[K^+]_o$ did not change significantly, but the results could be quantitatively explained by changes in $[K^+]_i$. For example, a mean outward K^+ current of 1 nA for 2 s could decrease $[K^+]_i$ from 10 mM to 3 mM in a 10 pF cell. Dialysis by the recording pipette reduced but did not fully prevent changes in $[K^+]_i$. With 10 mM K_o^+ , 150 mM Na_i^+ , and 140 mM Na_o^+ , steps to +20 mV produced a positive shift in V_R , as expected from depletion of K_i^+ , but opposite to the shift expected from a decreased K^+/Na^+ selectivity. Long steps to V_R caused inactivation, but no change in V_R . We conclude that current-dependent changes in $[K^+]_i$ need to be carefully evaluated when studying large K^+ currents in small cells.

INTRODUCTION

Inactivated states are normally considered to be nonconducting conformations of a channel. Nevertheless, several recent studies have challenged that assumption. For example, slowly inactivated *Shaker* channels have been reported to be permeable to Na^+ (Starkus et al., 1997, 1998). Similarly, it has been reported that as Kv2.1 inactivates, it first passes through a state with decreased selectivity for K^+ before finally entering a truly nonconducting state (Kiss et al., 1999). For Kv2.1, the primary evidence was a systematic change in the reversal potential (V_R) toward E_{Na} during the inactivation process (Kiss et al., 1999). We have collected similar data, and originally favored the same interpretation (Frazier et al., 1998). However, these studies on Kv2.1 have relied on whole-cell patch clamp recording from a mammalian cell line (HEK293 cells) transfected with Kv2.1. In such experiments, small cell size (10–15 pF), large current amplitudes (often >1 nA), long depolarizations (seconds), and low $[K^+]$ (2–10 mM) combine to create a situation where undesired changes in the K^+ gradient may occur.

We report here that accumulation of $[K^+]_o$ is not significant in our conditions, but depletion or accumulation of $[K^+]_i$ does occur. Furthermore, the changes in $[K^+]_i$ lead to changes in V_R that closely mimic a change in the ionic selectivity of the channel. Finally, we have examined the possibility that an actual change in ionic selectivity of Kv2.1 does occur during the inactivation process, in conjunction with K^+ redistribution, but we were not able to find any evidence to that effect. We conclude that changes in $[K^+]_i$

can influence the interpretation of results from studies of K^+ channel inactivation in mammalian expression systems.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney (HEK293) cells stably transfected with Kv2.1 were generously provided by Dr. Arthur M. Brown. Cells were cultured in minimum essential medium (MEM, with L-glutamine) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.4–0.5 mg/ml geneticin (G418). The cultures were split at 80–90% confluence, and electrophysiological recording was conducted 1–3 days later.

Electrophysiology

All electrophysiological recordings were made in the whole-cell configuration using an Axopatch 200 (Axon Instruments, Foster City, CA) in voltage clamp mode. The holding potential was -80 mV. Resistance of the electrodes was 1.5–3.5 M Ω when filled with NaCl or KCl based internal solution. Series resistance varied between 3 and 10 M Ω , and was always compensated by >80%. Data were recorded to a microcomputer using pClamp (Clampex v. 6.0 or 7.0; Axon Instruments). Linear leakage and capacitive currents were subtracted on line (P/–4), with the “raw” unsubtracted data saved on a second A-D channel. Data were analyzed with pClamp (Clampfit v. 6.0 or 7.0) and spreadsheet programs. Values are expressed as mean \pm SEM.

The standard internal solution (denoted as “ Na_i ” throughout this manuscript) contained, in mM: 120 NaCl, 1 CaCl₂, 11 ethyleneglycol-bis-(α -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 4 ATP (Mg^{2+} salt), and 10 HEPES (free acid). The standard external solution (Na_o) contained, in mM: 145 NaCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (free acid). Where noted (10 K_i + Na_i or 10 K_o + Na_o) 10 mM K^+ was added to the solution in exchange for 10 mM Na^+ . For the experiments in Fig. 7 an external solution was used (10 K_o + Na_o) in which external Na^+ was replaced by NMG⁺ (*N*-methyl-D-glucamine). All solutions were titrated to pH 7.3 using NaOH or NMG⁺ base where appropriate. The total Na^+ concentration was 159.5 mM for “ Na_i ” and 149 mM for “ Na_o .” Before recording, the electrode current was adjusted to zero in Na_o (or Na_o + 10 K_o). Voltages were not corrected for the resulting junction potential, calculated to be +2.3 to +2.9 mV. External solutions were delivered via a gravity-driven

Received for publication 11 August 1999 and in final form 30 December 1999.

Address reprint requests to Charles J. Frazier, Dept. of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106. Tel.: 216-368-5526; Fax: 216-368-3952; E-mail: cjf2@po.cwru.edu.

© 2000 by the Biophysical Society

0006-3495/00/04/1872/09 \$2.00

bath flow system, controlled by solenoid valves. Before recording data, the flow was turned on and cells were lifted off the surface of the culture dish.

Permeability ratios were calculated from Goldman-Hodgkin-Katz theory. With two ions A and B of any charge (z_A, z_B), where each ion may be present on both sides of the membrane, the permeability ratio (P_A/P_B) can be calculated directly from an observed reversal potential (V_R), using an expression derived from the Goldman-Hodgkin-Katz current equation, Eq. 13-5 of Hille (1992):

$$P_A/P_B = \frac{-z_B^2([B]_i - [B]_o e^{-\nu_B})(1 - e^{-\nu_A})}{z_A^2([A]_i - [A]_o e^{-\nu_A})(1 - e^{-\nu_B})} \quad (1)$$

where $\nu_A = z_A V_R F/RT$ and $\nu_B = z_B V_R F/RT$. If $z_A = z_B$, this reduces to:

$$P_A/P_B = -([B]_i - [B]_o e^{-\nu})/([A]_i - [A]_o e^{-\nu}) \quad (2)$$

Diffusion calculations

Two conditions were considered, $[K^+]_o$ near the membrane resulting from a constant outward current, and changes in $[K^+]_i$ resulting from an outward current (Fig. 5). Both calculations solved the diffusion equation in spherical geometry with flux at the cell membrane, using a forward time, central space finite difference scheme (Crank, 1956; Strikwerda, 1989).

Current simulations

The effects of changes in $[K^+]_i$ on the kinetics of macroscopic Kv2.1 currents were simulated based on the model of Klemic et al. (1998) for gating of Kv2.1. The model was modified as follows: activation was shifted to more negative voltages, as observed for Kv2.1 in low $[K^+]$, by multiplying the rates for channel opening and voltage sensor activation by 2.0, and dividing the rate constants for channel closing and voltage sensor deactivation by the same factor. Inactivated states were deleted from the model. The Goldman-Hodgkin-Katz current equations were used to describe K⁺ and Na⁺ currents through the open channel, with $P_{Na}/P_K = 0.03$. P_K was chosen to give current amplitudes comparable to those observed experimentally. For each time point, changes in $[K^+]_i$ and $[Na^+]_i$ were calculated from the simulated K⁺ and Na⁺ currents, for a spherical 10 pF cell (assuming 1 $\mu F/cm^2$), and dialysis from the pipette (with $\tau_d = 4$ s, or as noted). As discussed further below, spatial gradients of $[K^+]_i$ and changes in $[K^+]_o$ were not included. Simulations were performed with the SCOP simulation package (v. 3.51; Simulation Resources, Berrien Springs, MI).

RESULTS

Kv2.1 is selective for K⁺, but is permeable to Na⁺ in the absence of K⁺. In the presence of both Na⁺ and K⁺, current will be carried through the channel by a mixture of both ions (Korn and Ikeda, 1995). With 10 mM K_i⁺ and 0 mM K_o⁺, in otherwise Na⁺-based solutions, tail currents recorded following brief (25 ms) depolarizations to +20 mV reversed at -26.3 ± 2.4 mV ($n = 3$; $P_{Na}/P_K = 0.037$). In the opposite condition (0 mM K_i⁺ and 10 mM K_o⁺), $V_R = +26.3 \pm 1.3$ mV ($n = 6$; $P_{Na}/P_K = 0.032$). The P_{Na}/P_K ratios are somewhat larger than previously reported for Kv2.1 in physiological solutions (Korn and Ikeda, 1995), but still indicate a clear K⁺ selectivity under resting conditions, even with low $[K^+]$.

Following a long (2 s) depolarization to +20 mV with 10 mM K_i⁺, V_R shifted by $+13 \pm 6$ mV ($n = 3$). Fig. 1, A and C illustrate a similar experiment, for a 2 s depolarization to -10 mV. With 10 mM K_o⁺, the shift was -7.4 ± 1.4 mV ($n = 5$) (Fig. 1, B and D). In each case the shift in V_R was toward E_{Na} , and represents an apparent two to threefold increase in P_{Na}/P_K . With 10 mM K_i⁺, a voltage step between 0 and -20 mV will produce an outward driving force on K⁺ and an inward driving force on Na⁺. Currents measured in that voltage range begin as outward currents presumably carried by K⁺ and end as inward currents presumably carried by Na⁺ (Figs. 1 A, 2 A). This basic phenomenon was also observed when the K⁺ concentration gradient, and the driving force on K⁺ and Na⁺, were reversed (Fig. 2 B). These results are similar to those of Kiss et al. (1999), who interpreted them as evidence that inactivation of Kv2.1 involves progression through a state with increased Na⁺ permeability before reaching a final nonconducting conformation.

In some conditions, the current during a depolarizing step seemed to inactivate fairly rapidly (e.g., Fig. 1 B). However, the change in V_R will also affect the current amplitude, by changing the driving force. That is most obvious in records where the current crosses zero. More subtly, changes in the instantaneous $I-V$ relationship following long depolarizations were not always as would be expected if the decay in macroscopic current resulted from channel inactivation. In Fig. 1, C and D, the slope or chord conductances changed little during depolarizations lasting 2 s, despite the dramatic change in current during the depolarization. Furthermore, when the experiment in Fig. 1 A was repeated using a depolarizing pulse positive to both E_{Na} and E_K (+20 mV), there was no apparent macroscopic inactivation, and the conductance was actually increased following a 2 s depolarization (Fig. 2 C). These observations call into question whether the changes in current amplitude and reversal potential truly result from channel inactivation.

Theoretically, changes in the K⁺ gradient could also explain the results of Figs. 1 and 2, even in the complete absence of inactivation. For example, with 10 mM K_i⁺ (Fig. 1 A), an outward K⁺ current would tend to increase K_o⁺ and decrease K_i⁺, which would shift V_R toward 0 mV, as observed in Fig. 1 C. Although the extent of K⁺ redistribution necessary to explain the results may seem prohibitive, there are several factors present in this system (small cell size, large current amplitude, low $[K^+]$, and long depolarization) that act together to accentuate the problem. Therefore, before concluding that P_{Na}/P_K is altered during inactivation, it is essential to determine whether the K⁺ gradient is changing significantly.

Changes in external [K⁺] are not significant

Accumulation of external K⁺ during an outward current is a well-known phenomenon (Frankenhaeuser and Hodgkin,

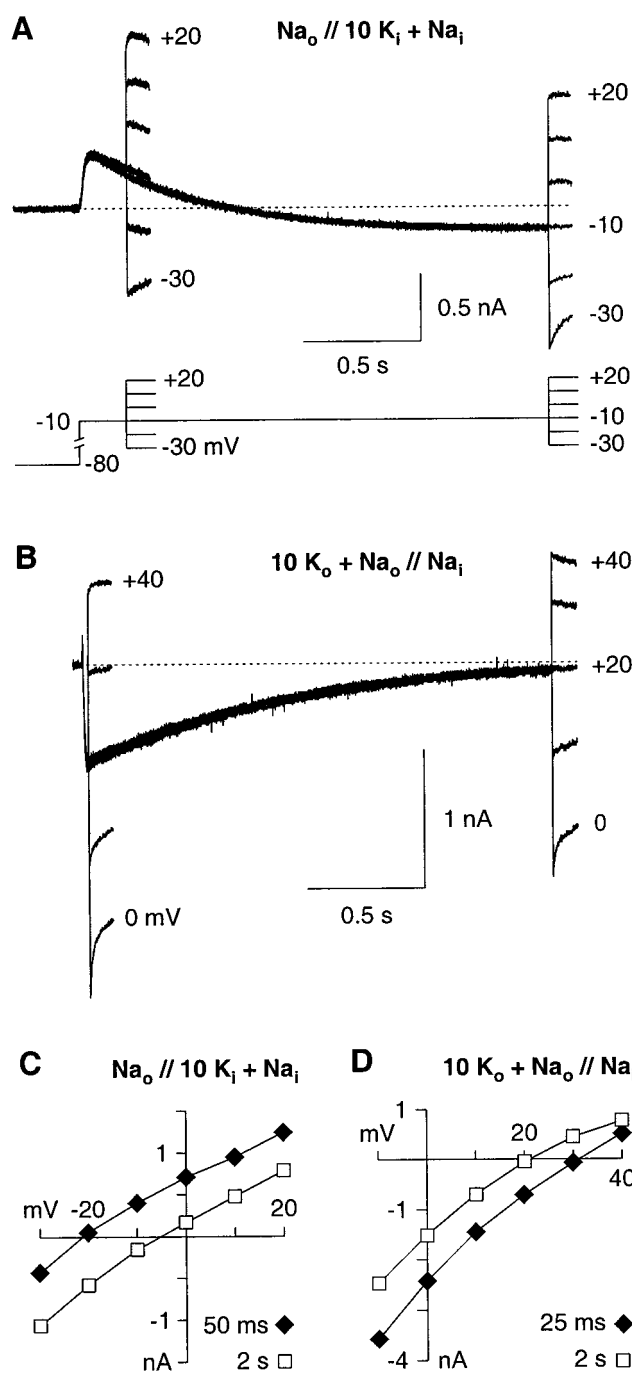


FIGURE 1 Changes in reversal potential (V_R) during long depolarizations. (A) Current records are superimposed from two experimental runs, where steps to -10 mV lasting either 200 or 2000 ms were followed by brief steps to voltages from -30 to $+20$ mV (see illustration of the voltage protocol below). (B) Records shown as in A, but for 25 ms or 2 s steps to $+20$ mV, in a cell with 10 mM K_o^+ rather than 10 mM K_i^+ . (C) Instantaneous current-voltage (I - V) relationships measured from initial tail current amplitudes in the same cell as A, for 50 ms or 2 s prepulses to -10 mV. Reversal potentials in this cell were -21 mV after 50 ms, -17 mV after 200 ms, and -5 mV after 2 s. (D) Instantaneous I - V relations from the experiment in B, with $V_R = +31$ after 25 ms and $+21$ after 2 s.

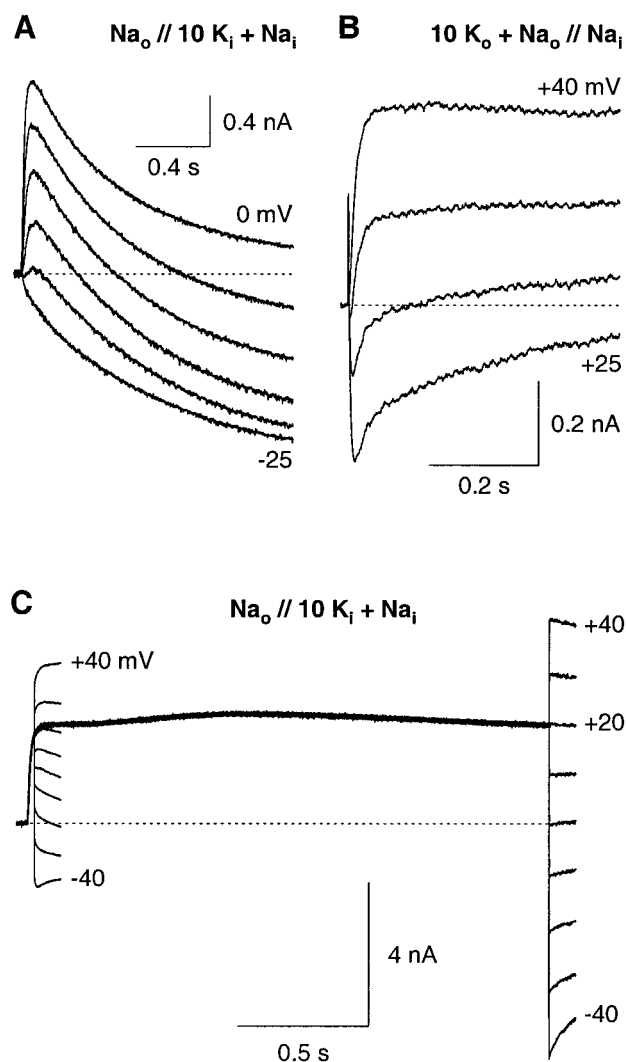
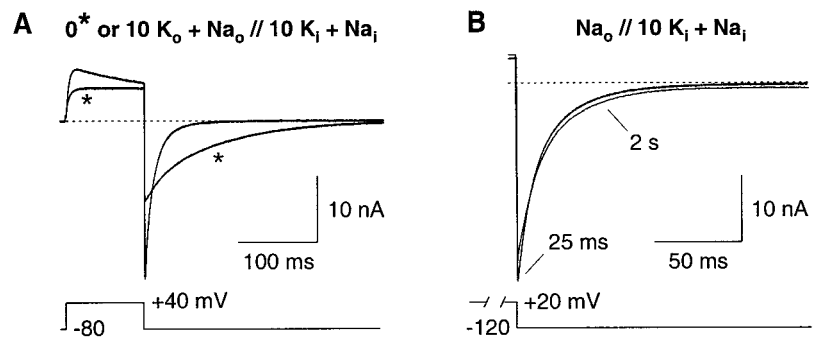


FIGURE 2 Changes in direction of current during long depolarizations to voltages near V_R . Records are shown with 10 mM K_i^+ (A) or 10 mM K_o^+ (B), to the voltages indicated near each record (5 mV increments). (C) Prepulses to $+20$ mV lasting 50 ms or 2 s were followed by steps to voltages from -40 to $+40$ mV (10 mV increments).

1956). To minimize the risk of accumulation, during these experiments cells were continuously superfused with external solution and were routinely lifted off the dish before data were recorded.

To test for changes in $[\text{K}^+]_o$, we used the effect of K_o^+ on tail current kinetics (Korn and Ikeda, 1995). With 10 mM K_i^+ and 0 mM K_o^+ , decay of tail currents was quite slow. When 10 mM K^+ was added to the external solution, inward current amplitude increased and deactivation became much faster (Fig. 3 A). Nevertheless, when tail current kinetics were compared following 25 ms and 2 s steps to $+20$ mV with 10 mM K_i^+ and 0 mM K_o^+ , no significant change in amplitude or kinetics was observed (Fig. 3 B). An accumulation of $[\text{K}^+]_o$ sufficient to produce the $+13$ mV shift in V_R observed in that condition ($\sim 3 \text{ mM}$) should have

FIGURE 3 Evaluation of changes in $[K^+]_o$. (A) Effect of K_o^+ on deactivation of Kv2.1. Superimposed records taken with only 10 mM K_i^+ (*), or with 10 mM K_i^+ and K_o^+ . (B) Tail currents recorded with K_i^+ and nominally 0 $[K^+]_o$, following depolarizations to +20 mV lasting 25 ms or 2 s. In this cell, V_R shifted from -26 to -13 mV during the 2 s depolarizations, although the current amplitude at +20 mV changed little (see Fig. 2 C). Also note that the tail current deactivation rate is faster in C than in B because the repolarization voltage is more negative (-120 vs. -80 mV).



also produced a clear increase in the rate of deactivation (Korn and Ikeda, 1995). Therefore, in agreement with Kiss et al. (1999), we conclude that accumulation of $[K^+]_o$ does not contribute significantly to the reversal potential shifts observed for Kv2.1 following long depolarizations. This conclusion is supported by diffusion calculations, assuming free radial diffusion of K_o^+ away from the membrane (not shown).

Changes in intracellular $[K^+]$ are significant

We next attempted to determine whether depletion or accumulation of K_i^+ could be occurring in our experiments. As a first step, we looked for V_R shifts following long depolarizations with 10 mM K^+ on both sides of the membrane, in otherwise NMG⁺-containing solutions. Assuming that NMG⁺ cannot permeate Kv2.1 channels, K^+ is the only charge carrier. Because K^+ is present in equal amounts on both sides of the membrane, V_R is near 0 mV. If there is no change in the K^+ gradient following a long depolarization,

there should be no shift in V_R , regardless of any changes in the selectivity of the channel that may occur. Alternatively, a shift in V_R would indicate a change in $[K^+]_i$ (since changes in $[K^+]_o$ have been ruled out above). Experimentally, V_R was shifted in the positive direction by a 2 s pulse to a voltage positive to E_K (+20 mV; Fig. 4, Table 1). That is the expected result if depletion of $[K^+]_i$ is produced by the outward current observed during the depolarization. Complementary results (indicating accumulation of K_i^+) were produced by inward currents observed during long voltage steps negative to E_K , yet still sufficiently depolarized to open the channels (e.g., -20 mV; data not shown).

The problem can also be detected by comparing two different measures of inactivation in Na⁺-free solutions. In Fig. 4 A the current decayed from a peak value of 2.48 nA to 0.45 nA at the end of the 2 s depolarization to +20 mV, indicating an apparent inactivation of 81%. In contrast, the conductance calculated from Fig. 4 B decreased only 24% between 25 ms and 2 s, from 71 to 54 nS. This comparison is similar to the classical "envelope test" for inactivation

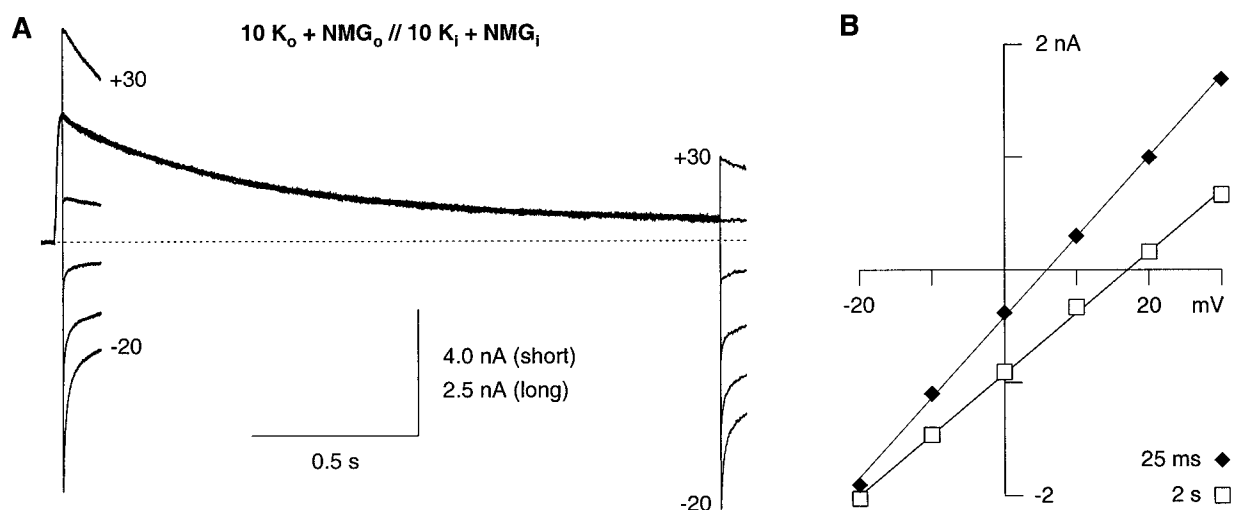


FIGURE 4 Changes in V_R in the absence of Na⁺. (A) Prepulses lasting 25 ms or 2 s were followed by voltage steps to -20 to +30 mV, in 10 mV increments. The currents were normalized to the peak value during the prepulse. Because of rundown, the absolute current levels were smaller for the longer pulses (which were run at a later time), as indicated by the scale bars. (B) Instantaneous I - V relations from the experiment of A. V_R = +6 mV after 25 ms, +17 mV after 2 s.

TABLE 1 Changes in V_R and $[K^+]_i$ resulting from long (2 s) depolarizations

	V_R (mV)	$[K^+]_i$ (mM)
Observed*	$+11.3 \pm 0.6$	4.8 ± 0.2
Predicted: Integrated current [†]	$+10.7 \pm 2.7$	5.4 ± 0.8
Predicted: Exponential decay, with dialysis [‡]	$+15.6 \pm 1.6$	3.5 ± 0.5
Predicted: Exponential decay, no dialysis [§]	$+20.1 \pm 2.3$	2.4 ± 0.6

Values are for the experimental condition of Fig. 4 (symmetrical 10 K + NMG), thus $[K^+]_i = 10$ mM at $t = 0$; $n = 9$.

*“Observed” values are the experimentally measured shift in V_R (the V_R measured after 2 s at +20 mV, minus V_R after 25 ms at +20 mV), and the corresponding $[K^+]_i$ calculated from the Nernst equation.

[†]The change in $[K^+]_i$ was calculated from the integrated current, neglecting dialysis from the recording pipette.

[‡]The current was assumed to decay exponentially, resulting from a combination of K^+ efflux and dialysis from the recording pipette (Eq. 4).

[§]The current was assumed to decay exponentially, resulting from K^+ efflux only (Eq. 4, with $\tau_K = \tau_m$ and $f = 0$).

(Hodgkin and Huxley, 1952), which Kv2.1 fails under these recording conditions. Because the time course of macroscopic current decay is affected not only by true inactivation, but also by any changes in single channel current (here, secondary to changes in $[K^+]_i$), disagreement between the extent of current decay during a depolarization and the change in conductance measured from tail currents indicates that the time course of current decay does not accurately reflect the time course of inactivation. Furthermore, because the conductance is also affected both by gating (inactivation) and permeation (single-channel conductance), changes in conductance also may not accurately reflect inactivation. For example, the increased conductance apparent in the records of Fig. 2 C could result from relief of K^+ block of Na^+ current as $[K^+]_i$ decreases, since Kv2.1 exhibits a strong anomalous mole fraction effect between Na^+ and K^+ (Korn and Ikeda, 1995).

Calculation of the extent of K^+ redistribution

So far, we have argued that $[K^+]_i$ is changing by demonstrating changes in V_R under conditions where other plausible explanations can be excluded, but are the changes in $[K^+]_i$ really large enough to quantitatively explain the observed changes in V_R ? To address that question, we calculated the change in V_R expected from the observed K^+ currents.

Our first calculation simply integrated the observed K^+ current during a depolarization and calculated the corresponding change in $[K^+]_i$. The cell was assumed to be a single well-mixed compartment, and (for reasons discussed above) changes in $[K^+]_o$ were not considered. The volume of the cell was calculated from the measured cell capacitance, assuming a spherical cell with $1 \mu F/cm^2$ of surface membrane (i.e., no membrane infoldings), and all of the cell volume was assumed to be accessible to K^+ . This calculation

does not account for K^+ entering the cell by dialysis from the recording pipette, which is considered in detail below. The predicted shift in reversal potential (ΔV_R) was calculated from the Nernst equation using the calculated $[K^+]_i$, and was compared to the observed ΔV_R .

These calculations were performed for nine cells where V_R was measured following both short (25 ms) and long (2 s) depolarizations to +20 mV in solutions that contained 10 mM K^+ on both sides of the membrane, with no other permeant ions. The results of the calculations are presented in Table 1. The calculated $[K^+]_i$ decreased from 10 mM to 5.4 ± 0.8 mM, representing a total change in $[K^+]_i$ of 4.6 mM, and an expected reversal potential shift of $+10.7 \pm 2.7$ mV. The experimentally measured ΔV_R was $+11.3 \pm 0.6$ mV, which corresponds to a decrease in $[K^+]_i$ to 4.8 ± 0.2 mM. The agreement between calculated and measured values suggests that $[K^+]_i$ redistribution can fully explain the ΔV_R observed under these ionic conditions.

Effect of dialysis from the recording pipette

Changes in $[K^+]_i$ will be limited, to some extent, by dialysis of the cell by the recording pipette. The time constant (τ_d) for dialysis of K^+ ions was estimated according to Eq. 11 of Pusch and Neher (1988), based on their observed “normalized diffusion rate” for K^+ , corrected for cell volume:

$$\tau_d = 0.0267 R_s C_m^{1.5} \quad (3)$$

With the series resistance (R_s) in $M\Omega$, and membrane capacitance (C_m) in pF, τ_d is in seconds. For the nine cells of Table 1, $\tau_d = 5.1 \pm 1.1$ s (range, 2.2–13.3 s). Qualitatively, that is considerably longer than the 2 s voltage steps that were used, which suggests that dialysis from the pipette is too slow to fully prevent changes in $[K^+]_i$. Quantitatively, the time course and extent of changes in $[K^+]_i$ can be estimated by considering the combined effects of dialysis and a membrane current (Mathias et al., 1990). In response to sudden activation of a K^+ conductance by depolarization, assuming that $[K^+]_i$ is initially equal to $[K^+]_p$ in the pipette ($[K^+]_p$), the time course of the change in $[K^+]_i$ is given by

$$[K^+]_i = (1 - f)[K^+]_p e^{-t/\tau_K} + f[K^+]_p \quad (4)$$

$[K^+]_i$ changes with time constant $\tau_K = \tau_d \tau_m / (\tau_d + \tau_m)$, where τ_m is the time constant for flux across the membrane. We assume that $\tau_m = Q_0/I_0$, where Q_0 is the total amount of charge on K^+ ions in the cell before the voltage step, and I_0 is the peak current (before changes in $[K^+]_i$). That calculation of τ_m neglects channel inactivation. The ratio of steady-state to initial $[K^+]_i$ is $f = \tau_m / (\tau_m + \tau_d)$.

Average values were $\tau_m = 1.5 \pm 0.3$ s, $\tau_K = 1.1 \pm 0.2$ s, and $f = 0.23 \pm 0.02$. That predicts a $[K^+]_i$ of 3.5 ± 0.5 mM at the end of a 2 s depolarization (Table 1), somewhat lower than the value calculated from the experimentally observed ΔV_R (4.8 ± 0.2 mM). That discrepancy may exist because

the calculation of τ_m does not allow for channel inactivation, which would also act to limit changes in $[K^+]_i$. We conclude that large changes in $[K^+]_i$ are possible, even considering dialysis from the recording pipette.

Spatial gradients within the cell

We considered the possibility of local $[K^+]_i$ depletion near the membrane during a maintained outward current. Assuming diffusion in a spherical cell, with $[K^+]_i$ initially uniform at 10 mM, a 1 nA current activated at $t = 0$ would produce a $[K^+]_i$ gradient between the bulk solution and the cell membrane of only 17 μ M (Fig. 5). The gradient would develop within 10 ms of the onset of the current, and would be maintained until bulk $[K^+]_i$ begins to be depleted, at times >100 ms (Fig. 5). In other words, the ratio of $[K^+]_i$ at the membrane to bulk $[K^+]_i$ would decrease from 1.0 at $t = 0$ to 0.9983 during the first 10 ms, and that ratio would be constant thereafter. These results support the assumption, used in the calculations described above, that the interior of the cell can be considered a single well-mixed compartment.

Simulation of the effects of changes in $[K^+]_i$ on K^+ currents

Considering the close agreement between the experimentally observed ΔV_R and the ΔV_R calculated from the expected change in $[K^+]_i$, we conclude that no other mechanism in addition to changes in $[K^+]_i$ is required to fully explain our results with K^+ as the only charge carrier. Since the ΔV_R values under these ionic conditions are similar to those observed in the presence of Na^+ , we suspect that

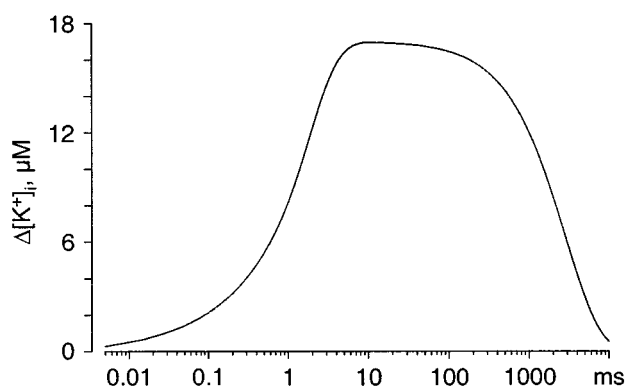


FIGURE 5 Calculation of the K^+ gradient inside a cell during an outward current. $\Delta[K^+]_i$ is the difference between $[K^+]_i$ at the center of the cell and $[K^+]_i$ at the cell membrane. The gradient is steeper near the membrane (i.e., the concentration is most uniform near the center of the cell; calculations not shown). For this calculation, $[K^+]_i$ was initially 10 mM, and a 1 nA current (at the surface of a 10 pF cell) was suddenly activated at $t = 0$. The current then declined in proportion to $[K^+]_i$ at the membrane. The calculation assumed free diffusion and spherical geometry (see Materials and Methods). The integration interval was 5 μ s with 50 radial shells (of 0.18 μ m width, for a 10 pF cell).

redistribution of K^+ can also account for the ΔV_R in solutions containing both Na^+ and K^+ . However, the calculations presented above are not possible when Na^+ is present, since there is no model-independent way to separate the observed current into K^+ and Na^+ components.

To illustrate how changes in $[K^+]_i$ might affect K^+ currents, we simulated currents using a model incorporating channel gating, current-dependent changes in $[K^+]_i$, and dialysis from the recording pipette. The model of Klemic et al. (1998) for gating of Kv2.1 was used, with modifications (see Materials and Methods). Notably, no inactivation was allowed, to demonstrate that changes in $[K^+]_i$ can produce apparent inactivation.

Fig. 6, *A* and *B*, illustrate simulated currents for the conditions of Fig. 1, *A* and *B*, assuming dialysis from the pipette with $\tau_d = 4$ s. For illustration, the prepulse potential was adjusted to produce currents that reverse direction during the 2 s depolarizations. In Fig. 6 *A*, $[K^+]_i$ decreased from 10 mM to 5.6 mM at the end of the 2 s prepulse, and $[Na^+]_i$ increased from 140 mM to 144.3 mM, producing $\Delta V_R = +9.8$ mV. In Fig. 6 *B*, $[K^+]_i$ increased from 0 to 3.7 mM, while $[Na^+]_i$ decreased from 140 to 135.3 mM, $\Delta V_R = -15.5$ mV. Note that the net current is small near the reversal potential, but significant changes in $[K^+]_i$ and V_R can still occur.

To evaluate the effect of dialysis from the pipette, the simulation was run with different values of τ_d . Fig. 6, *C* and *D* show the resulting currents during 2 s prepulses for the same prepulse voltages and ionic conditions as *A* and *B*. The changes in $[K^+]_i$, and the consequent time-dependent changes in the observed currents, were reduced but not eliminated by dialysis, as expected from the calculations presented above (Table 1). Notably, even with relatively fast dialysis, changes in $[K^+]_i$ could still lead to a reversal in the direction of the current.

These simulations support the hypothesis that the experimentally observed changes in current amplitudes and V_R (e.g., Fig. 1) result from changes in $[K^+]_i$. The main limitation of the simulation is the use of Goldman-Hodgkin-Katz theory to describe permeation in this channel. Since Kv2.1 exhibits an anomalous mole fraction effect for K^+ and Na^+ , the contributions of K^+ and Na^+ to the observed current could depend on voltage and time in complex ways. Actually, some of the detailed differences between the simulation and the experimental results may be attributable to ion-ion interactions. Specifically, in experiments with 10 mM K_o^+ and nominally 0 mM K_i^+ , there is often a rapid initial change in current (Fig. 2 *B*; see also Kiss and Korn, 1999), not observed in the reversed ionic condition (Fig. 2 *A*). A change in $[K^+]_i$ from 0 to a low value (~ 1 mM) could have a greater effect than a corresponding decrease (10 mM to ~ 9 mM). We next turned our attention toward finding an experimental approach that could distinguish a change in selectivity of Kv2.1 from changes in $[K^+]_i$, under mixed ionic conditions.

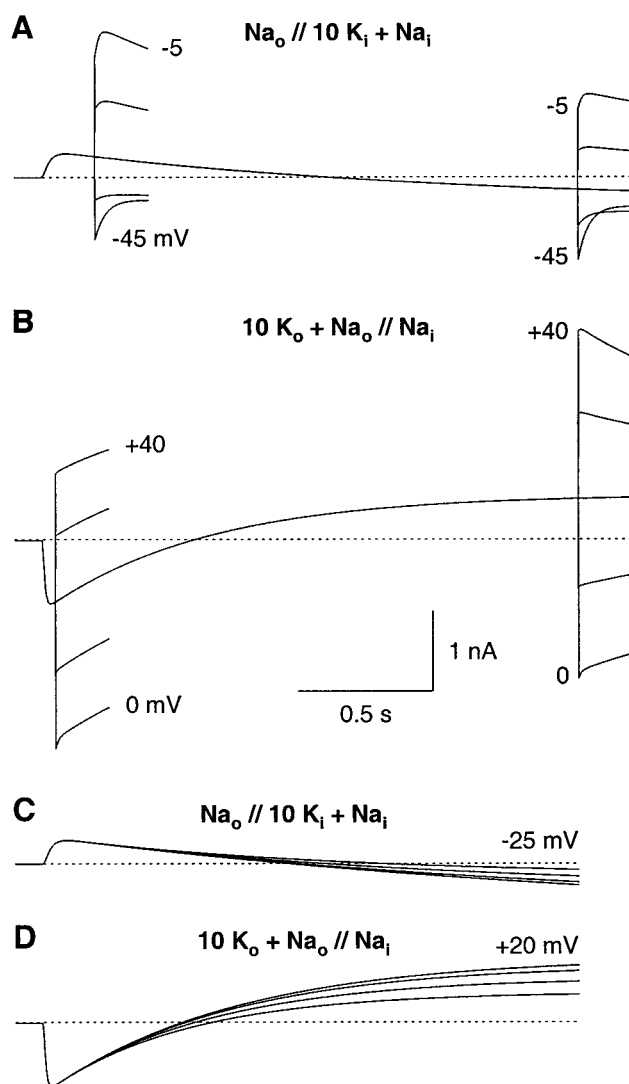


FIGURE 6 Simulation of changes in V_R . Activation kinetics of Kv2.1 were simulated based on Klemic et al. (1998) (see Materials and Methods for modifications to the model). *A* and *B*, currents simulated according to the protocol of Fig. 1, *A* and *B*, except that the 2 s voltage step was to -25 mV rather than -10 mV in *A*. Dialysis from the pipette was included with $\tau_d = 4$ s. The scale bar in *B* applies to all parts of this figure. *C* and *D*, effect of dialysis from the recording pipette on the time course of macroscopic currents. The simulations were run with $\tau_d = 10,000$ s (i.e., effectively no dialysis), 13 s, 4 s, and 2 s, to cover the range observed in our experiments. As τ_d increased, the currents crossed zero at earlier times, and the change in steady-state current was larger, reflecting larger changes in $[K^+]_i$. Voltage steps were from the holding potential of -80 mV to the voltages indicated.

Does an actual change in selectivity occur, in addition to K^+ redistribution?

The fundamental difficulty in interpreting the experiments in Figs. 1 and 2, and in previous studies, is that either a change in $[K^+]_i$ or an increase in P_{Na}/P_K would shift V_R in the same direction. Thus, given that $[K^+]_i$ does change, it is

difficult to determine whether a change in selectivity also occurs. To approach the problem we created a situation where an increase in P_{Na}/P_K would produce a negative ΔV_R , but redistribution of K^+ would produce a positive ΔV_R . That was accomplished by using 10 mM K^+ on both sides of the membrane, but with 150 mM Na^+ inside the cell and 140 mM NMG $^+$ outside. In that situation V_R would be near 0 mV for a K^+ -selective channel, but would be extremely negative for an Na^+ -selective channel. Under these conditions, V_R was near 0 mV following brief (25 ms) depolarizations. We reasoned that following a long depolarization to $+20$ mV, ΔV_R would be positive if redistribution was the predominant effect (depletion of $[K^+]_i$ by an outward current), but ΔV_R would be negative if a change in selectivity in favor of Na^+ was the predominant effect (since E_{Na} is extremely negative). Not only was ΔV_R positive under those conditions, V_R was shifted nearly to the prepulse potential of $+20$ mV (Fig. 7 *A*). In fact, V_R approached the prepulse potential within 2 s whether that potential was positive or negative to E_K (Fig. 7, *A*, *B*, and *D*). That result strongly suggests that changes in $[K^+]_i$ contribute much more to the observed ΔV_R than any possible change in selectivity of the channel.

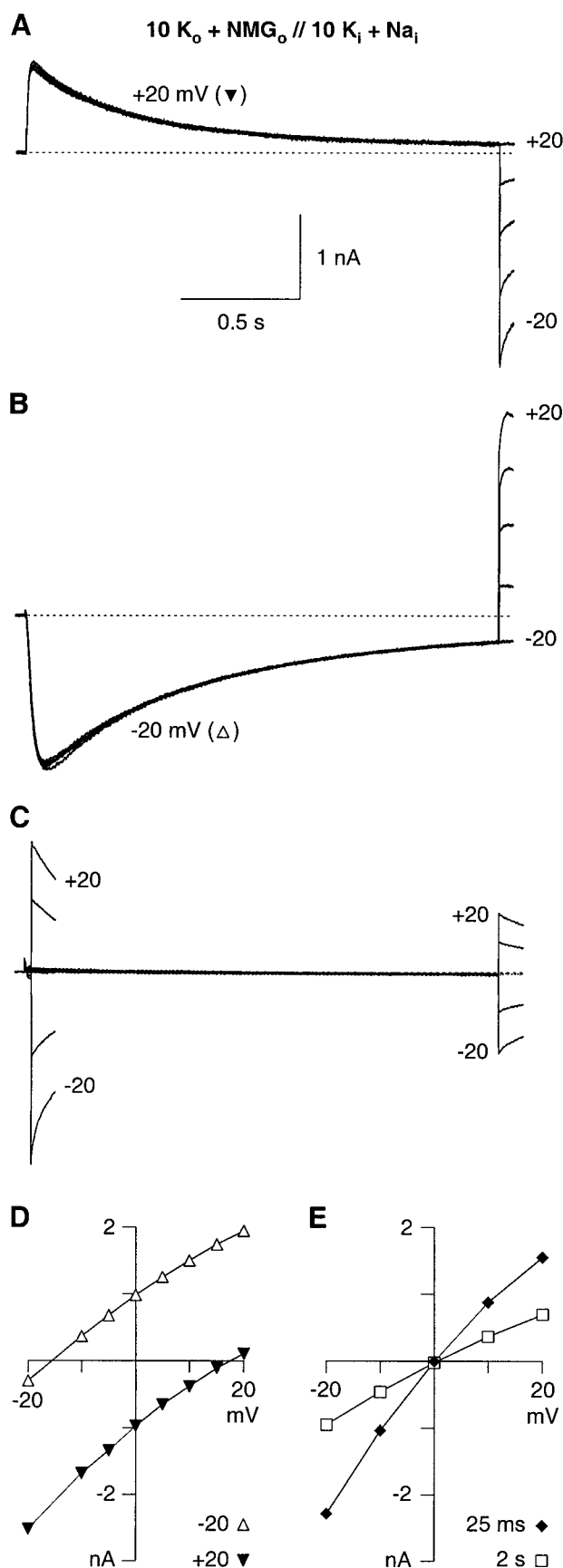
To maximize our ability to detect a change in selectivity, we minimized K^+ redistribution by activating the channels with a prepulse to the reversal potential (Fig. 7 *C*). Because there was very little net current during the 2 s depolarization, even a small change in selectivity in favor of Na^+ should have been detectable as a net negative ΔV_R . However, in the absence of significant K^+ redistribution, no ΔV_R was observed (Fig. 7, *C* and *E*). Furthermore, the lack of a change in V_R was not due to an absence of inactivation, as the conductance was clearly decreased (Fig. 7 *E*). (In this case, with no evidence for changes in selectivity or $[K^+]_i$, the change in slope can be interpreted as inactivation.)

In summary, we conclude that the shifts in V_R that we observe can be explained entirely by changes in $[K^+]_i$. Thus, our results do not support the proposal that Kv2.1 channels exhibit a change in selectivity during the inactivation process.

DISCUSSION

We find that large changes in $[K^+]_i$ are possible in whole-cell recording conditions, especially in small cells with relatively large currents. These changes in $[K^+]_i$ can artifactually mimic channel inactivation and reduction in K^+/Na^+ selectivity.

Current-dependent changes in $[K^+]_o$ have long been recognized, especially in multicellular preparations with restricted extracellular spaces (Frankenhaeuser and Hodgkin, 1956; Orkand, 1980). Changes in $[K^+]_i$ have received less attention, especially in whole-cell recording, where dialysis from the recording pipette allows equilibration of small molecules on a time scale of a few seconds (Pusch and



Neher, 1988). Mathias et al. (1990) previously considered the case where plasma membrane ion transporters compete with diffusion from a pipette for control of intracellular ion concentrations, and much of their analysis applies to the situation here, where large membrane currents flow through ion channels.

Several quantitative factors combined to make changes in $[K^+]_i$ significant in our experimental conditions: (1) large currents, (2) small cells, (3) long depolarizations, and (4) low $[K^+]_i$. Factors (1) and (2) reflect the high channel density possible with overexpression of cloned channels in mammalian cell lines. It is worth noting that the relevant ratio here is current/volume, not current/cell surface area (as often used for normalization of current levels across cells). Since the cell volume depends on the third power of the cell radius, K^+ depletion increases dramatically for smaller cells. Factors (3) and (4) result from our attempt to study slow inactivation of K^+ channels, and its dependence on $[K^+]_i$. Of course, these effects can happen for ions other than K^+ . Changes in $[Ca^{2+}]_i$ are well recognized, but the relevant factors are somewhat different because of exogenous and endogenous Ca^{2+} buffers (Ríos and Stern, 1997).

In retrospect, changes in $[K^+]_i$ may have seriously affected some previous studies of K^+ channel inactivation. Specifically, the results of Kiss et al. (1999) can be explained by changes in $[K^+]_i$, so their conclusion that Kv2.1 changes selectivity during inactivation appears to be incorrect. The effects of low $[K^+]_i$ on slow inactivation of Kv1.5 (Fedida et al., 1999) may also need to be reexamined.

Experiments on inside-out patches have suggested that the *Shaker* K^+ channel loses K^+ selectivity upon inactivation (Starkus et al., 1997, 1998). In those studies, it is clear that substantial Na^+ currents remain following long depolarizations, but the shifts in V_R observed in mixed ionic conditions ($Na^+ + K^+$) could in principle be explained by changes in $[K^+]_i$. Our results and calculations are not directly applicable to inside-out patches, which are very different geometrically from the whole-cell configuration. However, intracellular ion depletion has been observed in cell-attached patches, where the depletion occurs in a poorly defined local space near the inside of the patch (Wang et al., 1998). Similar effects should be considered for inside-out patches, especially when macroscopic currents are recorded, implying very high current densities.

FIGURE 7 Distinction between change in $[K^+]_i$ and change in selectivity. Records shown are 2 s prepulses, followed by voltage steps in the range -20 to $+20$ mV, for prepulses to $+20$ mV (A), -20 mV (B), or 0 mV (C). Prepulses of 25 ms are also shown in C only. The scale bars in A apply to A = C. A and B are from the same cell. (D) Instantaneous I - V relations from A and B, measured following 2 s prepulses. $V_R = -16$ mV for prepulses to -20 mV, and $+18$ mV for prepulses to $+20$ mV. (E) Instantaneous I - V relations from C, measured after 25 ms or 2 s. $V_R = +0.1$ mV (25 ms), $+0.5$ mV (2 s). The changes in V_R are as expected for a change in $[K^+]_i$, with no change in selectivity.

To some extent, the possibility of artifacts resulting from ion accumulation and depletion can be assessed by calculation and simulation. However, control experiments are at least as important, especially in conditions where some key parameters needed for the calculations are uncertain. Simplification of the ionic conditions (e.g., replacement of the variably permeant ion Na^+ with the impermeant NMG^+) should be generally useful. When a change in ion selectivity is suspected, it can be revealing to use ionic conditions where ion redistribution would shift V_R in the opposite direction. Comparison of results obtained over a wide range of channel expression levels could also be used to evaluate ion redistribution artifacts.

We thank Dr. Miklós Gratzl for helpful discussions about diffusion calculations and Dr. Ben W. Strowbridge for valuable comments on experimental design.

This work was supported by National Institutes of Health Grant NS24471 (to S.W.J.), National Institutes of Health postdoctoral fellowship NS10828 (to C.J.F.), and a Howard Hughes Medical Institute grant to Case Western Reserve University School of Medicine.

REFERENCES

- Crank, J. 1956. *The Mathematics of Diffusion*. Clarendon Press, Oxford.
- Fedida, D., N. D. Maruoka, and S. Lin. 1999. Modulation of slow inactivation in human cardiac Kv1.5 channels by extra- and intracellular permeant cations. *J. Physiol. (Lond.)* 515:315–329.
- Frankenhaeuser, B., and A. L. Hodgkin. 1956. The after-effects of impulses in the giant nerve fibers of *Loligo*. *J. Physiol. (Lond.)* 131:341–376.
- Frazier, C. J., E. G. George, and S. W. Jones. 1998. Time-dependent change in K^+/Na^+ selectivity of the Kv2.1 potassium channel in low K^+ . *Soc. Neurosci. Abstr.* 24:2034.
- Hille, B. 1992. *Ionic Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA.
- Hodgkin, A. L., and A. F. Huxley. 1952. The components of membrane conductance in the giant axon of *Loligo*. *J. Physiol. (Lond.)* 116:473–496.
- Kiss, L., J. LoTurco, and S. J. Korn. 1999. Contribution of the selectivity filter to inactivation in potassium channels. *Biophys. J.* 76:253–263.
- Klemic, K. G., C. C. Shieh, G. E. Kirsch, and S. W. Jones. 1998. Inactivation of Kv2.1 potassium channels. *Biophys. J.* 74:1779–1789.
- Korn, S. J., and S. R. Ikeda. 1995. Permeation selectivity by competition in a delayed rectifier potassium channel. *Science* 269:410–412.
- Mathias, R. T., I. S. Cohen, and C. Oliva. 1990. Limitations of the whole cell patch clamp technique in the control of intracellular concentrations. *Biophys. J.* 58:759–770.
- Orkand, R. K. 1980. Extracellular potassium accumulation in the nervous system. *Fed. Proc.* 39:1515–1518.
- Pusch, M., and E. Neher. 1988. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Arch.* 411:204–211.
- Ríos, E., and M. D. Stern. 1997. Calcium in close quarters: microdomain feedback in excitation-contraction coupling and other cell biological phenomena. *Annu. Rev. Biophys. Biomol. Struct.* 26:47–82.
- Starkus, J. G., L. Kuschel, M. D. Rayner, and S. H. Heinemann. 1997. Ion conduction through C-type inactivated *Shaker* channels. *J. Gen. Physiol.* 110:539–550.
- Starkus, J. G., L. Kuschel, M. D. Rayner, and S. H. Heinemann. 1998. Macroscopic Na^+ currents in the “nonconducting” *Shaker* potassium channel mutant W434F. *J. Gen. Physiol.* 112:85–93.
- Strikwerda, J. C. 1989. *Finite Difference Schemes and Partial Differential Equations*. Wadsworth and Brooks/Cole, Pacific Grove, CA.
- Wang, L. Y., L. Gan, T. M. Perney, I. Schwartz, and L. K. Kaczmarek. 1998. Activation of Kv3.1 channels in neuronal spine-like structures may induce local potassium ion depletion. *Proc. Natl. Acad. Sci. USA* 95:1882–1887.