Divalent Ion Trapping Inside Potassium Channels of Human T Lymphocytes

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ABSTRACT Using the patch-clamp whole-cell recording technique, we investigated the influence of external Ca^{2+}, Ba^{2+}, K^+, Rb^+, and internal Ca^{2+} on the rate of K^+ channel inactivation in the human T lymphocyte-derived cell line, Jurkat E6-1. Raising external Ca^{2+} or Ba^{2+}, or reducing external K^+, accelerated the rate of the K^+ current decay during a depolarizing voltage pulse. External Ba^{2+} also produced a use-dependent block of the K^+ channels by entering the open channel and becoming trapped inside. Raising internal Ca^{2+} accelerated inactivation at lower concentrations than external Ca^{2+}, but increasing the Ca^{2+} buffering with BAPTA did not affect inactivation. Raising $[K^+]_o$ or adding Rb^+ slowed inactivation by competing with divalent ions. External Rb^+ also produced a use-dependent removal of block of K^+ channels loaded with Ba^{2+} or Ca^{2+}. From the removal of this block we found that under normal conditions ~25% of the channels were loaded with Ca^{2+}, whereas under conditions with 10 μM internal Ca^{2+} the proportion of channels loaded with Ca^{2+} increased to ~50%. Removing all the divalent cations from the external and internal solution resulted in the induction of a nonselective, voltage-independent conductance. We conclude that Ca^{2+} ions from the outside or the inside can bind to a site at the K^+ channel and thereby block the channel or accelerate inactivation.

INTRODUCTION

Voltage-dependent K^+ channels in human T lymphocytes inactivate almost completely over a period of hundreds of milliseconds during depolarizing voltage steps (DeCoursey et al., 1984; Matteson and Deutsch, 1984). The inactivation rate is sensitive to the presence of divalent ions in the bathing solution; elevated external Ca^{2+} or added lanthanum increase, whereas manganese and cobalt decrease, the rate of inactivation (DeCoursey et al., 1985). One possible explanation for these results is that Ca^{2+} or lanthanum can gain access to a site that inactivates the channel, whereas manganese and cobalt may prevent Ca^{2+} from gaining access to this site. Increasing $[Ca^{2+}]_i$ also reduces the K^+ conductance of T cells (Bregestovski et al., 1986) and accelerates inactivation of K^+ channels of B cells (Choquet et al., 1987). Thus, both external and internal Ca^{2+} regulate the effective number of K^+ channels.

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The purpose of the experiments reported here is to define mechanisms of ion interaction with K^+ channels. The similarity of effects when raising external or internal [Ca^{2+}] suggests a common site of action. In some nerve preparations, inactivation of voltage-gated Ca^{2+} channels depends on Ca^{2+} entry through open Ca^{2+} channels and intracellular accumulation, rather than on membrane potential alone, a phenomenon known as Ca^{2+}-dependent inactivation of the Ca^{2+} channel (for review see Eckert and Chad, 1984). An analogous mechanism in which sparingly permeant Ca^{2+} ions pass through the K^+ channel and lead to inactivation might account for the effects of external di- and polyvalent cations on the rate of K^+ channel inactivation described above. We therefore investigated whether the model of Ca^{2+}-dependent inactivation of Ca^{2+} channels could be also applied to K^+ channel inactivation of human T lymphocytes. Our experiments suggest that rather than modifying a site accessible to intracellular Ca^{2+} buffers, divalent ions can become trapped inside K^+ channels. Since $[Ca^{2+}]_i$ is known to rise shortly after mitogen addition, the interaction of ions within the K^+ channel may be relevant to signal transduction mechanisms. Some of the results have been reported in preliminary communications (Grissmer and Cahalan, 1987, 1988).

METHODS

The experiments were carried out on single cells of a human T lymphocyte-derived cell line, Jurkat E6-1, using the whole-cell recording mode of the patch-clamp technique (Hamill et al., 1981). All experiments were done at room temperature (22–26°C).

Solutions

The cells under investigation were bathed in normal Ringer solution. External solutions containing varying concentrations of Ca^{2+}, Ba^{2+}, K^+, and Rb^+ are listed in Table I. The chamber volume contained ~250 μl of solution which could be totally exchanged during the recordings by bath perfusion within 15–20 s. The patch-pipette usually contained 160 mM K-aspartate with [Ca^{2+}] buffered to 10^{-8} M (see Table I). In some experiments the pipette solution contained higher [Ca^{2+}].

Data Acquisition

The holding potential was adjusted in all experiments to $E = -80$ mV. The patch-clamp amplifier (either List L/M-EPC 7, Adams and List Associates, Ltd., Great Neck, NY; or Axon Instruments, Inc., Axopatch, Burlingame, CA) was used in the voltage-clamp mode without series resistance compensation. Electrodes were pulled from Accufill 90 Micropets (Becton, Dickinson & Co., Parsippany, NJ) in three stages, coated with Sylgard (Dow Corning Corp., Midland, MI) and fire polished to resistances, measured in the bath, of 2–7 MΩ. K^+ channel inactivation was not different if hard glass was used as pipette glass (compare Cota and Armstrong, 1988; Furman and Tanaka, 1988).

In all experiments, the command input of the patch-clamp amplifier was controlled by a computer (PDP 11/73) via a digital-analog converter, and membrane currents were recorded at a bandwidth of 2 kHz. Correction for linear leakage and capacitative currents was achieved by analog subtraction and by digital subtraction of an appropriately scaled mean current associated with eight pulses delivered from a hyperpolarized potential. All potentials were corrected for the liquid junction potential that develops at the tip of the pipette if the pipette solution is different from that of the bath. The liquid junction potential between the normal internal (pipette) and external (bath) solution was −13 mV.

TABLE I

Solutions

External Cl^- anion (pH 7.4)	Na^+	K^+	Ca^{2+}	Mg^{2+}	HEPES	EGTA	Ba^{2+}	Rb^+
Normal ringer	160	4.5	2	1	5	—	—	—
Ca^{2+}								
"0"	160	4.5	—	3	5	1	—	—
0.5	160	4.5	0.5	2.5	5	—	—	—
4.6	156	4.5	4.6	1	5	—	—	—
12	144	4.5	12.4	1	5	—	—	—
54	80	4.5	53.8	1	5	—	—	—
106	—	4.5	105.7	1	5	—	—	—
K^+								
0	164.5	—	2	1	5	—	—	—
2.2	162.2	2.2	2	1	5	—	—	—
10	154.5	10	2	1	5	—	—	—
20	144.5	20	2	1	5	—	—	—
40	124.5	40	2	1	5	—	—	—
80	84.5	80	2	1	5	—	—	—
133	31.5	133	2	1	5	—	—	—
140	24.5	140	2	1	5	—	—	—
160	4.5	160	2	1	5	—	—	—
Rb^+-Ringer	4.5	—	2	1	5	—	—	160
Ba^{2+}								
0.3	160	4.5	—	2.7	5	—	0.3	—
2	160	4.5	—	1	5	—	2.0	—
10	144	4.5	2	1	5	—	10.4	—
54	80	4.5	2	1	5	—	53.8	—
106	—	4.5	2	1	5	—	105.7	—

Internal (pH 7.2)	K^+	Ca^{2+}	Mg^{2+}	HEPES	EGTA	Cl^-	Asp^-
$[Ca^{2+}]_i = 10^{-8}$ M	160	0.1	2	5	1.1	4.2	160
$[Ca^{2+}]_i = 10^{-5}$ M	160	1.1	2	5	1.1	4.2	160
$[Ca^{2+}]_i = 2 \times 10^{-5}$	160	2.0	1	5	—	166	—

The measured osmolarity of the solutions was 290–320 mosmol. Asp^-, aspartate-; HEPES, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid. $[Ca^{2+}]_i$ was calculated assuming a dissociation constant for EGTA and Ca^{2+} at pH 7.2 of 10^{-7} M according to Portzehl et al., 1964; in some experiments the intracellular Ca^{2+} buffering was increased by including 55 mM 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) and 5 mM $CaCl_2$ (free $[Ca^{2+}]_i = 10^{-8}$ M) in the pipette solution. All concentrations are in millimolar.

Analysis

Exponentials were fitted to the decay of the K^+ current as described in more detail by Cahalan et al. (1985) by inputting values through potentiometers. Attempts to fit the decay of the K^+ current in high $[K^+]_o$ with a single exponential failed to describe the time course of

the K^+ channel inactivation sufficiently. The fit, however, was improved by including a certain amount of baseline offset. This is consistent with the idea that there are K^+ channels that do not inactivate during a depolarizing pulse, or that there is always a certain number of open K^+ channels during depolarizations due to an equilibrium between the rate of activation and inactivation. Even with this fitting procedure, including an offset, the decay of the K^+ current could not be adequately characterized, especially during the first 300–500 ms after the onset of the depolarizing pulse. Therefore, the decay of the K^+ current in high $[K^+]_o$ was described with a double exponential function.

RESULTS

External Ca^{2+} and Ba^{2+} Enhance Inactivation

Raising $[Ca^{2+}]_o$ reversibly increases the rate of K^+ channel inactivation in Jurkat cells, as illustrated by K^+ currents in Fig. 1. Raising $[Ca^{2+}]_o$ from 2 to 12 mM Ca^{2+} consistently reduced the peak current amplitude ($I_{K\text{peak}}$, from 1,314 to 1,200 pA in the cell of Fig. 1), as well as speeding the K^+ current decay. The values for the inactivation time constants τ_h, obtained by single exponential fitting (see Methods), in normal Ringer solution before and after the Ca^{2+} treatment were 230 and 226

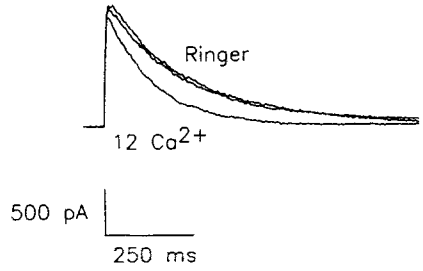

FIGURE 1. K^+ currents in a Jurkat cell in normal Ringer solution before, during, and after application of 12 Ca^{2+} Ringer. $[Ca^{2+}]_i = 10^{-8}$ M. K^+ outward currents associated with 2-s pulses to +40 mV from a holding potential of −80 mV. Cell 94.

ms, respectively; τ_h in Ringer solution with 12 mM Ca^{2+} was 129 ms. While $I_{K\text{peak}}$ in 12 mM Ca^{2+} Ringer is reduced to ~90% of the control value, τ_h is almost 1.8 times faster than in normal Ringer solution. Adding external Ba^{2+} results in similar effects on the time course of inactivation, but also induces a long-lasting "use-dependent" block of K^+ currents, as described below.

Raising $[Ca^{2+}]_o$ may change the negative surface potential of the cell by neutralizing surface charges, thereby shifting the voltage dependence of K^+ channel gating to more depolarized potentials. To assess the surface potential effect, $I_K(E)$ curves were measured and the corresponding $g_K(E)$ curves were calculated. These two curves are shown in Fig. 2, *A* and *B*. The $g_K(E)$ curve in 12 mM Ca^{2+} is shifted by 12 mV to more depolarized potentials, and $g_{K\text{max}}$ is reduced to 93% of the control values before and after the Ca^{2+} treatment.

To quantitate the influence of raising $[Ca^{2+}]_o$ on the inactivation of the K^+ channel, we measured K^+ currents associated with pulses of different amplitudes. The inactivation time constants, τ_h, shown in Fig. 3 were slightly voltage dependent in normal Ringer, confirming earlier measurements on human T lymphocytes (see Fig. 6 of Cahalan et al., 1985). Raising Ca^{2+} shifts the voltage dependence of τ_h by an

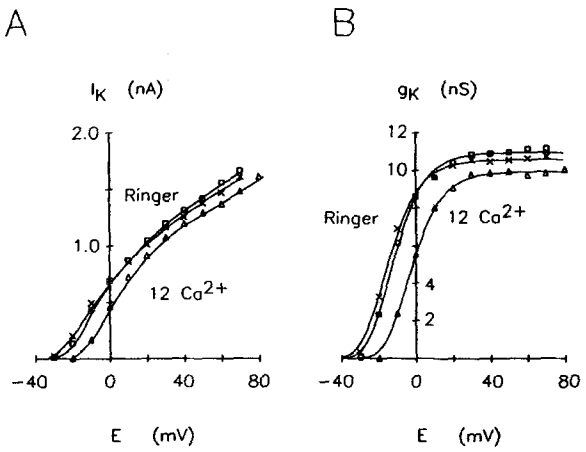

FIGURE 2. Shift in K^+ channel activation in 12 Ca^{2+} Ringer. (*A*) Current-voltage relations before (□), during (△), and after (X) application of 12 Ca^{2+} Ringer. $[Ca^{2+}]_i = 10^{-8}$ M. Peak K^+ currents obtained with 2-s depolarizing pulses are plotted against the absolute membrane potential, *E*, in mV. The lines through the points were drawn by eye for clarity. (*B*) Peak K^+ conductance-voltage relations before (□), during (△) and after (X) application of 12 Ca^{2+} Ringer. The lines through the points were fitted with the Boltzmann equation:

$$g_K(E) = g_{K\,max}/\{1 + \exp[(E_n - E)/k]\}^4$$

with the following parameters:

	Before	During Ca^{2+} treatment	After
$g_{Kmax}(nS)$	10.97	9.95	10.59
$E_{1/2}(mV)$	−11.22	−1.86	−14.27
$k(mV)$	9.89	9.90	10.11

Same cell as in Fig. 1.

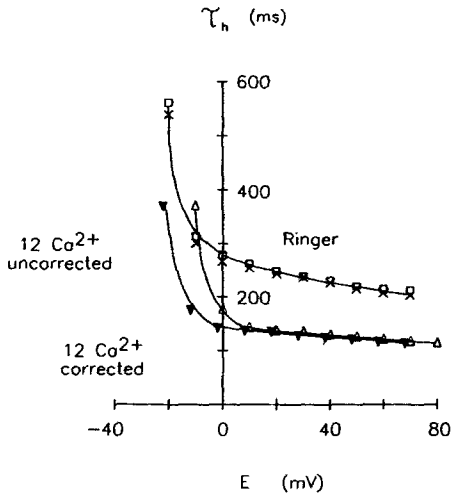

FIGURE 3. Inactivation time constants τ_h of K^+ currents before (□), during (△), and after (X) application of 12 Ca^{2+} Ringer. $[Ca^{2+}]_i = 10^{-8}$ M. Pulses were given every minute to allow complete recovery from inactivation. The time constants were obtained by fitting a single exponential to the decay of the K^+ currents. The ▼ were obtained by correcting the τ_h values measured in 12 Ca^{2+} for a shift along the voltage axis determined from the shift of the conductance-voltage curve in 12 Ca^{2+} compared with normal Ringer solution. The lines through the points were drawn by eye for clarity. Same cell as in Figs. 1 and 2.

amount comparable to the shift of activation shown in Fig. 2. In addition to this shift, τ_h is reduced in 12 mM Ca^{2+}-Ringer. The combination of both effects causes crossover of the control curve and the curve obtained in 12 mM Ca^{2+}. To correct for surface potential effects, the τ_h values obtained in 12 mM Ca^{2+} (labeled "12 Ca uncorrected") were shifted to more hyperpolarized potentials by the same amount that the $g_K(E)$ curve was shifted to more depolarized potentials (12 mV) when $[Ca^{2+}]_o$ was raised from 2 to 12 mM ("12 Ca corrected"). The reduction of τ_h was then determined by calculating the ratios of the control τ_h and the corrected τ_h in 12 mM Ca^{2+}. At E = 10 mV the reduction was to 52% of the control value, whereas at E = 50 mV the reduction was to 57% of the control value. This means that, aside from a shift, the Ca^{2+} effect on the inactivation time constant shows very little, if any, voltage dependence.

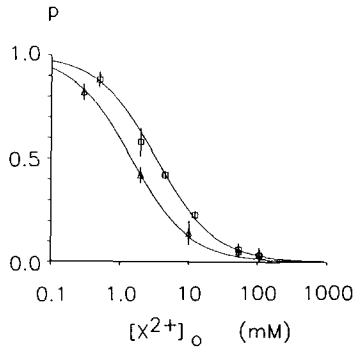

FIGURE 4. The change p of the inactivation time constant τ_h in varying concentrations of Ca^{2+} (□) or Ba^{2+} (△). All external solutions contain 4.5 mM $[K^+]_o$. $[Ca^{2+}]_i = 10^{-8}$ M. p is the normalized change of τ_h, defined as

$$p = (\tau_h - \tau_{hmin})/(\tau_{hmax} - \tau_{hmin})$$

with τ_{hmax} = 430 ms and τ_{hmin} = 65 ms. Divalent ion concentration $[X^{2+}]_o$ is plotted on a logarithmic scale. The bars on the points reflect the standard deviation, each point reflects the mean of at least three measurements. The curve through the points was calculated assuming that p is proportional to $K_d/([X^{2+}]_o + K_d)$ with K_d = 3.5 mM for Ca^{2+}, and K_d = 1.5 mM for Ba^{2+}.

Fig. 4 shows the change in τ_h (p, as defined in the legend) in Ringer solutions with different $[Ca^{2+}]_o$ or $[Ba^{2+}]_o$. The results indicate that raising $[Ca^{2+}]_o$ from 10^{-9} M to 100 mM accelerates the rate of inactivation more than fivefold. The change is normalized such that p = 1 corresponds to the maximum inactivation time constant, τ_{hmax}, of 430 ms; p = 0 corresponds to the minimum inactivation time constant, τ_{hmin}, of 65 ms. The estimate of τ_{hmax} was obtained by measuring τ_h in Ringer solutions with $[Ca^{2+}]_o = 10^{-9}$ M and it was found to be 422 ± 17 ms (n = 3), whereas τ_{hmin} was determined by fitting the calculated curve to the measured data points. τ_{hmin} was the same for either Ca^{2+} or Ba^{2+}. It is concluded that Ca^{2+} or Ba^{2+} can bind with apparent K_d's of 3.5 and 1.5 mM to a site with one-to-one stoichiometry (Hill plots of these curves yield a slope of 1.03 and 1.01, respectively), and thus influence K^+ channel inactivation.

In addition to accelerating the rate of inactivation, Ba^{2+} ions result in progressive "use-dependent" block of K^+ current. Fig. 5 *A* shows a K^+ current in normal Ringer associated with a 1-s test pulse to 40 mV from a holding potential of −100 mV. Immediately after recording this trace, the bath solution was changed to 10 mM Ba^{2+} and K^+ currents elicited by identical test pulses were recorded. The first trace in 10 mM Ba^{2+} shows an acceleration in the decay of the K^+ current, whereas the peak current amplitude is hardly affected, which is similar to the effect of raising external Ca^{2+} shown in Fig. 1. Subsequent test pulses result in a progressive reduction of the peak current amplitude. This type of "use-dependent block" is similar to the effects of external Ba^{2+} on the delayed outward K^+ currents in muscle fibers (Werman and Grundfest, 1961; Sperelakis et al., 1967), squid axons (Armstrong and Taylor, 1980), and myelinated nerve fibers (Arhem, 1980). The peak K^+ current

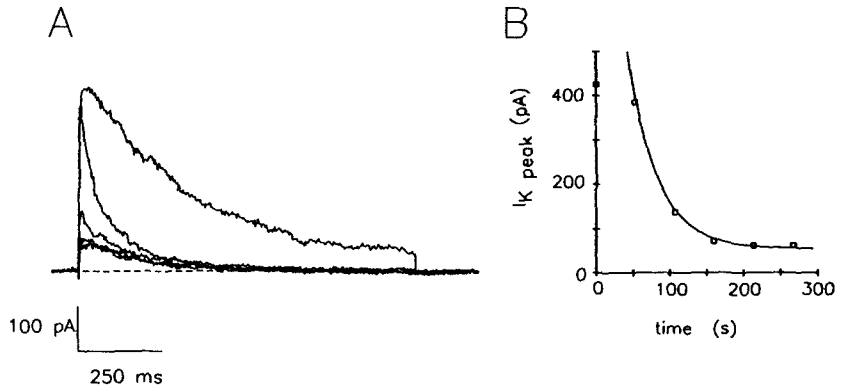

FIGURE 5. Block of K^+ currents by 10 mM Ba^{2+}. (*A*) K^+ currents associated with 1-s depolarizing test pulses to 40 mV from a holding potential of −100 mV in normal Ringer solution (control) and immediately after application of a Ringer solution containing 10 mM Ba^{2+}. $[Ca^{2+}]_i = 10^{-8}$ M. The interval between test pulses was 53 s, long enough to allow complete recovery from inactivation in normal Ringer solution. (*B*) Peak current amplitudes of the K^+ currents ($I_{K\,peak}$), shown in *A*, at different times after the solution change from normal Ringer to 10 mM Ba^{2+}. Cell 603.

amplitudes are plotted in Fig. 5 *B*. A single exponential fit to these points yields an apparent time course for blocking the K^+ channels by Ba^{2+}. This apparent time course strongly depends on the interval between test pulses (data not shown), suggesting that Ba^{2+} can block only if the channel is open.

Fig. 6 illustrates that removal of the Ba^{2+} block also depends on channel opening. After changing the bath solution from 10 mM Ba^{2+} to normal Ringer solution (shown at the arrow), the Ba^{2+} block of K^+ current is slowly removed as the depolarizing pulses are continued; peak K^+ current recovers almost to the original level, ~1,400 pA. The time constant for washing out the Ba^{2+} using this particular protocol was ~80 s. If, on the other hand, pulses are not delivered after washing out the Ba^{2+}, the channels remain blocked, as illustrated in the right-hand side of the figure. This time, after establishing the Ba^{2+} block in 10 mM Ba^{2+} and then restoring the

bath solution to normal Ringer solution, the membrane potential was held constant at −80 mV for 10 min without pulses. When the same sequence of depolarizing pulses was then delivered, the time course for washing out the Ba^{2+} was the same as the one on the left side, except for the 10-min delay in which channels remained closed. Ba^{2+} can therefore be trapped within the closed channel and cannot dissociate from the blocking site until after the channel opens. The time course for washing out the Ba^{2+} also depends very strongly on the interval between pulses (data not shown), suggesting that the longer the channels are held open, the faster the washout. This mechanism is similar to Ba^{2+} block of delayed outward K^+ current in squid axons and myelinated nerve fibers (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982; Woll, 1982) and of voltage-dependent Ca^{2+}-

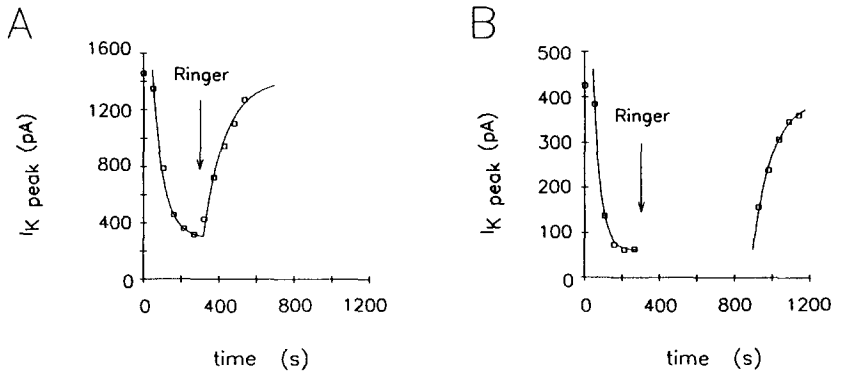

FIGURE 6. Removal of the Ba^{2+} block. K^+ currents were elicited with the same pulse protocol as described (legend of Fig. 5), and peak K^+ currents ($I_{K\,peak}$) were plotted at different times after the solution change from normal Ringer to 10 mM Ba^{2+}. $[Ca^{2+}]_i = 10^{-8}$ M. The bath solutions were changed from 10 mM Ba^{2+} to normal Ringer solution at the arrows. (*A*) The block of the K^+ current is removed without delay, and the peak K^+ current recovers almost to the original level before the Ba^{2+} treatment if depolarizing pulses are given every 53 s. Cell 604. (*B*) No recovery of $I_{K\,peak}$ occurred if the membrane was not depolarized. After 10 min in normal Ringer solution the membrane was depolarized with the same pulse protocol used for monitoring the Ba^{2+} washout in *A*. Cell 603.

activated K^+ channels from skeletal muscle (Vergara and Latorre, 1983; Miller, 1987; Miller et al., 1987).

Internal Ca^{2+} Mimics External Ca^{2+}

Bregestovski et al. (1986), as well as Choquet et al. (1987), suggested that internal Ca^{2+} may play a role in regulating the number of K^+ channels capable of being activated. In further experiments, we increased the intracellular Ca^{2+} concentration and monitored the inactivation time constant, τ_h, at +40 mV after breaking into the cells with pipettes filled with either $[Ca^{2+}]_i = 10^{-8}$ M, 10^{-5} M, or 2 mM, as illustrated in Fig. 7. With pipettes containing 10^{-8} M Ca^{2+} (squares) there is a small reduction of τ_h 10 min after breaking into the cell. This is probably due to the grad-

ual disappearance of a junction potential between the pipette solution and the cytoplasm (Marty and Neher, 1983). When the patch pipettes were filled with $[Ca^{2+}]_i$ = 10^{-5} M there was a much more pronounced effect of τ_h after breaking into the cell (triangles). After 10 min, the τ_h values with $[Ca^{2+}]_i = 10^{-5}$ M correspond to a 1.7-fold increase in the rate of inactivation, which was somewhat less than the threefold decrease in half-decay time reported by Choquet et al. (1987), and possibly due to the different test pulse voltages used. If the patch pipettes contained 2 mM $[Ca^{2+}]_i$, inactivation accelerated even further after breaking into the cell (circles). After 5 min, the τ_h values with $[Ca^{2+}]_i$ = 2 mM corresponded to a fivefold increase in the

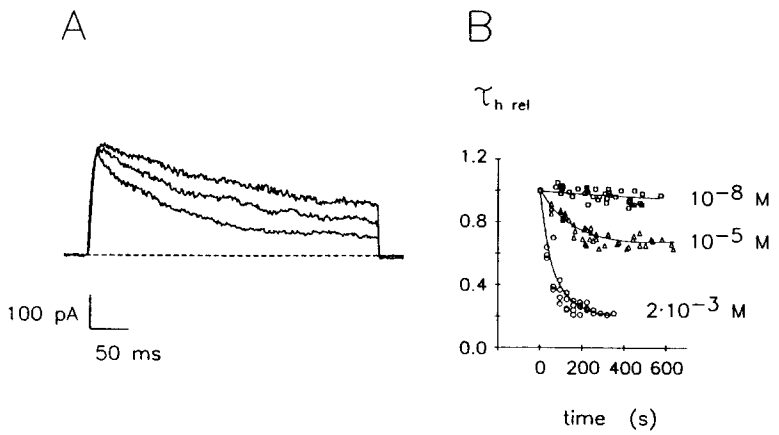

FIGURE 7. K^+ currents measured in normal Ringer solution at different times t after breaking into the cell with varying $[Ca^{2+}]$ in the pipette solution. (*A*) K^+ currents were associated with depolarizing pulses to +40 mV from a holding potential of −80 mV at time 0, 53 s, and 106 s (time 0 means ~20 s after reaching the whole-cell configuration). The pipette solution contained 2×10^{-3} M Ca^{2+}. (*B*) τ_h values were obtained by fitting single exponentials to the decay of the K^+ currents obtained from current records similar to the one shown in *A*. τ_h values were normalized to the τ_h values measured at time 0, meaning ~15 to 45 s after reaching the whole-cell configuration. The squares represent ten experiments with $[Ca^{2+}]_i = 10^{-8}$ M, the triangles represent nine experiments with $[Ca^{2+}]_i = 10^{-5}$ M, the circles represent four experiments with $[Ca^{2+}]_i$ = 2 mM. The lines through the points were drawn by eye for clarity.

rate of inactivation. With such a high internal Ca^{2+} concentration it was impossible to record longer than 6 min after breaking into the cell.

If the binding site for Ca^{2+} that influences the rate of K^+ channel inactivation is located within the membrane field, and Ca^{2+} is able to reach this site from either side of the membrane, raising $[Ca^{2+}]_i$ may alter the voltage dependence of the effect of $[Ca^{2+}]_o$ on τ_h. To assess this we did experiments of the type shown in Figs. 1–3, but with $[Ca^{2+}]_i = 10^{-5}$ M. The τ_h values obtained in normal Ringer with $[Ca^{2+}]_i$ = 10^{-5} M were plotted against the membrane potential, E, as shown in Fig. 8 (open squares). Raising $[Ca^{2+}]_o$ from 2 to 12 mM with $[Ca^{2+}]_i = 10^{-5}$ M shifted the $I_K(E)$ and $g_K(E)$ curves of the K^+ current by 10 mV to more depolarized potentials (not

shown). Therefore, it was necessary to correct the τ_h values measured with $[Ca^{2+}]_o$ = 12 mM and $[Ca^{2+}]_i = 10^{-5}$ M (filled triangles), in a manner similar to that shown in Fig. 3. The reduction of τ_h M Ca^{2+} was then determined by calculating the ratios of τ_h with normal Ringer outside and the corrected τ_h in Ringer with 12 mM Ca^{2+} outside. At E = 10 mV, the reduction was to 63% of the control value, whereas at E = 50 mV the reduction was to ~72% of the control value. This means that the effect of raising $[Ca^{2+}]_o$ on the inactivation time constant shows little voltage dependence. Comparing the effect of increased $[Ca^{2+}]_o$ on τ_h with $[Ca^{2+}]_i = 10^{-8}$ or 10^{-5} M yields two results. First, the voltage dependence of increased $[Ca^{2+}]_o$ on the inactivation time constant is steeper with $[Ca^{2+}]_i = 10^{-5}$ M compared with 10^{-8} M. Second, the change in τ_h produced by raising $[Ca^{2+}]_o$ is smaller when $[Ca^{2+}]_i = 10^{-5}$ M compared with 10^{-8} M, which suggests competition between $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ for the same site. In addition, this series of experiments provides further evidence that the lymphocyte K^+ channel is not activated by intracellular Ca^{2+}, as we did not

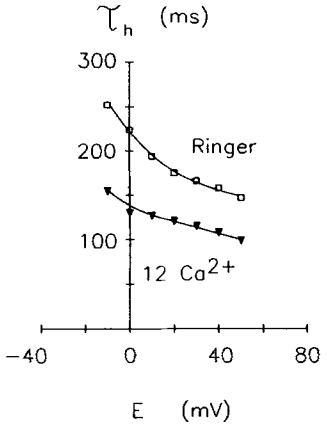

FIGURE 8. Inactivation time constants τ_h of K^+ currents in a Jurkat cell in normal Ringer (□) and in 12 Ca^{2+} Ringer (▼) with $[Ca^{2+}]_i = 10^{-5}$ M in both cases. The ▼ values were obtained by correcting the τ_h values measured in 12 Ca^{2+} ($[Ca^{2+}]_i = 10^{-5}$ M) for a shift along the voltage axis due to a change in surface potential, a correction similar to the one in Fig. 3. The lines through the points were drawn by eye for clarity. Cell 133.

observe any significant shift of the activation parameters to more hyperpolarized potentials with $[Ca^{2+}]_i = 10^{-5}$ M, compared with $[Ca^{2+}]_i = 10^{-8}$ M.

If there is a binding site located on the cytoplasmic side of the membrane, which can be accessed by Ca^{2+} ions from the outside moving through the channel, then increasing the intracellular Ca^{2+} buffering may reduce the effect of extracellular Ca^{2+}, by analogy with Ca^{2+}-dependent inactivation of Ca^{2+} channels (Eckert and Chad, 1984). However, increasing cytoplasmic Ca^{2+} buffering by including 55 mM BAPTA and 5 mM Ca^{2+} in the pipette (free $[Ca^{2+}]_i = 10^{-8}$ M) did not change the effect of Ca^{2+} on τ_{hf} (data not shown). These results suggest either that the site at which $[Ca^{2+}]_o$ binds is inside the K^+ channel and not accessible to cytoplasmic Ca^{2+} buffers, or that there are two sites, one external and one internal.

K^+ and Ca^{2+} Have Opposing Effects on K^+ Channel Inactivation

Reducing $[K^+]_o$ from 4.5 to 0 mM has almost the same effect on the K^+ current as does increasing $[Ca^{2+}]_o$, as shown in Fig. 9 *A*. The time course of the decay of the

K^+ current is faster in 0 mM K^+ compared with normal Ringer solution, with τ_h values of 200 and 285 ms, respectively. Thus, removing K^+ from the Ringer accelerated the rate of inactivation by a factor of 1.4. Fig. 9 *B* illustrates the effect of increasing $[K^+]_o$ from 4.5 to 160 mM. The reduction of the K^+ current amplitude in high $[K^+]_o$ is due to a change in reversal potential E_K for potassium ions; thus, the driving force of these ions at +40 mV is reduced. In addition to slowing channel closing during tail currents (Cahalan et al., 1985), raising $[K^+]_o$ slows down inactivation. The time course of the inactivation in 160 mM K^+ Ringer could be well described by a double exponential with fast and slow inactivation time constants, τ_{hf} and τ_{hs}. The value for τ_{hf} was 320 ms and was 1.6 times larger than the τ_h value

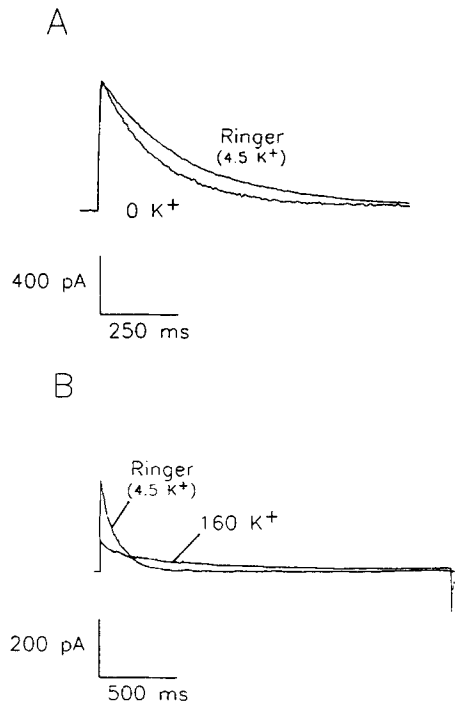

FIGURE 9. Effects of varying $[K^+]_o$ on K^+ channel inactivation. $[Ca^{2+}]_i = 10^{-8}$ M. (*A*) K^+ currents associated with 5-s depolarizing pulses to +40 mV from a holding potential of −80 mV in normal Ringer solution (4.5 mM K^+) and after application of a Ringer solution containing 0 mM K^+. Cell 119. (*B*) K^+ currents in a Jurkat cell in normal Ringer solution (4.5 mM K^+) and after application of a K^+-Ringer solution (160 mM K^+). K^+ outward currents associated with 3.8-s pulses to +40 mV. Cell 80.

found in normal Ringer solution (200 ms). The value for τ_{hs} was ~4 s, a slow inactivation not seen in normal Ringer.

A dose-response curve demonstrating the effect of $[K^+]_o$ on the fast component, τ_{hf}, of K^+ channel inactivation is shown in Fig. 10. The change in the fast inactivation time constant was normalized as was done previously with Ca^{2+}. A value of 1 corresponds to the maximum inactivation time constant of 430 ms. This τ_h value was assumed to be the same as in the experiments with $[Ca^{2+}]_o = 10^{-9}$ M. A value of 0 corresponds to the inactivation time constant of 200 ms and was obtained in experiments with $[K^+]_o = 0$ mM as shown in Fig. 9 *A*. Fits of these data to the Hill equation yielded a slope of 1.1, which is consistent with the idea that only a single potassium ion is required to slow τ_{hf}. A calculation of an adsorption isotherm to the dose-

response curve (line in Fig. 10) is in agreement with the data with a value of 13 mM for the apparent dissociation constant of K^+ at its site of action.

Three explanations for the opposing influences of Ca^{2+} and K^+ on inactivation are possible. (*a*) Two different receptors at the K^+ channel interact allosterically; Ca^{2+} bound to its receptor results in faster inactivation and K^+ bound to its receptor results in a loss of affinity of the Ca^{2+} receptor for Ca^{2+}. (*b*) Ca^{2+} can bind to a site where is can speed inactivation, and K^+ either competes with Ca^{2+} for this site or prevents Ca^{2+} from reaching it. (*c*) There are two separate and independent binding sites for Ca^{2+} and K^+; Ca^{2+} binding will result in a faster inactivation, whereas K^+ binding will result in slower inactivation. The agreement of $\tau_{h\,max}$ with $[Ca^{2+}]_o = 10^{-9}$ M and of $\tau_{hf\,max}$ with $[K^+]_o = 160$ mM (compare Figs. 4 and 10) argues against this idea of independent sites. Table II shows calculations of apparent dissociation constants of the Ca^{2+} site reaction (*a*) for the case of an affinity change at the Ca^{2+} binding site and (*b*) for the case of competition. Values of K_d, reflecting the change in affinity of the site for Ca^{2+} under (*a*), change almost two orders of magnitude (compare $K_d = 1$ mM for $[K^+]_o = 0$ mM with $K_d = 79$ mM for $[K^+]_o = 160$ mM).

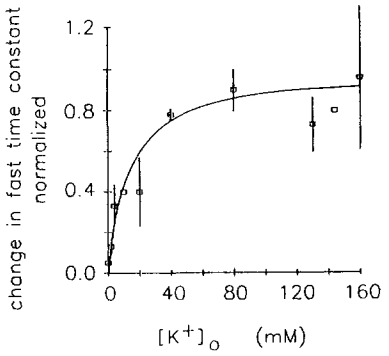

FIGURE 10. Dose-response curve of $[K^+]_o$ on the change p of the fast inactivation time constant τ_{hf} in Ringer solutions with $[Ca^{2+}]_o = 2$ mM. $[Ca^{2+}]_i = 10^{-8}$ M. p is the normalized change of τ_{hf}, with $\tau_{hf\,min} = 200$ ms and $\tau_{hf\,max} = 430$ ms, as discussed in the legend of Fig. 4. The curve through the points was calculated assuming that p is proportional to $K_d/([K^+]_o + K_d)$ with $K_d = 13$ mM.

This change in K_d seems to be too large to be explained by a loss in affinity of the Ca^{2+} binding site for Ca^{2+}. For the case of competition (*b*), the calculated dissociation constant of Ca^{2+} and its receptor, in the presence of the competing K^+, should be independent of $[K^+]_o$, as indicated in Table II assuming that the true dissociation constant for the reaction of K^+ with the receptor, i.e., K_K, is ~2 mM. From these calculations we favor the idea that Ca^{2+} and K^+ compete for the same binding site.

External Rb^+ and Divalent Trapping

The effects of replacing NaCl and KCl in normal Ringer with RbCl were similar to those shown in Fig. 9 *B* in which $[K^+]_o$ is increased from 4.5 to 160 mM. Interestingly, however, the first trace, after the application of Rb^+-Ringer, was consistently different in the time course of activation from the second and following traces in Rb^+-Ringer, as illustrated in Fig. 11 *A*. One can see that the K^+ current associated with the first test pulse to +40 mV in Rb^+-Ringer activates very rapidly (within 5 ms) to 767 pA, then activates further very slowly to reach a peak after 100 ms. It

then inactivates with a time course similar to the slow inactivation seen in 160 mM K^+-Ringer. The K^+ currents associated with the second and following test pulses in Rb^+-Ringer do not show the slow activation; the peak K^+ current is reached within 10 ms. The K^+ current decays associated with the first, second, and subsequent test pulses in Rb^+-Ringer are superimposable. In addition, tail currents in Rb^+-Ringer are prolonged compared with normal, as observed previously (Cahalan et al., 1985). We believe that the slow phase of activation represents the removal of divalent ions

TABLE II

Calculated Dissociation Constant, K_d, of Ca^{2+} Binding Site

$[Ca^{2+}]_o$	$[K^+]_o$	n	K_d calculated for assumptions (a)*	(b)‡
mM	*mM*		*mM*	*mM*
2.0	0.0	5	1.0	1.0
2.0	2.2	2	1.7	0.8
2.0	4.5	58	2.8	0.9
2.0	10.0	1	3.5	0.6
2.0	20.0	1	7.4	0.7
2.0	40.0	2	24.1	1.1
2.0	80.0	2	31.2	0.8
2.0	144.4	6	79.1	1.1
2.0	160	5	79.1	1.0
0.5	4.5	2	3.7	1.1
4.6	4.5	2	3.3	1.0
12.4	4.5	4	3.7	1.1
53.8	4.5	5	3.4	1.1
105.7	4.5	3	3.3	1.0

Apparent dissociation constant, K_d, of Ca^{2+} site reaction calculated for the case of (*a*) exclusive change of affinity of the Ca^{2+} binding site by K^+, and for the case of (*b*) competition. The equations used are from Ulbricht and Wagner (1975) and Grissmer (1984) with little modification. The equations are:

$$^*K_d = [Ca^{2+}]_o/(1/p - 1)$$

(p is defined in legend of Fig. 4) and

$$^‡K_d = [Ca^{2+}]_o/(1/p - 1)(1 + [K^+]_o/K_K)$$

K_K is the dissociation constant for the reaction of K^+ with the Ca^{2+} binding site, assuming that there is no direct influence of K^+ on the fast inactivation with K_K = 2 mM.

from K^+ channels, for reasons described below. On this assumption, the difference between the first and second current traces after applying Rb^+-Ringer represents the time course of unblocking. The peak amplitude of the difference current was 20% of the amplitude of the scaled second current trace, and the time course of the decay of this difference current was 72 ms. In a total of five experiments we obtained a relative peak amplitude of the difference current of 24 ± 3% (mean ± SD); the time constant of the decay of these difference currents was 75 ± 6 ms.

Pretreatment with 10 mM $BaCl_2$ increased the amplitude of the difference cur-

rent between the first and second current traces in Rb^+-Ringer to 76% of the peak, without changing the time constant of the decay of this difference current (70 ms), compared with that without Ba^{2+} pretreatment. This result, illustrated in Fig. 11 *B*, indicates that the fraction of channels containing trapped divalent ions is correlated with the amplitude of the difference current between the first and second current traces in Rb^+-Ringer. It also suggests that the relative amplitude of the difference current under normal conditions (i.e., no Ba^{2+} pretreatment) reflects the percentage of channels that have Ca^{2+} trapped inside.

To test whether the fraction of channels with trapped Ca^{2+} can be modified, we varied external and internal [Ca^+]. Increasing $[Ca^{2+}]_o$ to 106 mM Ca^{2+} accelerated inactivation, but changed neither the relative amplitude of the difference current

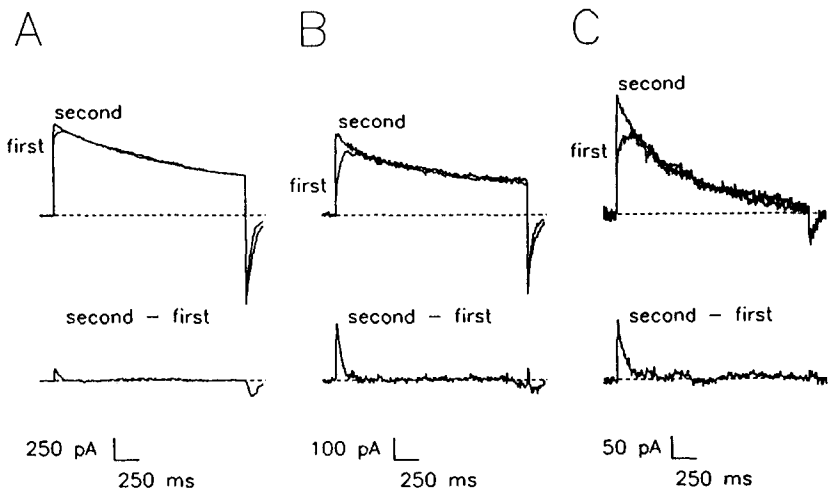

FIGURE 11. K^+ currents associated with the first and second 2-s depolarizing test pulses to +40 mV (holding potential −80 mV) after changing to Rb^+-Ringer. Pulse interval was 53 s. The bath solution was totally exchanged before delivering the pulses. (*Upper row*) Bath solution was changed from normal Ringer to Rb^+-Ringer ($[Ca^{2+}]_i = 10^{-8}$ M) in *A*, from a Ringer solution with 10 mM Ba^{2+} to Rb^+-Ringer ($[Ca^{2+}]_i = 10^{-8}$ M) in *B*, and from normal Ringer to Rb^+-Ringer ($[Ca^{2+}]_i = 10^{-5}$ M) in *C*; records were taken after the solution change. (*Lower row*) Difference current of the first and second current trace in Rb^+-Ringer.

between the first and second current traces in Rb^+-Ringer nor its decay. This suggests that Ca^{2+} loaded from the outside during the pulse may have been released to the inside during the 1-min pulse interval between the last current trace in 106 mM Ca^{2+} and the first trace in Rb^+-Ringer. This led us to attempt to determine whether we could increase the number of channels that have Ca^{2+} trapped inside by increasing $[Ca^{2+}]_i$. For this purpose we used patch-pipettes filled with $[Ca^{2+}]_i = 10^{-5}$ M. After breaking into the cell, we waited at least 5 min to attain equilibrium between the pipette solution and the cell cytoplasm. The pipette solution was then changed to Rb^+-Ringer. The difference between the first and second current trace obtained under these conditions is shown in Fig. 11 *C*. The peak amplitude of the difference current was 57% of the second current trace; the decay of the difference current

was 71 ms. In a total of five experiments, we obtained 52 ± 8% for the relative amplitude of the difference current; the time course for the decay of these difference currents was 76 ± 4 ms. We conclude that it is possible to load the channels with Ca^{2+} from the inside, and that this Ca^{2+} can be released to the outside.

Effects of Removing All Divalent Cations

To see whether the residual inactivation rate of K^+ channels (which corresponds to $\tau_{h\,max}$ in low external and internal $[Ca^{2+}]$ is due to block by Mg^{2+} ions, which would be analogous to the Mg^{2+} block of inward rectifier K^+ channels (Matsuda et al., 1987; Vandenberg, 1987), experiments were performed using internal solutions with no added Mg^{2+} ($[Ca^{2+}]_i = 10^{-8}$ M). After breaking into a cell we recorded normal inactivating K^+ currents even after 15 min with normal Ringer outside. If we changed to a Ringer solution with no added Mg^{2+} and $[Ca^{2+}]_o = 10^{-9}$ M, changes in the membrane current that were similar to those described by Armstrong and Lopez-Barneo (1987) occurred. We observed the following results. (*a*) The inward holding current increased. (*b*) When the voltage was stepped to $E = +40$ mV from a holding potential of -80 mV, there was an instantaneous jump in current from inward to outward. (*c*) The K^+ current became smaller, with slower decay of the current (comparable with the experiments with $[Ca^{2+}]_o = 10^{-9}$ M), and disappeared at last. Extending these results, which are similar to those found previously on squid neurons (Armstrong and Lopez-Barneo, 1987), we found that: (*d*) the induction of the new "leakage" conductance could be prevented by 2 mM Mg^{2+} inside. (*e*) This conductance could be blocked by either 1 mM Ca^{2+} or 1 mM Mg^{2+} outside. (*f*) Ba^{2+} could not only prevent the induction of this conductance but could also block it after its development with a K_d around 3 mM. (*g*) The induction of this conductance could not be prevented by totally blocking the K^+ channels with 10 nM charybdotoxin, a potent blocker of the K^+ channel (Sands et al., 1988), either before or during treatment with zero-divalent external and internal solutions. Although raising $[Mg^{2+}]_o$ to 12 mM has little effect on the K^+ channel inactivation rate (data not shown), even 1 mM Mg^{2+} can "protect" the K^+ channels while also preventing the increased leakage current seen in zero divalent. We are uncertain whether this conductance is related to the K^+ channel protein, or, alternatively, represents a nonspecific conductance induced by zero divalent.

DISCUSSION

It is well established that ions can alter the gating of voltage-dependent channels. One mechanism involves electrostatic attraction and binding of ions to negative surface charges at each side of the membrane (for review, see Hille, 1984), thereby influencing the voltage dependence of channel gating. Several observations, however, cannot be explained by surface potential theory alone. One example is that externally applied Zn^{2+} ions slow activation without altering the time course of deactivation in squid axons (Gilly and Armstrong, 1982). State-dependent binding mechanisms, in which the ion binds more strongly to a particular channel conformation, provide an explanation for effects of divalent ions on channel gating. A special case of state-dependent mechanisms involves ion binding to open channels. For example,

occupancy of an open channel by either a permeant or blocking ion has been hypothesized to hinder channel closing for both acetylcholine receptors and K^+ channels (Marchais and Marty, 1979; Swenson and Armstrong, 1981; Cahalan and Pappone, 1983; Matteson and Swenson, 1986; Armstrong and Matteson, 1986). The effects of permeant monovalent, divalent, and blocking ions on T cell K^+ channels are further examples of state-dependent channel mechanisms. A novel aspect of the results reported here is that we have focused on the effects of ions on channel inactivation gating.

Divalent Effects on Inactivation: Block or Modulation

We have shown that the lymphocyte K^+ channel inactivation rate is strongly influenced by external Ca^{2+} (or Ba^{2+}), as well as internal Ca^{2+}. There are two possible mechanisms that should be considered. Ca^{2+} binding to a site in the channel may result in (or modulate the rate of) a conformational change that inactivates the channel. A second possibility is that block of the K^+ channel by a Ca^{2+} ions is the inactivation mechanism. In this case, depolarization to open the channel would also enable entry and block by divalent ions; the slow recovery from inactivation would represent the unbinding of trapped Ca^{2+}. In either mechanism, if Ca^{2+} entry were decreased (*a*) by reducing $[Ca^{2+}]_o$, (*b*) by competition with an external permeant ion, or (*c*) by a K^+ channel blocker, then inactivation should become slower. We now consider experimental evidence on each of these points. (*a*) As $[Ca^{2+}]_o$ is reduced, the time constant of inactivation approaches a maximum value, $t_{h\,max}$, of ~430 ms. This residual inactivation observed in essentially Ca^{2+}-free internal and external solutions may be due to an intrinsic inactivation mechanism, or due to block by another ion present, for example Mg^{2+}. The induction of "leak" conductance and disappearance of K^+ conductance upon the removal of all divalents prevents a clear test of this hypothesis. (*b*) When $[K^+]_o$ is elevated, inactivation becomes slower and incomplete, even during pulses lasting 4 s (Fig. 9 *B*). Table II describes the interaction between $[K^+]_o$ and $[Ca^{2+}]_o$ on the fast component of inactivation; permeant monovalent ions may compete with Ca^{2+} for entry into the channel. A slow component of inactivation with a time constant greater than $t_{h\,max}$ is revealed when $[K^+]_o$ is raised. This may indicate that $t_{h\,max}$ is not due to an intrinsic inactivation mechanism. (*c*) External tetraethylammonium ($[TEA^+]_o$) slows the time constant of inactivation as it reduces the current, resulting in crossover of the K^+ currents in the presence and absence of TEA^+ and a constant current integral during long depolarizing pulses as $[TEA^+]_o$ is elevated (Grissmer and Cahalan, 1989). Similar crossover of K^+ current due to channel block, combined with slowing of inactivation, was also observed with external Mn^{2+} or Co^{2+} ions (DeCoursey et al., 1985). The kinetic effects of TEA^+ on inactivation were modeled by a scheme in which channels blocked by TEA^+ cannot inactivate (Grissmer and Cahalan, 1989). TEA^+ (or Mn^{2+} or Co^{2+}) blocking the K^+ channel may prevent inactivation by preventing Ca^{2+} entry. If this is true, then the fact that TEA^+ results in t_h values far greater than $t_{h\,max}$ might argue against an intrinsic t_h of 430 ms, as discussed in (*a*) above, and in favor of divalent entry being a prerequisite for inactivation. Either divalent block or a conformational change induced by a divalent ion within the channel would be consistent with the evidence described above.

The concentration dependence of the Ca^{2+} (or Ba^{2+}) effect on inactivation is apparently inconsistent with a simple divalent-block hypothesis; $t_{h\,min}$ saturates at a minimum value of 65 ms as $[Ca^{2+}]_o$ or $[Ba^{2+}]_o$ is elevated. According to a simple divalent-block mechanism for inactivation, one would expect the time constant of inactivation to decrease monotonically as the concentration of divalent is raised, as is the case for internal Ba^{2+} block of squid axon K^+ channels (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982). The effects of both $[Ca^{2+}]_o$ and $[Ba^{2+}]_o$ on lymphocyte K^+ channel inactivation can be described by a simple 1:1 binding isotherm, with saturation at high $[Ca^{2+}]_o$ or $[Ba^{2+}]_o$ at the same value of $t_{h\,min}$ (Fig. 4). This result suggests that divalent binding to a site inside the channel induces a conformational change that inactivates the channel with a limiting rate corresponding to $t_{h\,min}$. A more complicated blocking model involving extremely slow single filing of Ca^{2+} (or Ba^{2+}) between an outermost saturable site and a blocking site within the channel might also account for $t_{h\,min}$. Regardless of the exact mechanism, the acceleration of inactivation by divalent ions parallels the idea that channel closing (deactivation) is more rapid when channels contain Ca^{2+} than when they contain monovalent ions, as proposed for squid axon K^+ channels (Armstrong and Matteson, 1986).

Blocking Model for $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$

Raising either internal or external $[Ca^{2+}]$ accelerates the rate of inactivation. Competition between internal and external Ca^{2+} ions for the same binding site within the channel may result in the diminished effect of $[Ca^{2+}]_o$ on τ_h when $[Ca^{2+}]_i$ is raised (Figs. 3 and 8). We modeled the change in τ_h by assuming that Ca^{2+} can block the open channel nearly irreversibly, and that Ca^{2+} may reach the blocking site from either side of the membrane. Under this assumption one can separate τ_h due to an intrinsic inactivation process (measured in $[Ca^{2+}]_o = 10^{-9}$ M and $[Ca^{2+}]_i = 10^{-8}$ M) from τ_{Ca} due to Ca^{2+} reaching the site. These τ_{Ca} values measured in different $[Ca^{2+}]_o$ and different $[Ca^{2+}]_i$ were plotted against the absolute membrane potential E as shown in Fig. 12 (open squares). The voltage dependent of τ_{Ca} changes from a small increase of τ_{Ca} with more depolarization in $[Ca^{2+}]_i = 10^{-8}$ M, to a marked reduction of τ_{Ca} with depolarization in $[Ca^{2+}]_i = 10^{-5}$ M. The lines through the points are least-squares fits to the data considering that the K^+ channel has one local free energy minimum (site) that can either be empty or occupied by Ca^{2+}. Ca^{2+} moves from either side of the membrane to the site over an intervening energy maximum (barrier). The data could be reasonably described by a free energy profile shown in Fig. 13, which illustrates a two-barrier one-site model. In this model the binding site for Ca^{2+} is almost at the extracellular side of the membrane, perhaps near the channel mouth. The reason for the very broad plateau of the inner barrier is the weak voltage dependence of τ_{Ca} in all the solutions with different Ca^{2+} concentrations. Alternatively, a second two-barrier one-site model, with the plateau at the outer barrier, is indistinguishable from the one shown in Fig. 13. The Ca^{2+} binding site would then be about two thirds of the way across the electric field from the outside.

The similar effects raising internal and external $[Ca^{2+}]$ on K^+ channel inactivation (Figs. 3 and 8), and the unblocking of divalent cations from K^+ channels (Fig. 11),

are results consistent with Ca^{2+} ions being sparingly permeant through K^+ channels, as suggested by the energy profile of Fig. 13. A calculation of the Ca^{2+} flux through an open K^+ channel using the rate constants for leaving the Ca^{2+} binding site (obtained from the barrier heights and well depth given in legend of Fig. 13) shows that this Ca^{2+} flux is ~0.6 ions/channel per s at 0 mV. Even with all K^+ channels open, the maximum Ca^{2+} flux in a human T cell with 400 channels would be ~250 ions/cell per s, at least 2 orders of magnitude smaller than estimates of Ca^{2+} entry from tracer flux measurements which were reported by Metcalfe et al. (1980) and Hesketh et al. (1983) to be 24,000–130,000 Ca^{2+} ions/cell per s. The calculated flux

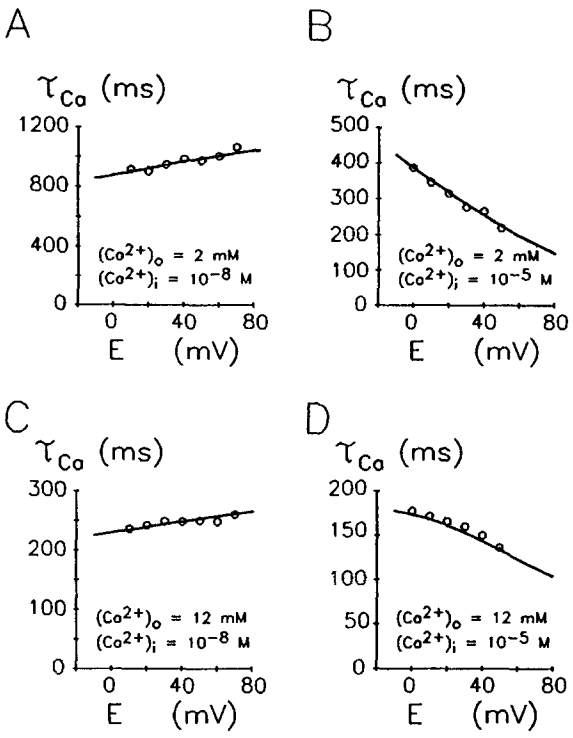

FIGURE 12. Time constants τ_{Ca} for Ca^{2+} reaching the site at the K^+ channel at four different combinations of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$. (*A*) $[Ca^{2+}]_o$ = 2 mM, $[Ca^{2+}]_i$ = 10^{-8} M. (*B*) $[Ca^{2+}]_o$ = 2 mM, $[Ca^{2+}]_i$ = 10^{-5} mM. (*C*) $[Ca^{2+}]_o$ = 12 mM, $[Ca^{2+}]_i$ = 10^{-8} M. (*D*) $[Ca^{2+}]_o$ = 12 mM, $[Ca^{2+}]_i$ = 10^{-5} M. τ_{Ca} was obtained by dividing the time course of the decay of the K^+ current with $[Ca^{2+}]_o$ = 10^{-9} M by the time course of the decay of the K^+ current in the different solutions. The newly created time course reflects the time course of Ca^{2+} reaching its site at the channel. The lines through the points were least-squares fits to the data with values for the inner and outer barrier; the well and the electrical distances are given in legend of Fig. 13.

is based upon the blocking model with a static energy profile as shown in Fig. 13. As discussed above, the changes in inactivation may result from modulation of an intrinsic inactivation process as Ca^{2+} ions from either side of the membrane bind to a site within the membrane. We cannot estimate the flux in this case.

In single channel studies of the Ca^{2+}-activated K^+ channel, Neyton and Miller (1988*b*, *c*) described Ba^{2+} block from either the *cis* or *trans* side of the membrane in terms of a single site within the channel that can bind Ba^{2+}. In their model to account for interactions between Ba^{2+} and K^+, three additional sites can bind K^+ ions (Neyton and Miller, 1988*c*), but when $[K^+]_o$ is lowered a second Ba^{2+} ion can also bind from the outside (C. Miller, personal communication). The asymmetric

concentration dependence and rates of Ba^{2+} block in the Ca^{2+}-activated K^+ channel are similar to the Ca^{2+} effect on lymphocyte K^+ channel inactivation described here. Although a single-site model may account for the divalent effects on inactivation, we cannot rule out additional divalent binding sites.

Trapping of Divalent Ions

The data presented in Figs. 5, 6, and 11 illustrate some of the dynamics of divalent trapping in the K^+ channel. Fig. 5 demonstrates that Ba^{2+} ions cannot enter the K^+ channel until it is opened. The channels must be opened in order for recovery from Ba^{2+} block to occur (Fig. 6). The recovery rate strongly depends on the interval between test pulses, suggesting that if the channels could be held open longer, the recovery from Ba^{2+} block would be faster. However, the channels inactivate during depolarizing pulses, and so Ba^{2+} ions do not dissociate from all the channels during a single depolarizing pulse. If one could keep the channels open for a very long time

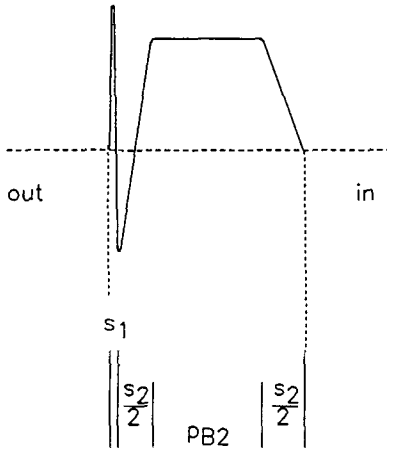

FIGURE 13. Energy profile for Ca^{2+} in the K^+ channel. This energy profile was obtained by the least-squares fit shown in Fig. 12. The heights, B, and position of the barriers and the well are: B_i = 21 RT, well = −14.7 RT, B_o = 15.7 RT, electrical distance of the well from the outside s_1 = 0.04, the length of the plateau of B_{out} (p_{B2}) is 0.52, and $s_2/2$ is therefore 0.22.

by preventing inactivation one might see the real time course for Ba^{2+} leaving the channel with a single depolarizing test pulse. We succeeded in reducing the rate of inactivation by changing to high Rb^+-Ringer, and we were able to measure the time course for Ba^{2+} and Ca^{2+} leaving the K^+ channel (see Fig. 11). The time course for leaving the K^+ channel, reflected in the decay of the difference current shown in Fig. 11, is the same for either Ba^{2+} or Ca^{2+}. This similarity in the time course of the difference current may result from the dissociation of Rb ions being rate limiting; if Rb^+ binds to a site and can prevent the removal of a Ca^{2+} or Ba^{2+} ion, the time course of the difference current would be limited by the time course for Rb^+ leaving its site. This assumption could also explain the failure to observe a difference in the first and second current trace in 160 mM K^+ Ringer. Under normal conditions ~25% of all channels contain a Ca^{2+} ion. Surprisingly, raising $[Ca^{2+}]_o$ did not increase this fraction, as indicated by the amplitude of the difference current between the first and second current trace in Rb^+-Ringer. An explanation for this

behavior could be that Ca^{2+} is able to leave its binding site even if the channel is closed. The latter possibility is supported by the experiments with an increase in $[Ca^{2+}]_i$ to 10^{-5} M, which accelerates inactivation and increases the difference current to >50%.

Several lines of evidence are presented in this paper which indicate that Ca^{2+} and Ba^{2+} are able to enter open K^+ channels from the outside. If the channel closes, Ca^{2+} and Ba^{2+} become trapped inside the channel. Ba^{2+} remains trapped, whereas Ca^{2+} is thought to cross the membrane to the inside during the interval between test pulses. Ba^{2+} trapping has been described previously for the high conductance Ca^{2+}-activated K^+ channel from rabbit or rat muscle incorporated into planar lipid bilayers (Vergara and Latorre, 1983; Miller, 1987; Miller et al., 1987; Neyton and Miller, 1988*a*). The existence of a Ba^{2+}-trapping mechanism, as well as the previously described sensitivity to charybdotoxin of the Ca^{2+}-activated "maxi"-K^+ channel and the lymphocyte K^+ channel (Miller et al., 1985; Sands et al., 1988), implies structural similarities between these two channel types and raises the question of whether the K^+ channel in T lymphocytes might be Ca^{2+} activated. Our experiments with high internal Ca^{2+} of up to 2 mM show no apparent activation of this channel; instead, raising $[Ca^{2+}]_i$ speeds up inactivation. One conclusion from our experiments on Ba^{2+} trapping is that divalent ions in the internal or external solution must wait for the channel to open before entering the pore. If the channel closes, Ba^{2+} is trapped inside the channel and remains trapped even in the absence of extracellular and intracellular Ba^{2+}. From the sidedness of the Ba^{2+} action, one has to conclude that both the Ca^{2+}-activated K^+ channel and the voltage-gated K^+ channel in T lymphocytes have a gating mechanism at the external side that can shield the inside of the channel from externally applied divalent ions, as well as a gating mechanism at the cytoplasmic side of the membrane that shields the inside of the channel from internal blockers (cf. Armstrong, 1971).

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