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Potassium Channels in the Plasmalemma of *Chara corallina* are Multi-Ion Pores: Voltage-Dependent Blockade by Cs^+ and Anomalous Permeabilities

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Summary. The outer membranes of plant cells contain channels which are highly selective for K^+. In the giant-celled green alga *Chara corallina*, K^+ currents in the plasmalemma were measured when the cell was depolarized to the K^+ equilibrium potential in relatively high external K^+ concentrations. K^+ current was reduced by externally added Cs^+. Cs^+ mainly inhibited inward K^+ current, in a strongly voltage-dependent manner; the effective valence of the blocking reaction was often greater than 1, increasing with higher external Cs^+ concentrations and with lower K^+ concentrations. This is consistent with the channels being single-file, multi-ion pores. Outward current could also be inhibited by Cs^+, when external K^+ concentrations were low relative to Cs^+ concentrations. As the ratio of K^+/Tl^+ was changed (keeping the sum of the two ions equal), both the resting potential and plasmalemma conductance went through minimums; this is the so-called "anomalous mole fraction effect," and is consistent with a channel whose pore can be multiply occupied. These effects together strongly suggest that the K^+ channels found in the plasmalemma of *Chara* are multi-ion pores.

Key Words K^+ · blockade · ion channel · current-voltage curves · channel structure · voltage clamp

Introduction

In the plasmalemma of giant cells of the green alga *Chara corallina*, there are K^+ selective channels which dominate membrane conductance when the cell is depolarized in solutions with a high K^+/Ca^{2+} ratio, when the PD stays near the K^+ equilibrium potential ("K-state": Keifer & Lucas, 1982; Beilby, 1985). It is thought that these channels may be similar to high unitary conductance Ca^{2+}-activated K^+ channels (high G K^+ (Ca^{2+})) which are widespread in animal cells (*see* Tester, 1988*a*).

Blockade of the *Chara* K^+ channels by Cs^+ was shown in the previous paper, but in the present study, the details of this blockade are investigated. The properties are compared with those of various channels found in animal cells, and provide a preliminary investigation of the structure of the plant plasmalemma K^+ channel.

Externally added Cs^+ is a well-known voltage-dependent blocker of inward K^+ currents of many types of K^+ channel in animal cells (e.g. Hagiwara, Miyazaki & Rosenthal, 1976), including of high G K^+ (Ca^{2+}) channels (Gorman, Woolum & Cornwall, 1982; Cecchi et al., 1987). The blockade by Cs^+ is often strongly potential dependent (i.e. the blockade increases with membrane hyperpolarization), and has been fitted for various channels to a model of ion blockade developed by Woodhull (1973) and Hille and Schwarz (1978). The fraction of blocked channels (R_B) to unblocked channels ($1 - R_B$) is

$$\frac{R_B}{1 - R_B} = \frac{[Cs]}{K_{Cs}} \cdot e^{-z'FE/RT},$$

where K_{Cs} is the dissociation constant at 0 mV of the Cs^+ binding, z' is the effective valence of the blocking reaction by externally added Cs^+, and F, E, R and T have their usual meanings (*see* Hille & Schwarz, 1978).

If the potential dependence of the blockade is anomalously strong, than this suggests that ions are interacting within the channel pore, and this is taken as evidence for the channels being able to contain more than one ion at a time—i.e. a multi-ion pore (Hille & Schwarz, 1978). Blockade of K^+ conductance by Cs^+ has been described in plant tissues (Sokolik & Yurin, 1981, 1986; Schauf & Wilson, 1987*a,b;* Findlay, Tyerman & Paterson, 1988), but usually not in detail.

In this work, the blockade by Cs^+ in *Chara* is shown to be consistent with the channels being multi-ion pores, and this conclusion is supported by showing that the channels display the so-called "anomalous mole fraction" effect. This is when both resting potential and conductance go through a

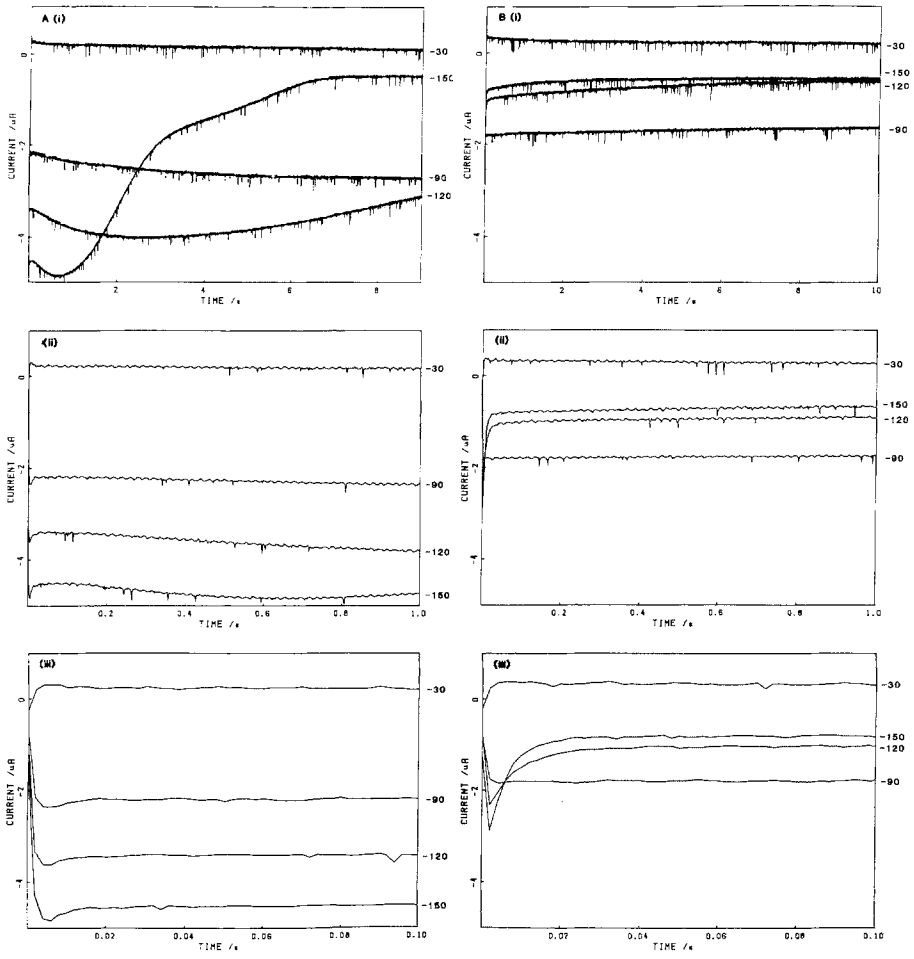

Fig. 1. Change of current with time ($I - t$) after jumps in voltage from the resting PD (−47 mV) to that shown next to each trace. Current was sampled every 2 msec and the clamp circuit settle time was 2 to 4 msec; channels remained open for at least 1 sec after the voltage jump. (*A*) Cell in 10K solution only, with the clamp held for 10 sec (i). Parts (ii) and (iii) the first second and first 100 msec, respectively, of the traces in part (i). (*B*) As in *a*, but cell with 0.3 mM CsCl added. The *I/V* relations plotted in Fig. 3 are from the same cell; cell surface area 1.3×10^{-5} m^2

minimum as the ratio of K^+/Tl^+ is changed (while keeping the sum of the two ions equal), and is only consistent with a channel whose pore can be multiply occupied. Anomalous permeabilities have been described for several types of channel (Hagiwara et al., 1977; Hille & Schwarz, 1978), including the high G K^+ (Ca^{2+}) channel (Eisenman, Latorre & Miller, 1986), but are not known to have been investigated previously in plant cells.

Abbreviations: APW, artificial pond water; *I/V*, current-voltage; PD, potential difference.

Materials and Methods

Current-voltage (*I/V*) relations of the plasmalemma of small leaf cells of *Chara corallina* were used to measure the K^+ and "leak" conductances, and most of the procedures used have been described in the previous paper (Tester, 1988*b*). All the results described here and in the previous paper were obtained during the same series of experiments.

The fraction of blocked to unblocked channels was calculated using the K^+ conductance, G_K obtained from *I/V* curves viz.:

$$\frac{R_B}{1 - R_B} = \frac{G_K \text{ in the control solution}}{G_K \text{ in solution with inhibitor added}} - 1.$$

The change in current required to clamp the plasmalemma to various potentials after a PD jump from the resting potential was measured using the programmes COMSET and DIPLOG of Beilby and Beilby (1983), but no sine wave was superimposed upon the baseline clamp potential. The clamp was held for 10 sec, and current was sampled every 2 msec.

TlCl (from Aldrich) was used in the anomalous permeabilities experiments, and was soluble at 5 mM, but was not used at higher concentrations due to its low solubility. Solutions containing TlCl were made on the day of the experiment and stored in the dark, although the solution was exposed to the light for the short time when in the cell chamber.

Results

Blockade by Cs^+

The change in current required to clamp the plasmalemma to various potentials after a PD jump from the resting potential was measured in cells either in a 10K solution only (Fig. 1*A*) or with 0.3 mM CsCl added (Fig. 1*B*). In the 10K solution, the channels

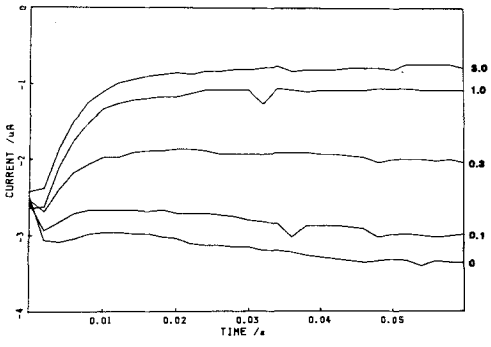

Fig. 2. $I - t$ relations as in Fig. 1, for the first 60 msec after a jump in voltage from the resting potential (−64 mV) to −120 mV. Cell in either a 10K solution only (0), or with 0.1, 0.3, 1.0 or 3.0 mM CsCl added. Cell surface area 1.5×10^{-5} m^2

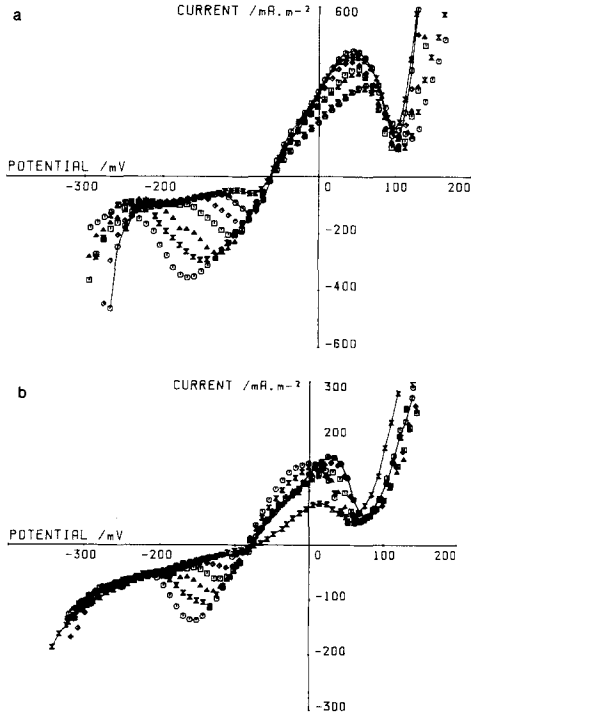

Fig. 3. Effect of Cs^+ on K^+ conductance across the plasmalemma of *Chara corallina*. (*a*) *I/V* relations measured with the cell in a 3K solution only (○) or with 0.01 (×), 0.03 (△), 0.1 (□), 0.3 (◇), 1.0 (⊖) or 3.0 (⊗) mM CsCl added. (*b*) Cell in a 10K solution only (○) and or with 0.01 (×) to 3.0 (⊗) mM CsCl added as above

were mostly open at the resting PD, and small jumps in potential led to a rapid initial settling in the current (Figs. 1*Aii* and 1*Aiii*). However, further changes in current occurred after several seconds (Fig. 1*Ai*); these changes were small with only small shifts in potential (to, for example, −30 or −90 mV), but with jumps to more negative potentials, the changes could be considerable (Fig. 1*Ai*); at −150 mV, the channels closed slowly, and completely, over several seconds ($t_{0.5}$ approx. 2.5 sec). With shifts to even more negative potentials, this rate of closure became very rapid, occurring over several milliseconds (*data not shown*). When the cell was in a 10K solution only, the time at which the maximal current occurred after the potential jump varied with the clamp potential, so analyses as done by Hagiwara et al. (1976) for the starfish egg membrane inward rectifier could not be done here. Similar long-term changes in membrane resistance have also been recorded in *Chara* by other workers (e.g. Bisson, 1984; Homble & Jenard, 1984).

For further *I/V* analysis, current was measured 60 msec after potential jumps because, a) the blockade of current by Cs^+ was fully complete by this time (Fig. 1*Biii*), and b) shorter clamp times allowed a wider voltage span across which current could be measured before the time- and voltage-dependent closure of the channels complicated calculations. (As it was, calculations were only done for potentials between the reversal potential and −150 mV.) Also, as the current mainly increased after 60 msec only for cells in control solutions, and not for those in the presence of Cs^+, an earlier measurement of current will lead to an underestimation of the relative degree of blockade by Cs^+, and thus of z', the "effective valence of the blocking reaction" (Hille & Schwarz, 1978); this is preferable in the present investigation, where a major question is whether or not the channels are multi-ion pores. As an ideal analysis cannot be done, because of the complex voltage and time interactions upon channel kinetics, it was thought better to err on the side of an underestimation of z', rather than risk calculations becoming limited by slow channel closures.

It is assumed that Cs^+ does not alter the gating characteristics of the channel—i.e. that the reduced current measured upon addition of Cs^+ is due to a blockade of the channel, and not due to an increased rate of channel closure. This possibility is best studied with patch-clamp techniques, although "instantaneous" *I/V* curves may also help. It should be noted that although single-channel studies would be able to distinguish between channel conductivity and channel on-off times, such work would be limited by the narrower voltage range than that possible in whole-cell studies. Although neither method is ideal, it is believed that the whole-cell method presented here is suitable for the problem addressed here.

Addition of Cs^+ clearly reduced inward K^+ fluxes in a concentration-dependent manner. An example of the effect of different concentrations of Cs^+ on tail currents is shown in Fig. 2. Only the first 60 msec after the potential jump are shown, and the current after 60 msec is that used to plot the *I/V* curves, which illustrate the blockade by different concentrations of Cs^+ over a wide range of potentials (Fig. 3). The reduction of the inward K^+ cur-

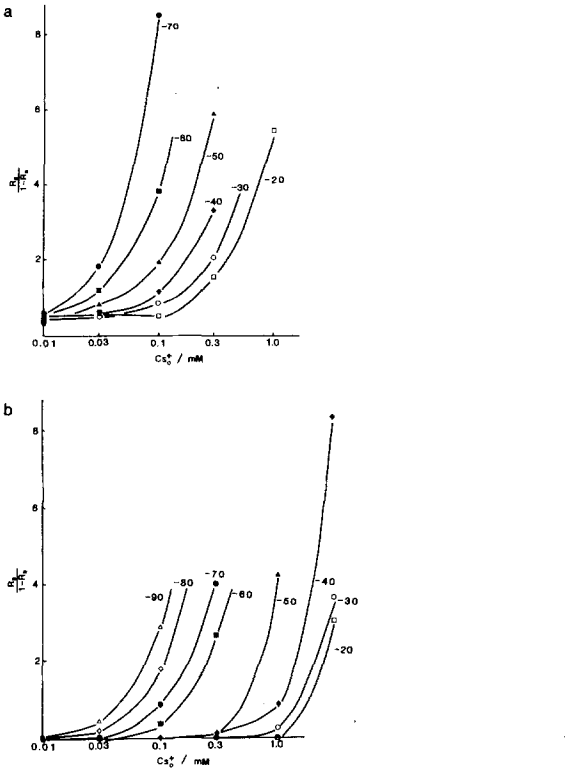

Fig. 4. Dose-response curves for suppression by Cs^+ of inward K^+ current at different ΔPD's (i.e. the difference between the clamped membrane potential and the reversal potential, indicated next to each curve in mV). Semi-log plots of the fraction of blocked to unblocked channels against external Cs^+ concentration. (*a*) With 3 mM K^+ externally. (*b*) With 10 mM K^+

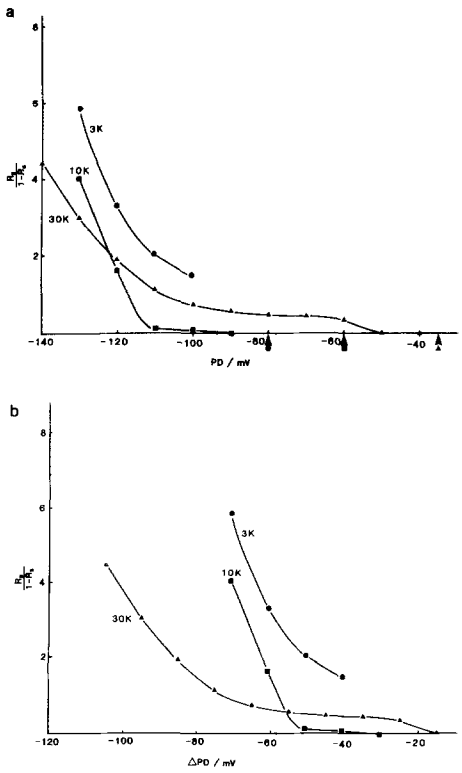

Fig. 5. Effect of external K^+ on blockade of K^+ channels by 0.3 mM external Cs^+. (*a*) Fraction of blocked to unblocked channels plotted against clamped membrane potential (PD) with 3, 10 and 30 mM K^+ externally. The arrows on the abscissa indicate the reversal potential (or E_K) across the cell membrane in the three K^+ concentrations. (*b*) As in *a*, but plotted against the *difference* between the clamped potential and E_K (ΔPD)

rent was completely reversible. High concentrations of CsCl also inhibited outward as well as inward currents when the cells were in 3K solutions (Fig. 3*a*), but this blockade did not appear to be voltage dependent. Conversely, with higher concentrations of external K^+, addition of Cs^+ *increased* the (net) outward K^+ current (Fig. 3*b*). This is in agreement with a report by Sokolik and Yurin (1986) of an increase by 20 to 30% of outward current upon addition of up to 5 mM Cs^+ in an external solution containing 5 mM K^+, but the reason for this is unknown. This increase in outward current was also observed upon addition of low concentrations of Ba^{2+} (*see* Fig. 4 in Tester, 1988*b*). Blockade of inward K^+ current was strongly voltage-dependent; this dependence is analyzed in more detail below.

Analysis of Cs^+ Blockade

Blockade of G_K by Cs^+ was strongly PD dependent, in contrast to the blockade by TEA^+, and the application of Cs^+ caused a marked rectification in the *I/V* profile between the two regions of negative-slope conductance (Fig. 3). As a result, dose-response curves of blockade by Cs^+ were drawn for several different voltages (Fig. 4). (For this and subsequent Figures, the measure of blockade by Cs^+ is given by the fraction of blocked to unblocked channels, $R_B/1 - R_B$, as used by Woodhull (1973) and Hille and Schwarz (1978) in their model of potential-dependent ion blockade.) The dose-response curves clearly show the strengthening of the blockade with hyperpolarization away from the reversal potential (Fig. 4). The family of curves also appears to be moved to the right with higher external K^+ concentration—i.e. as external K^+ concentration increases, more Cs^+ is required to block a channel.

The evidence for competition between K^+ and Cs^+ is clarified in Fig. 5, where the fraction of blocked to unblocked channels in 0.3 mM Cs^+ is plotted against clamped membrane potential (PD) for different external K^+ concentrations (Fig. 5*a*). In this Figure, it can be seen that an increase in K^+ never causes more than a small reduction in block by Cs^+, and for many potentials, blockade by Cs^+ is

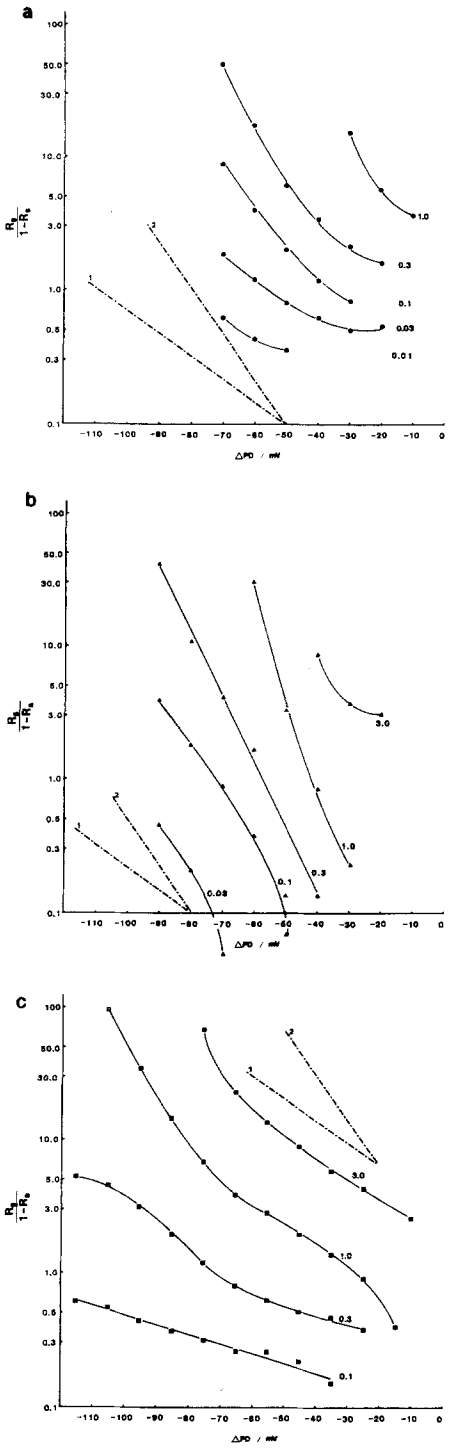

Fig. 6. Semi-log plot of the fraction of blocked to unblocked channels versus the difference between the clamped potential and E_K (ΔPD) with different external concentrations of Cs^+ and K^+; the slopes of the curves are proportional to the "effective valence of the blocking reaction," z'. (*a*) Cell in 3K solution, with 0.01 to 1.0 mM CsCl added. (*b*) Cell in 10K solution with 0.03 to 3.0 mM CsCl added. (*c*) Cell in 30K solution with 0.1 to 3.0 mM CsCl added. All values calculated from *I/V* curves measured on the same cell. The broken lines represent curves with arbitrary K_D's but of slopes equivalent to effective valences of 1 and 2

greater in the 30K than in the 10K solutions, an effect which is intuitively most unlikely. However, when the fraction of blocked to unblocked channels is plotted against the difference between the clamped potential and E_K (ΔPD), a clear competitive effect of K^+ on Cs^+ blockade is apparent, as evidenced by a reduction in the fraction of blocked to unblocked channels at a given PD with increasing external K^+ concentration (Fig. 5*b*), especially at more negative potentials, where the blockade by Cs^+ is stronger. This is a similar effect to that described by Hagiwara et al. (1976) for the inward rectifier.

Evidence for a multi-ion channel pore can be obtained from the fitting to a Boltzmann distribution of the fraction of blocked to unblocked channels at different membrane potentials (Hille & Schwarz, 1978). If the apparent valence of the blocking reaction is greater than unity (i.e. when the voltage dependence of the blockade becomes anomalously strong), ions must be interacting within the channel pore (Woodhull, 1973; Hille & Schwarz, 1978). In Fig. 6, semi-log plots of the fraction of blocked to unblocked channels against potential are plotted for different external concentrations of Cs^+ and K^+. The slopes of the curves are proportional to the "effective valence of the blocking reaction," z'. The broken lines in Fig. 6 are of slopes corresponding to values for z' of 1 and 2.

Curve fitting to obtain z' from Woodhull's (1973) model was not done, as it was not considered certain that the blocking reaction satisfactorily fitted the potential-dependent ion blockade model; sets of data, especially in the 3K and 10K solutions (Figs. 6*a* and 6*b*) showed consistent curvilinear trends, which may or may not be significant. For the moment, the points are joined by arbitrary curves fitted by eye, and on each graph lines are drawn of slopes corresponding to effective valences of 1 and 2; the steeper the curve, the greater is the value for z'. The reasons for a departure from the model of blockade are not known.

Several trends can nevertheless be clearly seen from the Figures:

i) The valence of blockade increases with increased Cs^+ concentration, as has been reported for delayed rectifiers (Adelman & French, 1978), high G K^+ (Ca^{2+}) channels (Cecchi et al., 1987) and for the K^+ channels in the *Nitella* plasmalemma (Sokolik & Yurin, 1986).

ii) As the K^+ concentration increases, so z' becomes smaller, which is likely to be a reflection of the competition between Cs^+ and K^+ described above.

iii) As the Cs^+/K^+ ratio increases, the shape of the relations between fraction of blocked channels and potential become more concave upwards—i.e.

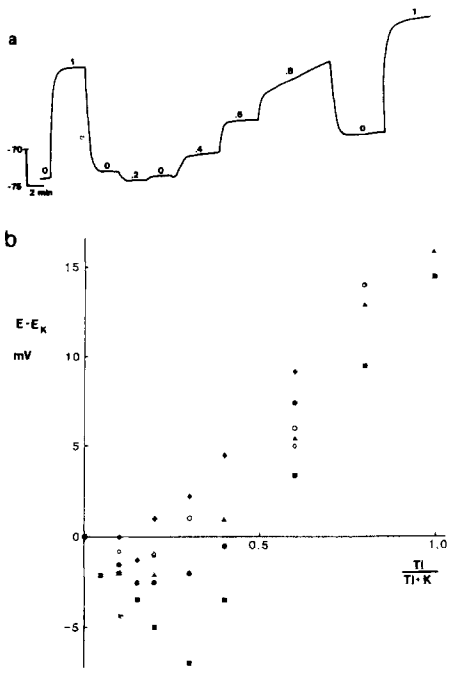

Fig. 7. Anomalous mole fraction effects on resting PD. (*a*) Potential changes across the plasmalemma of *Chara corallina* upon changes in $Tl^+/(Tl^+ + K^+)$, where the sum of $Tl^+ + K^+$ ions was kept constant at 5 mM. Vertical bar refers to potentials in mV; horizontal bar represents two minutes, with time moving from left to right. The numbers along the trace refer to different $Tl^+/(Tl^+ + K^+)$ ratios. (*b*) Summary of changes in resting PD from the PD in a 5K or a 10K solution upon changes in the external $Tl^+/(Tl^+ + K^+)$ ratio ($E - E_K$), from data such as shown in *a*. The total external concentration of $Tl^+ + K^+$ was either 5 or 10 mM (filled *vs.* open symbols); different symbols represent data from different cells

with more Cs^+ present relative to K^+, the blockade by Cs^+ becomes more strongly potential dependent.

iv) The blockade is such that z' is often greater than 1, and thus ions are interacting within the channel pore; this can only occur if more than one ion can occupy the channel at a time, and therefore this type of anomalously strong voltage dependence of blockade is evidence for the channel being a multi-ion pore.

It should be noted that there are many other models of ion movement through channels, such as the one developed by Hansen, Gradmann and their colleagues, which incorporates lazy (or inactive) states of the membrane protein. This model has been applied to various plant systems (Fisahn, Hansen & Gradmann, 1986; Bertl & Gradmann, 1987; Gradmann, Klieber & Hansen, 1987; Hansen & Fisahn, 1987). Although it would be interesting to know whether these models can explain the observed effects, analysis using this model is complex and is beyond the scope of the present study. Nevertheless, the similarity of some of the reaction schemes in Woodhull's and the lazy state models suggests that similar conclusions may be drawn from the two models, and any differences may be more in the physical interpretations than the mathematical abstractions. More realistic models incorporating stochastic (or Brownian) molecular dynamics (e.g. Läuger & Apell, 1982; Cooper, Jakobsson & Wolynes, 1985) have also not been considered, but it is thought that conclusions from such models would not be qualitatively different, although they clearly would be quantitatively very different!

Anomalous Permeabilities

As the ratio of Tl^+/K^+ was increased (keeping the sum of the two ions equal), the resting potential initially moved by a couple of mV's more negative, and then, upon substitution by larger amounts of Tl^+, the resting potential moved to more positive potentials (Fig. 7*a*). This effect, although small, was repeatable, and the results from several cells are summarized in Fig. 7*b*. More spectacular was the change in conductance through the K^+ channels; upon substitution of small amounts of Tl^+, a large decrease in K^+ channel G was observed (Fig. 8). The conductance slowly increased as the Tl^+/K^+ ratio increased further, until G of a cell in a solution containing exclusively Tl^+ was almost as great as of a cell in a solution containing only K^+. That both resting potential changes and plasmalemma conductance went through minimums is consistent with a channel whose pore can be multiply occupied. As far as is known, this is the first time such a phenomenon has been demonstrated for a plant channel.

Discussion

Two different types of evidence are presented here which indicate that K^+ does not appear to move across the membrane in a way consistent with the independent movement of ions through single-ion channels. The channels are blocked by Cs^+ in an anomalously voltage-dependent manner, and exhibit clear anomalous permeabilities in Tl^+-K^+ mixtures. Both of these effects are consistent with the channel being occupied by more than one ion at a time.

The strongly voltage-dependent blockade of open K^+ channels by Cs^+ was the main focus of this study. The effective valence of the blocking reaction z' was greater than unity, and it increased with the external Cs^+ concentration. The only known

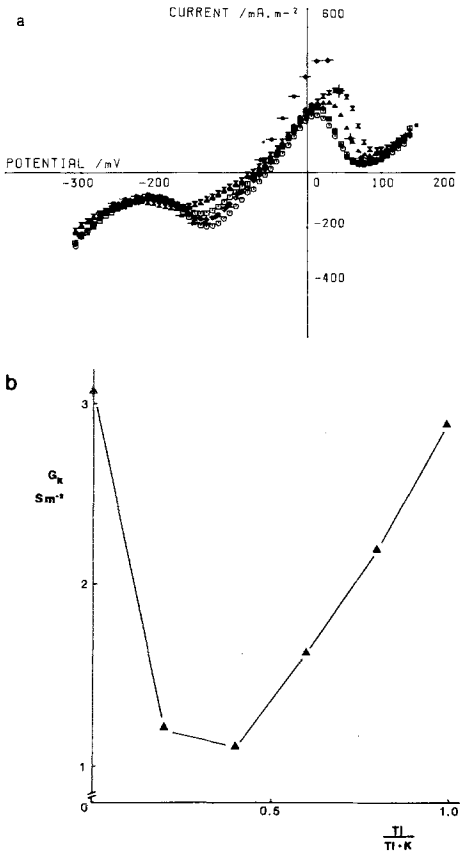

Fig. 8. Anomalous mole fraction effects on plasmalemma conductance. (*a*) *I/V* relations measured with the cell in either a 5K solution only (⊕), or with 1 to 5 mM TlCl replacing an equimolar amount of KCl—i.e. with $Tl^+/(Tl^+ + K^+)$ ratios of 0 (⊕), 0.2 (X), 0.4 (△), 0.6 (□), 0.8 (◇), or 1.0 (○). All curves were run at a baseline PD of −80 mV, held for 10 sec before starting the bipolar staircase of voltage-clamp commands. Control curve (⊕), the average of four curves run during the experiment, with values averaged over 15 mV potential spans (horizontal bars), with standard errors of the mean (vertical bars). (*b*) Conductance for inward current measured over approximately 30 mV more negative of the reversal potential, for different values of $Tl^+(Tl^+ + K^+)$, calculated from the *I/V* relations in *a*. A leak of 0.49 Sm^{-2} was subtracted for all curves

explanation for these results is that the K^+ channel must be able to be occupied by more than one ion at a time—i.e. that it is a multi-ion pore (cf. Hille & Schwarz, 1978). Sokolik and Yurin (1986) also found a strong voltage dependence of Cs^+ blockade of K^+ channels in the plasmalemma of the charophyte *Nitella flexilis*. The blockade was reversible, and did not decrease outward K^+ current. Sokolik and Yurin calculated an "electrical distance," z', of 0.9 to 2.4, increasing with a higher Cs^+ concentration. Sokolik and Yurin's calculations, however, were based on little data; often they were fitting an exponential through only three data points; and it was not clear if they were subtracting leak currents, nor whether the membrane currents and blockade had reached a pseudo-steady state. The experiments were repeated and extended here to take into account these criticisms; nevertheless, the results obtained in this study are in agreement with the basic results of Sokolik and Yurin.

It is also possible that the channel cannot become multiply occupied by K^+, but can be occupied by a K^+ ion and a Cs^+ ion at the same time. Such effects have been described for gramicidin A channels, where only one Na^+ ion can occupy the channel, whereas two Cs^+ ions can be found in channels at relatively low concentrations of CsCl (Finkelstein & Andersen, 1981).

Flux coupling measurements in cells of *Chara* (Smith, 1987; Smith, Smith & Walker, 1987) failed to show multiple occupancy of K^+ channels, but this does not contradict the results presented here. Multi-ion channels can give flux ratios equal to unity under a variety of conditions; for example, measurements of flux ratios through gramicidin channels can vary from 0.5 to 2 (Finkelstein & Andersen, 1981; Schagina, Grinfeldt & Lev, 1983), depending upon ionic conditions on each side of the pore. That Smith measured flux ratios systematically less than unity demonstrates the difficulty of such measurements. It is possible that the K^+ ions are moving through multi-ion pores which are infrequently occupied by more than one K^+ ion at a time, so the K^+ can move independently. This may occur if the concentrations of K^+ are relatively low on each side of the membrane, as was the case in Smith's work (he used about 10 mM externally and the cells probably had 100 mM K^+ internally). For external K^+ concentrations up to 30 mM, the plasmalemma K^+ conductance in *Chara* has been shown to increase in accordance with Goldman's constant field model (Beilby & Blatt, 1986), which is consistent with single occupancy of the channels by K^+ at the relatively low external K^+ concentrations used in these experiments. This suggests that the multiple occupancy characteristics described in this paper could be due to the higher affinity of binding to the channels by ions other than K^+, but that with higher concentrations of K^+, such as used in single-channel studies, multiple occupancy of the channel by K^+ could well occur.

In animal tissues, delayed rectifier (Adelman & French, 1978; Hille & Schwarz, 1978), inward rectifier (Hagiwara et al., 1976) and high G K^+ (Ca^{2+}) channels (Eisenman et al., 1986; Latorre, 1986; Cecchi et al., 1987) all appear to be multi-ion, single-file channels. In this paper is presented the best evidence to date for the existence of multi-ion K^+ channels in plants, and it is noteworthy to find similar properties and channel structure in the plant

channels studied here and different types of K^+ channels widespread in animal cells.

I thank Prof. E.A.C. MacRobbie and Dr. M.R. Blatt for helpful discussions; and M.J. and B.N. Beilby, for having designed and built such an excellent system with which to "stab" *Chara;* I am also indebted to Prof. C. Miller for his generously shared knowledge. Assistance from the Royal Commissioners for the Exhibition of 1851 is gratefully acknowledged.

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