EXTERNAL [K^+] AND THE BLOCK OF THE K^+ INWARD RECTIFIER BY EXTERNAL Cs^+ IN FROG SKELETAL MUSCLE

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ABSTRACT Frog skeletal muscle has a K^+ channel called the inward rectifier, which passes inward current more readily than outward current. Gay and Stanfield (1977) described a voltage-dependent block of inward K^+ currents through the inward rectifier by external Cs^+ in frog muscle. Here, frog single muscle fibers were voltage clamped using the vaseline-gap voltage-clamp technique to study the effect of external [K^+] on the voltage-dependent block of inward K^+ currents through the inward rectifier by external Cs^+. The block of inward K^+ currents through the channel by external Cs^+ was found to depend on external [K^+], such that increasing the external concentration of the permeant ion K^+ potentiated the block produced by the impermeant external Cs^+. These findings are not consistent with a one-ion channel model for the inward rectifier. The Eyring rate theory formalism for channels, viewed as single-file multi-ion pores (Hille and Schwarz, 1978), was used to develop a two-site multi-ion model for the inward rectifier. This model successfully reproduced the experimentally observed potentiation of the Cs^+ block of the channel by external K^+, thus lending further support to the view of the inward rectifier as a multi-ion channel.

INTRODUCTION

The potassium channel known as the inward rectifier was first described in frog skeletal muscle by Katz (1949), who found that in isotonic potassium sulfate, the conductance of frog muscle was much greater for inward currents than for outward currents. Inward rectification with similar properties has been described in a variety of other preparations, including starfish eggs (Hagiwara and Takahashi, 1974) and tunicate eggs (Ohmori, 1978), cardiac Purkinje fibers (Noble and Tsien, 1968), cat spinal motoneurons (Nelson and Frank, 1967), and guinea-pig olfactory cortex neurons (Constanti and Galvan, 1983).

In 1977 Gay and Stanfield described a voltage-dependent block of inward K^+ currents through the inward rectifier in frog skeletal muscle by external Cs^+. The block, which appeared to be instantaneous, increased with hyperpolarization of the membrane, as well as with the increase of external [Cs^+]. Outward K^+ currents were not affected. A similar (though time-dependent) block of inward currents through the inward rectifier by external Cs^+ was reported for starfish eggs by Hagiwara et al. (1976). Cs^+ itself was found to be essentially impermeant in both preparations. In the starfish eggs it was observed also that the block by external Cs^+ was sensitive to the [K^+] of the solution bathing the preparation, such that increasing the external [K^+] potentiated the block produced by a given external [Cs^+] at a particular membrane potential. A similar potentiation by K^+ of the inactivation or block produced by external Cs^+ was reported in the inward rectifier of tunicate eggs by Ohmori (1980).

Here experiments are described that demonstrate that in frog skeletal muscle, too, external K^+ potentiates the block of inward K^+ currents through the inward rectifier by external Cs^+. These findings are interesting especially in terms of their implications for modeling the inward rectifier channel; they are not consistent with a one-ion model for the channel, but they can be explained by a model in which the inward rectifier is viewed as a multi-ion channel. A two-site multi-ion model using the Eyring rate theory formalism discussed by Hille and Schwarz (1978) is described in this paper; the model successfully reproduces the experimental findings reported here.

METHODS

Clamp, Preparation, and Solutions

The Hille-Campbell vaseline-gap voltage-clamp technique (Hille and Campbell, 1976) was used in all experiments. This technique has been used previously to study the inward rectifier in frog skeletal muscle by Hestrin (1981), Schwarz et al. (1981), and Blatz (1984). The methods employed here closely resemble those described in the previous papers. I_E (as defined by Hille and Campbell) was used to measure current; in my circuit this was recorded as the voltage drop across a 1-K-ohm resistor in the current-injecting pathway. No corrections were made in the raw currents for capacity transients or leakage currents.

Single muscle fibers with diameters 80–100 μm were dissected from

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the dorsal head of the semitendinosus muscle of the northern grass frog *Rana pipiens*. Dissection and mounting in the experimental chamber were carried out in a solution containing 128 mM K-glutamate; 5 mM $MgSO_4$; 10 mM HEPES; 0.01 mM EGTA; adjusted to pH 7.2 with 3 mM KOH. During the experiments the cut fiber ends were bathed in a very similar solution, except that the K-glutamate concentration was 126 mM, the [EGTA] was raised to 1 mM, and 0.5 mM Na_2-ATP was added. The concentration of K^+ in the dissection and internal solutions resembled the internal $[K^+]$ of intact fibers. The presence of EGTA insured fiber relaxation during dissection and prevented fiber contraction during experiments. ATP was added to the internal solution (following Kovacs and Schneider, 1978) to aid in the maintenance of a healthy metabolic state in the preparation. The literature (e.g., Horowicz and Gerber, 1965) indicates that muscle metabolism and heat production increase under conditions of high $[K^+]_o$, and in my experiments external K^+ concentration was elevated.

The primary constituent of the external solutions was either 63 mM K_2SO_4 or a combination of K_2SO_4 and Na_2SO_4, totaling 63 mM (see Table I). Sulfate was used as the anion because it is impermeant (Horowicz, 1961). Sodium was substituted for potassium when $[K^+]_o$ was lowered. At the depolarized potentials used in these experiments, external Na^+ did not block the inward rectifier in the manner described by Standen and Stanfield (1979). (This was verified by several experiments in which the substitution of TMA_2SO_4 for K_2SO_4 was compared with the substitution of Na_2SO_4 for K_2SO_4.) Where Cs^+ was required in the external solutions, it was added as Cs_2SO_4 without substitution for K^+ or Na^+; thus the Cs^+-containing external solutions were in all other respects identical to the control external solutions. All of the external solutions contained 30 μM strophanthidin, a concentration sufficient to inactivate the Na^+-K^+ ATP-ase, which is stimulated under conditions of high $[K^+]_o$ (Horowicz and Gerber, 1965). All experiments were done at 13°C.

Procedure

Fibers were held at or near their calculated resting potentials (potassium equilibrium potentials), at either 0 or −17 mV. At these potentials other conductances in frog muscle are in nonconducting states (Adrian et al., 1970; Sanchez and Stefani, 1978), and with experimental solutions of the indicated ionic composition, the current measured was a summation of K^+ inward rectifier current and a linear leak component (see discussion of linear component in Results). From the holding potential, test pulses of 37 ms were applied at 2-s intervals, generating families of current signals, which were filtered at 1 KHz. The filtered current signals were sampled every 150 μs by an analog-to-digital converter controlled by a microcomputer. Data were stored on floppy disks.

Sampling of the current signal by the computer began 2 ms before the onset of each test pulse. The points sampled in this 2-ms interval were averaged, and this average of the baseline current was subtracted from the current signal collected during the test pulse.

Fig. 1 *A* shows a family of currents in control solution A1, containing 129 mM K^+ externally. Test pulses were applied from the holding potential of 0 mV. Currents for test pulses in the range from −43 to −85 mV have a detectable activation hump during the early part of the pulse. In several experiments performed in lower temperatures with lower $[K^+]_o$, this activation hump was more pronounced over a wider range of hyperpolarizing test pulses, which is consistent with the dependence of the activation of inward rectification on temperature and external $[K^+]$, as reported by Leech and Stanfield (1981).

Steady state current values were obtained by averaging the 10 points sampled from 20–21.4 ms after the onset of the test pulse. At this time in the test pulse even at the most negative test potentials, the effect of K^+ depletion from the T-tubules (Almers, 1972*a*, *b*) was negligible, while

TABLE I
COMPOSITION OF EXTERNAL SOLUTIONS

Reference	K^+	Na^+	Cs^+	SO_4^-
		mM		
A1	129	0	0	76
A2	129	0	0.25	76.125
A3	129	0	0.5	76.25
A4	129	0	1.0	76.5
A5	129	0	2.5	77.25
A6	129	0	5.0	78.5
B1	65	64	0	76
B2	65	64	0.25	76.125
B3	65	64	0.5	76.25
B4	65	64	1.0	76.5
B5	65	64	2.5	77.25
B6	65	64	5.0	78.5
C1	34	95	0	76
C2	34	95	0.25	76.125
C3	34	95	0.5	76.25
C4	34	95	1.0	76.5
C5	34	95	2.5	77.25
C6	34	95	5.0	78.5

In addition to the above, all external solutions contained 8 mM Ca^{++}; 5 mM Mg^{++}; 10 mM HEPES; 30 μM strophanthidin; 70 mM sucrose. pH 7.2.

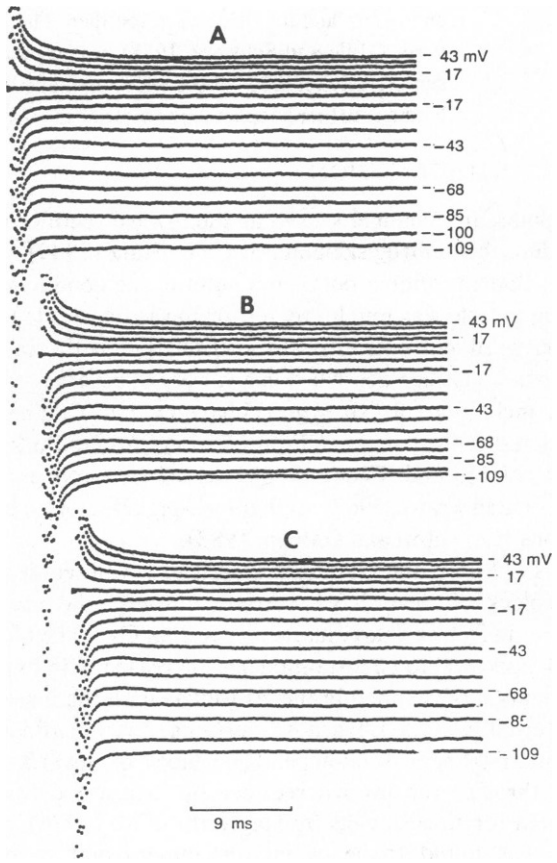

FIGURE 1 A set of current families from one fiber under voltage clamp in three successive external solutions. (*A*) 129 mM K^+ (control solution A1). (*B*) 129 mM K^+ and 1.0 mM Cs^+ (solution A4); current family taken 3 min after solution change, as in all cases following solution changes. (*C*) Return to solution A1. Holding potential: 0 mV. Command voltages for several of the pulses are indicated by numbers with dashes pointing to the appropriate current trace. Current magnitudes were in arbitrary units that have been omitted.

activation of the currents (Almers, 1971; Leech and Stanfield, 1981) was essentially complete; see Fig. 1. (For comparison, Blatz (1984) reports that in his experiments using the vaseline-gap clamp, inward rectifier currents achieved their steady state values in 5–10 ms.) Steady state current values were used here, as it was not the aim of these experiments to study either the kinetics of Cs^+ block or the possible effects of Cs^+ on inward rectifier activation kinetics. As pointed out by Schwarz et al. (1981), any attempt at the investigation of (macroscopic) kinetics in the presence of Cs^+ would have been complicated by the delay between the beginning of the test pulse and the achievement of voltage clamp of the T-tubules, as well as by the difficulty of separating capacity currents from ionic currents.

All the currents discussed in the Results section were measured in the steady state as described above. Since only relative current magnitudes were needed for analysis of the data, in most experiments currents were expressed in terms of arbitrary units.

The data analysis and modeling calculations were performed on the laboratory microcomputer.

RESULTS

In Fig. 1 raw currents are shown from a fiber that underwent two successive external solution changes, illustrating the effect of adding Cs^+ to the external solution and the subsequent recovery from this effect upon its removal. Fig. 1 *B* shows that inward currents were reduced markedly by the addition of Cs^+, while outward currents were not significantly affected. Upon return to the control solution, the inward currents recovered from the cesium-induced inhibition; the currents recorded in Fig. 1 *C* are almost indentical in magnitude to the initial control currents in Fig. 1 *A*.

Steady state currents (as described in Methods) are plotted in the current-voltage (*I-V*) curves shown in Fig. 2 *A* for the raw currents in Fig. 1 *A* and Fig. 1 *B*. In Fig. 2 *B* the steady state *I-V* curves for the three families of raw currents in Fig. 1 are shown after subtraction of a linear component of current, found for each *I-V* curve by taking the best least squares fit through the origin for a straight line that includes the points representing outward currents. Subtraction of this linear component, which is considered to be the leak superimposed on the current through the inward rectifier, was first suggested by Adrian and Freygang (1962); it is now standard procedure in the analysis of inward rectifier currents in frog muscle. The correspondence of this linear component of current to leak is substantiated by the current observed in one of my preparations when all the external K^+ was replaced by Cs^+, which is essentially impermeant through the inward rectifier (Gay and Stanfield, 1977; Standen and Stanfield, 1980). The current-voltage curves from this experiment are shown without subtraction in Fig. 3. It is apparent that the current measured in the presence of 129 mM Cs^+ externally is almost identical to the linear component that would be derived by taking the best-fitting line through the origin for the points representing outward currents.

Experiments were performed in such a way as to compare the effect of a particular external $[Cs^+]$ on the inward K^+ currents in a given fiber at two different external K^+ concentrations. A family of currents was collected in a control external solution, a corresponding cesium-containing solution, and again in the original control external solution after a 3-min recovery. Only those sets of current families in which good recovery (at least 80% of the initial control currents) was obtained in the second (bracketing) control were considered acceptable. In the second part of each experiment, the external control solution was changed to one containing a different $[K^+]$. The holding potential was adjusted to approximate the calculated resting potential in the new solution, and a set of current families was taken in the new $[K^+]_o$. In some experiments this was

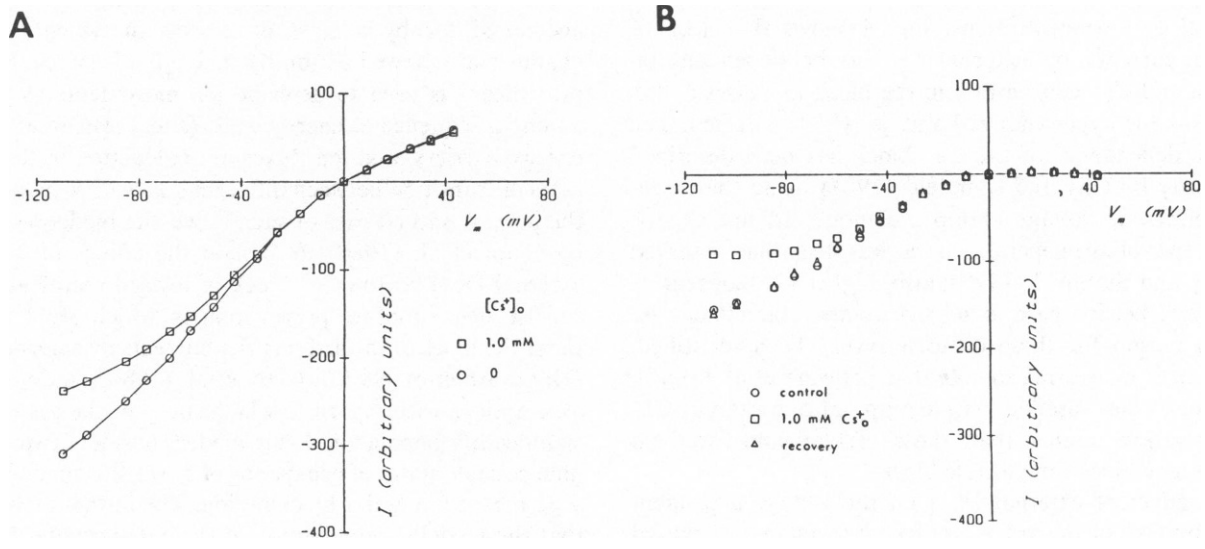

FIGURE 2 Current-voltage curves for steady state currents from the current families in Fig. 1. (*A*) Before subtraction of the linear component of current. (*B*) After subtraction of linear component. Circles: intial control (raw currents in Fig. 1 *A*). Squares: $[Cs^+]_o$ = 1.0 mM (Fig. 1 *B*). Triangles in *B*: return to control (Fig. 1 *C*).

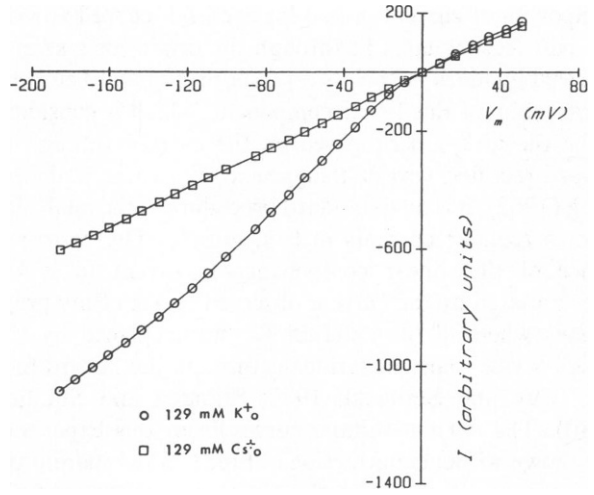

FIGURE 3 Steady state I–V curves from an experiment where 129 mM external K^+ (*circles*) was replaced by 129 mM Cs^+ (*squares*). $[K^+]_i$ = 127.5 mM, as in all experiments. Holding potential: 0 mV. No subtraction has been performed on these currents.

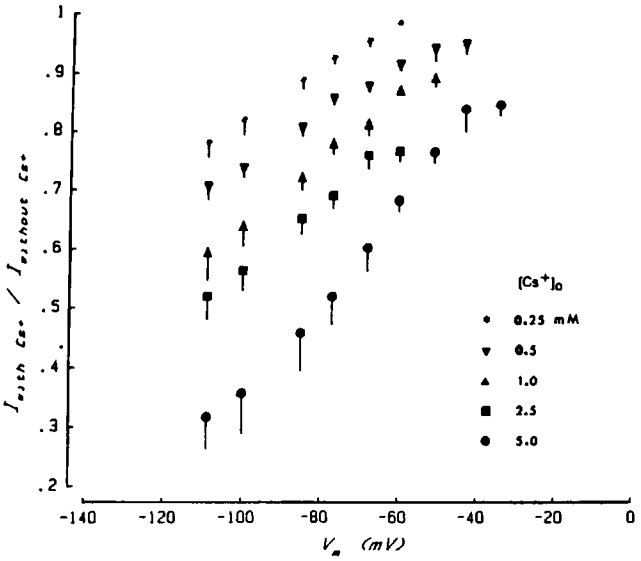

FIGURE 4 Fraction of current remaining in the presence of Cs^+ plotted against membrane potential. From experiments with 20 different fibers. $[K^+]_o$ = 129 mM. $[Cs^+]_o$ varied as shown in the symbol key. Each symbol represents an average of the ratios from several sets of current families. For $[Cs^+]_o$ of 0.25, 0.5, and 1.0 mM, 5 values were averaged for each symbol; 4 values were averaged from experiments with $[Cs^+]_o$ of 2.5 and 5.0 mM. Error bars represent the standard error of the mean.

followed by a return to the original control solution to repeat the first series of solutions.

The block of inward K^+ currents by external Cs^+ is expressed here in terms of a ratio of current (after linear component subtraction) at a given voltage in the presence of a particular $[Cs^+]_o$ to current at this voltage in the absence of Cs^+: Ratio = I with Cs^+/I without Cs^+. In each case the denominator is an average of the initial control and the recovery current values. Inward currents at the more positive hyperpolarizing test potentials were small, and their measurement was not consistently reliable; consequently, they were omitted from this analysis.

In Fig. 4 the averages of the ratios I with Cs^+/I without Cs^+ are plotted against V_m for a series of 23 sets of current families taken in 129 mM $[K^+]_o$, and one of five different external Cs^+ concentrations. Fig. 4 shows the block of inward currents by external Cs^+ to be dependent on voltage and Cs^+ concentration; the block increases as the membrane is hyperpolarized and as $[Cs^+]_o$ is raised. The voltage dependence of the Cs^+ block has been described previously by Gay and Stanfield (1977) using the three-microelectrode voltage clamp technique. In my experiments, the voltage dependence was less steep than observed by Gay and Stanfield (1977); also, higher Cs^+ concentrations were needed here to achieve comparable effects. As yet no reason for these differences has been identified, though it is interesting to note that Schwarz et al. (1981), who used the vaseline gap clamp, also required Cs^+ concentrations higher than those of Gay and Stanfield (1977) to achieve comparable block.

The effect of external $[K^+]$ on the voltage-dependent block by Cs^+ of inward K^+ currents through the inward rectifier is illustrated in Fig. 5 for three different Cs^+ concentrations. It is apparent from Fig. 5 that decreasing the external $[K^+]$ relieves the block produced by a particular $[Cs^+]_o$ at any given voltage. The fraction of current remaining in the presence of Cs^+ at any given V_m increases as external $[K^+]$ decreases from 129 to 34 mM.

MODEL

The results presented here clearly conflict with the predictions of a one-ion channel model for the inward rectifier. Hille and Schwarz (1978) have shown that many of the transport properties of delayed rectifier and inward rectifier K^+ channels in a variety of preparations can be accounted for by a class of models in which the K^+ channels are viewed as multi-ion single-file pores. Eyring rate theory is used to describe ion movements as jumps among a sequence of energy wells (sites) over intervening energy barriers, and ion fluxes are calculated in terms of rates of transition between different states of occupancy of the pore. A special case of such a two-site model was used by Ciani et al. (1980) to explain the effects of V_m and external $[K^+]$ on the Cs^+ block of inward rectification in starfish eggs, another preparation in which $[K^+]$ potentiates the block of inward rectifier currents by external Cs^+ (Hagiwara et al., 1976). Ciani et al. (1980) made certain assumptions with regard to which states of the system are significantly populated. Their model considers two of the nine possible states of occupancy of a two-site channel with a permeant ion and a blocking ion. The authors maintain that the model's reproduction of the enhancement of Cs^+ block by increasing concentrations of K^+ depends on their assumptions regarding the different states of occupancy. However, of their two main assumptions, one was found to

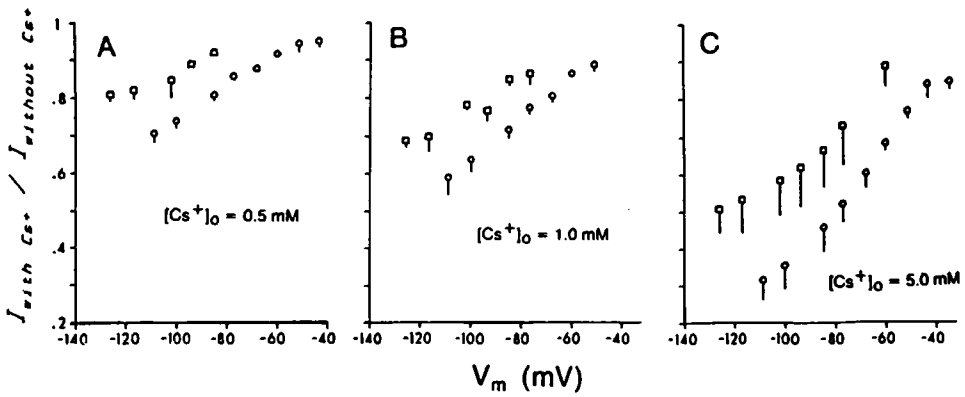

FIGURE 5 In each panel the fraction of current remaining in the presence of Cs^+ is plotted against membrane potential for a given $[Cs^+]_o$ with two different external $[K^+]$, 34 mM (*squares*) and 129 mM (*circles*). The symbols represent averages from several sets of current families. Circles are from experiments described in Fig. 4. Each square represents the average of two values. Error bars indicate the standard error of the mean.

be inconsistent with the model developed here, as described in the Discussion.

Schwarz et al. (1981), who studied K^+ current fluctuations in the presence of Cs^+ in the inward rectifier of frog muscle, also modeled the channel as a two-site multi-ion single-file pore, focusing, for their purposes, on a reaction scheme involving those states where the channel is unoccupied, or where one of the sites is occupied by Cs^+.

The purpose of the modeling described here was to determine whether a more general application of the

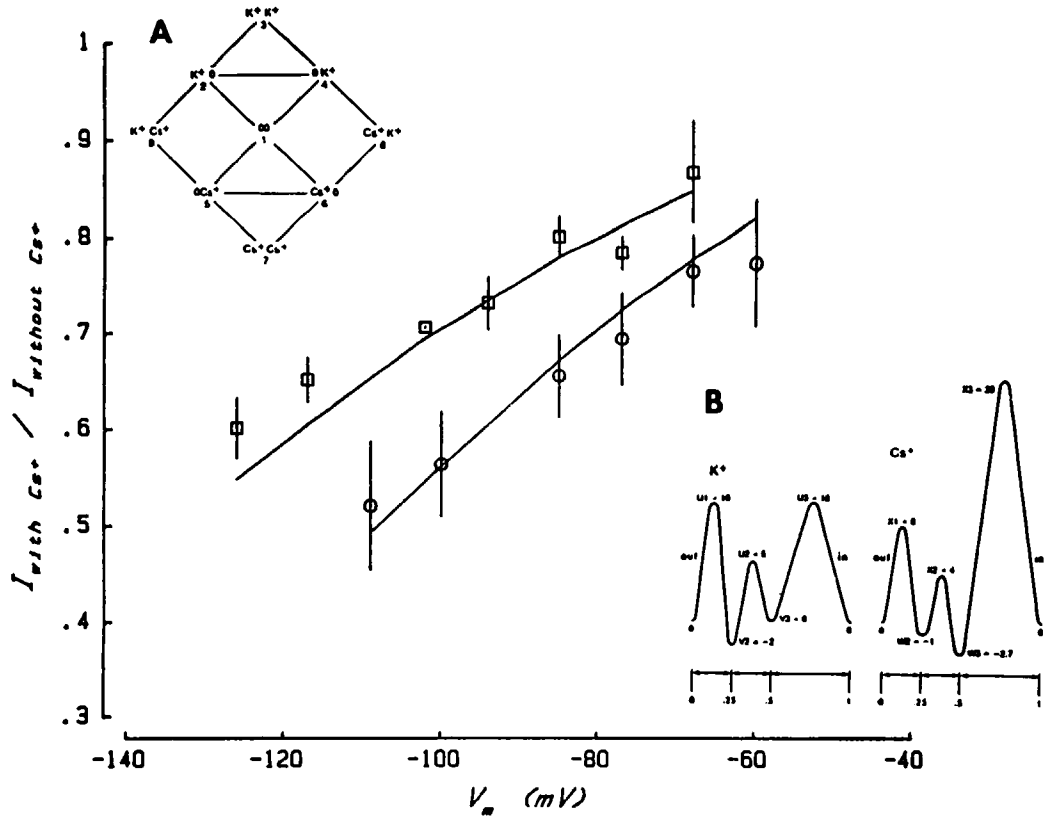

FIGURE 6 Fraction of current not affected by Cs^+ plotted against membrane potential. $[Cs^+]_o$ = 2.5 mM. Circles: $[K^+]_o$ = 129 mM; each symbol is the average of 4 values. Squares: $[K^+]_o$ = 34 mM; each symbol is the average of 2 values. Error bars represent ± the standard deviation. The solid lines were drawn through the corresponding values generated by the model. (*A*) The nine states of occupancy of the channel in the model. Permitted transitions are indicated by lines connecting the states. (*B*) The energy profiles for K^+ and Cs^+ used to generate the solid lines in this figure from the model. Barrier heights and well depths (in RT units) represent the chemical part of the free energies; electrical distance across the membrane is indicated by the horizontal arrows under each profile.

formalism used by Hille and Schwarz (1978) would account for the results reported here. A two-site multi-ion model for the channel was developed, allowing all nine of the possible states of occupancy of the channel in the presence of a permeant ion, K^+, and a blocking ion, Cs^+ (see Fig. 6, *inset A*). Forward and reverse rate constants derived from rate theory connect those states among which transitions are permitted. (Following Begenisich and Cahalan (1980), repulsion between ions was not included in the model.) K^+ is present on both sides of the membrane in the model, with the internal $[K^+]$ always at 129 mM. External $[K^+]$ was varied between 129 mM and 32 mM. Cs^+ was present externally in addition to K^+ when simulating the experimental runs. The probability of each of the nine possible states of occupancy of the channel in steady state was solved for by the method of Begenisich and Cahalan (1980). The barrier heights and well depths, as well as the electrical distance of the wells across the membrane, were varied until a combination of parameters which satisfactorily reproduced the data was found. The energy profiles in Fig. 6, *inset B* illustrate one of several such combinations of parameter values found in a limited search. Figure 6 shows how well this particular set of model parameter values reproduces the experimental data.

To get an intuitive understanding of the way in which this model reproduces the experimental findings, it is instructive to examine the fraction of channels in each of the nine states of occupancy of the system under a variety of conditions. The key to the model's ability to reproduce the potentiation of Cs^+ block by an increase in $[K^+]_o$ is in the probability of the state in which Cs^+ is in the inner well and K^+ is in the outer well of the pore (state 9 in Fig. 6, *inset A*). In this state, the pore is stopped up, since the Cs^+ ion is locked into the inner well by the presence of K^+ in the outer well and its high inner energy barrier. (The height of barrier X3 in Fig. 6, *inset B* reflects the fact that Cs^+ is known to be essentially impermeant.) In the presence of 2.5 mM external Cs^+, with the energy profiles in Fig. 6, the fraction of channels in this state increases about threefold when $[K^+]_o$ is raised from 32 to 129 mM. For example, at -100 mV, the probability of state 9 is 0.111 when $[K^+]_o$ is 32 mM, and it increases to 0.310 when $[K^+]_o$ is raised to 129 mM.

DISCUSSION

There is a substantial body of evidence for the multi-ion nature of the K^+ inward rectifier in frog skeletal muscle, as well as in other preparations, as summarized by Hille and Schwarz (1978). This paper provides additional support for this view of the channel. The model proposed successfully reproduced the data on a qualitative, and to some extent on a quantitative level. However, the model does not include the internal blocking particle suggested by Armstrong (1975) and used by Hille and Schwarz (1978) to produce the crossover effect that is characteristic of the inward rectifier. The introduction of this hypothetical blocking particle would have increased the number of free parameters significantly, thus complicating the calculations.

It is interesting to note that the model developed here, allowing all of the possible states of occupancy of the two-site channel in the presence of K^+ and Cs^+, is consistent with some of the assumptions made by Ciani et al. (1980) regarding the population of the different states. Adjustment of the free parameters of the present model to fit the data generally gave low steady state probabilities for all of the states with Cs^+ in the outer site, compared with the probability of the state where Cs^+ is in the inner site and K^+ in the outer site. This corresponds to the first assumption of Ciani et al. (1980). However, the second main assumption of Ciani et al. (1980), i.e. "pores containing K^+ as the sole ionic species ... are statistically negligible compared to those that are either empty or contain Cs^+ in the inner site only," does not follow from the present model. Even in the presence of Cs^+, a significant fraction of the channels (>20% under some conditions) was occupied by K^+ alone in the outer site, and at less hyperpolarized potentials this state tended to be at least twice as likely as the stopped up state with Cs^+ in the inner site and K^+ in the outer site. Ciani et al. (1980) themselves provided the caveat that their assumption notwithstanding, some pores may, in fact, "contain K^+ as the sole ionic species," but their assumption appears to have served well in the particular case of fitting the starfish egg data.

It is possible that a three-site model for the channel might have given even better reproduction of the experimental findings. The experimentally determined flux ratio exponent (n') for the inward rectifier has never exceeded 2 (Horowicz et al., 1968; Spalding et al., 1981), and though it is possible for a two-site channel to have an n' of 2, this value of the flux ratio exponent would be more likely for a channel with more than two sites (Begenisich and Smith, 1984; Hille and Schwarz, 1978). n' has a value of one for free-diffusion and one-ion pores, but it can be as large as the maximum number of ions allowed in a multi-ion pore (Begenisich and Smith, 1984). Hodgkin and Keynes (1955) first postulated the multi-occupancy of K^+ channels in nerve. Begenisich and De Weer (1980) found n' as high as 3.3 for the delayed rectifier in squid axon under certain conditions. Thus, there is evidence that some K^+ channels may have up to four sites. Further experiments on the inward rectifier in frog may help to elucidate the true number of ion sites in the channel, but the fact that the system is indeed a multi-ion channel now seems well established.

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