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A Tight-Seal Whole Cell Study of the Voltage-Dependent Gating Mechanism of K^+-Channels of Protoplasmic Droplets of *Chara corallina*[1]

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ABSTRACT

The biophysical properties of voltage-dependent K^+-channels of protoplasmic droplets of *Chara corallina* Klein ex Willd., em, R.D.W. were investigated using the tight-seal whole cell method. Two potassium currents were observed in voltage-clamp mode and they can be used to explain the transient membrane potential time course observed in current-clamp mode. The K^+-channels are identified by the effect of tetraethylammonium chloride which blocks both currents. A two-state, constant dipole moment model is used to fit the voltage-conductance curve. From this model the minimum equivalent gating charge involved in the gating mechanism of K^+-channels of *Chara* can be estimated.

For practical reasons most of the electrophysiological work on the biophysical properties of passive K^+ transport has been carried out on large aquatic plant cells. Time- and voltage-dependent K^+-channels have been studied by means of voltage- and current-clamp techniques on *Chara* (2–4, 11, 12), *Hydrodictyon africanum* (7), and *Nitella* (29). Many authors have published data showing that K^+-channels are involved in the excitation process of plant cells (*e.g.* 8, 19, 26, 27), and that potassium ions are the main species transported in the leakage current (2). K^+-channels also play a role in the light-induced membrane potential change in perfused *Chara corallina* cells (30) and in *Eremosphaera viridis* (17). Ion transpot through the plant cell membrane has also been studied by noise analysis of the membrane potential (6, 22–24). The minus two slope of the voltage noise power spectrum has been attributed to the activity of K^+-channels (6). More recently the patch-clamp technique has been used to study the properties of a single K^+-channel isolated from the plasmalemma of guard cells of *Vicia faba* (25) and of the membrane surrounding protoplasmic droplets of *C. corallina* cells (13).

In the present work the time- and voltage-dependence of K^+-channels were investigated on protoplasmic droplets by means of the tight-seal whole cell technique (9) which allows the intracellular ionic composition to be controlled. Two K^+-channel activities with different time- and voltage-dependent characteristics were identified.

MATERIALS AND METHODS

Protoplasmic Droplet. A protoplasmic droplet was obtained by gently cutting the lower end of a turgorless internodal cell of *Chara corallina* Kein ex Willd., em, R.D.W. maintained vertically and allowing the endoplasm to flow down from the open end into an artificial vacuolar solution (80 mM KCl, 50 mM NaCl, and 5 mM $CaCl_2$). In this solution protoplasmic droplets form spontaneously. We used protoplasmic droplets of 40 to 80 μm diameter containing no chloroplasts. Cell-attached patches were usually obtained about 30 min after collecting the endoplasm. When TEA[3] was used, sufficient TEA was added to the artificial vacuolar solution to make the concentration of TEA 20 mM and the concentration of NaCl was reduced to 30 mM to maintain the osmolarity constant.

Tight-Seal Whole Cell Mode. A high-resistance seal (about 1 Gohm) was established between the membrane of the droplet and a pipet isolating a patch of membrane. The transient capacitance caused by the patch when a voltage pulse is applied across the membrane was cancelled by means of a capacitance compensation circuit. A gentle suction was then applied to the pipet to break the membrane inside the pipet, establishing a tight-seal whole cell mode.

The pipet was pulled from borosilicate glass according to the method of Hamill *et al.* (9). It was filled with a low Ca^{2+} solution (80 mM KCl, 5 mM $CaCl_2$, and 10 mM EGTA-NaOH buffered with 10 mM Mes-NaOH to pH 6.0). EGTA was used to buffer the free Ca^{2+} concentration at $2 \cdot 10^{-5}$ M. The lack of ATP and the low pH ensured that only passive transport occurred through the membrane. If any change in the drop shape occurred after the tight-seal whole cell mode was established, then the drop was discarded.

Electrical Measurements. The experimental setup used in the present work was the same as that described in an earlier paper for single-channel measurements (13). The feedback resistor of the current to voltage converter was chosen to be 0.1 Gohm. Both voltage- and current-clamp modes were used in the present study. In each case a series resistance compensation was used to cancel the effect of the current flowing through the pipet which constitutes a resistance in series with the membrane of the droplet. The electrical signals were filtered through a low pass filter at 1 kHz.

Statistics. The data are presented in the form of the mean ± standard error (number of experiments). The probability that two data are or are not significantly different was calculated using the *t*-test method. The number of experiments refers to different

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[3] Abbreviations: TEA, tetraethylammonium chloride; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid. A m^{-2}, ampere per square meter; S m^{-2}, Siemen per square meter.

protoplasmic droplets each of them being formed from different internodal cells.

RESULTS

Resting State. The electrical characteristics of protoplasmic droplet are summarized in Table I. After the tight-seal whole cells mode had been established, the resting membrane potential of the droplet became more positive. The time needed to reach the steady state was about 30 min. This time corresponds to the time needed to perfuse the cytoplasmic phase with the pipet solution. The steady state membrane potential was +3.73 ± 1.95 mV (15). The membrane conductance calculated by measuring the current flowing through the membrane when it was clamped with a pulse to −5 mV was 14.36 ± 1.41 S m^{-2} (13).

In the presence of TEA the steady state resting membrane potential was not significantly different from that measured in its absence. However in the presence of TEA the resting membrane conductance was significantly decreased (Table I).

Voltage-Clamp Investigations. The time course of the inward current flowing through the membrane of a protoplasmic droplet voltage-clamped at different hyperpolarizing potentials is shown in Figure 1A. Near the resting state the membrane seemed to behave in a purely passive manner, *i.e.* the current flowing through the membrane was proportional to the voltage difference applied across it and was independent of time. At around −50 mV, the current flowing through the membrane decreased with time indicating that the membrane conductance decreased. At about −80 mV, after the first decrease in current was completed, an increase of current was observed, indicating that the membrane conductance increased. For practical purposes the first transient current will be called the early inward current and the second one will be called the delayed inward current. Over the range of potential investigated the delayed inward current displayed large fluctuations and always appeared after the time course of the early inward current had been completed. The time constant of the time course of both currents decreased with the applied voltage difference.

When TEA was present in the external solution both early and delayed inward currents were blocked and the membrane current was not time dependent (Fig. 1B). This result suggests that both early and delayed inward currents are carried by potassium ions since TEA is known to block K^+-channels of *Chara* (4, 12, 13, 30). In the presence of TEA and at membrane potentials for which the delayed inward current usually occurred, the current showed no large fluctuations, suggesting that the fluctuations observed in the absence of TEA were related to potassium transport.

The effect of varying the duration of voltage pulses to times large enough to increase the probability of closure of the channels is shown in Figure 2. In these experiments each voltage-clamp pulse was separated by a period of 30 s during which the membrane was clamped at the resting membrane potential.

Table I. *Electrical Characteristics of Protoplasmic Droplets of Chara in Resting State*

Superscript *i* and *f* refer to data obtained just after the tight-seal whole cell mode has been established and to the final steady state values, respectively. Subscript TEA refers to data recorded when 20 mM TEA is present in the external solution. *V* is the membrane potential and *G* the membrane conductance.

Parameter	Value	Unit
V^i	+7.95 ± 1.72 (15)	mV
V^f	+3.73 ± 1.95 (15)	mV
G^f	14.36 ± 1.41 (13)	Sm^{-2}
V^f_{TEA}	+2.54 ± 1.69 (8)	mV
G^f_{TEA}	7.46 ± 1.22 (8)	Sm^{-2}

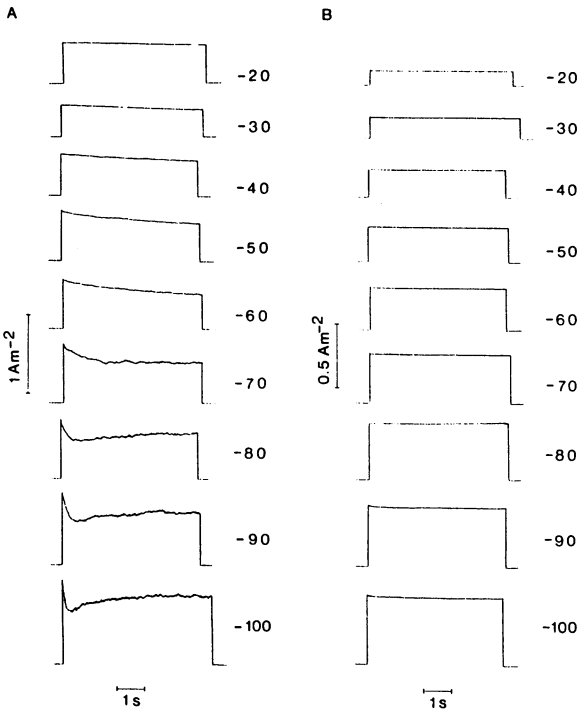

FIG. 1. Time course of the current flowing through the membrane of protoplasmic droplets of *Chara* clamped at different values of membrane potential (mV) as indicated on the right of each curve, (A) in the absence and (B) in the presence of 20 mM TEA.

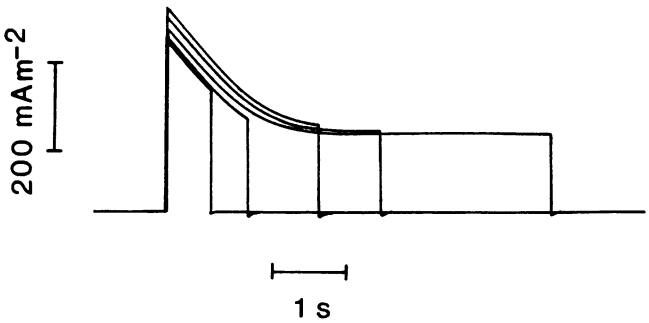

FIG. 2. Effect of the duration of the voltage-clamp pulse (−70 mV) on the inward K^+-current flowing through the membrane of protoplasmic droplets of *Chara*. At the end of each clamp the current quickly recovers its initial resting value after displaying a small transient overshoot.

Whatever the duration of the voltage-clamp pulse the current recovers to its initial value nearly instantaneously when the membrane is clamped back to the resting state.

Current-Clamp Investigations. Figure 3 shows the response of the membrane potential to inward current pulses of different magnitudes. At low current density a purely passive response was observed. At 0.25 A m^{-2} and above, a spontaneous increase in voltage occurred which was followed by a slight voltage decrease in which large fluctuations could be observed. These results are similar to those observed on intact cells of *C. corallina* (10), *Nitella* (16), and on protoplasmic droplets formed from internodal cells other than those of *C. corallina* (15, 20).

Voltage-Dependent Conductance. The voltage-dependences of the conductance associated with the early inward current and with the delayed inward current are shown in Figure 4. Over the range of voltage investigated in this study, the early inward current displayed a sigmoidal voltage-dependence conductance. The delayed inward current also displayed a nonlinear voltage-

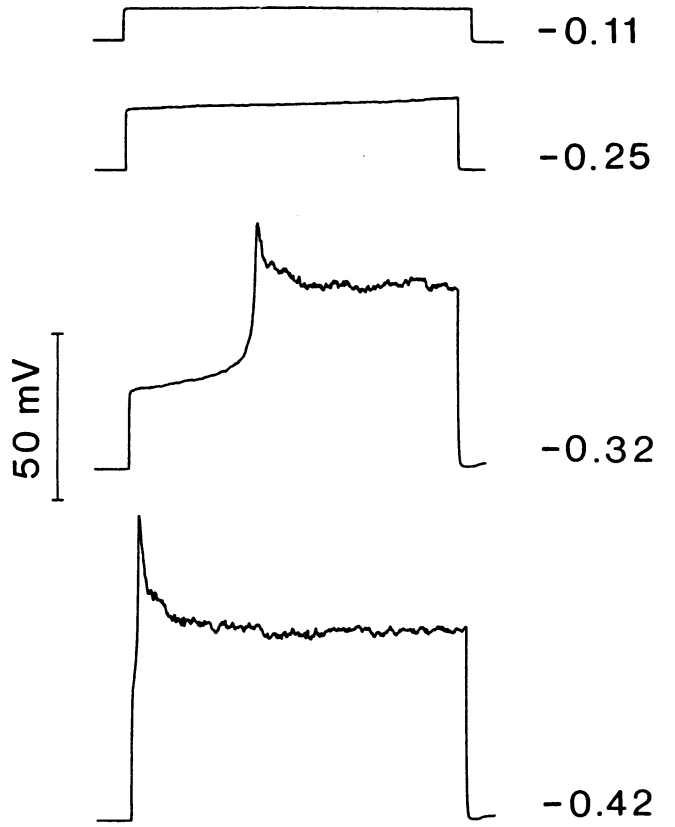

FIG. 3. Time-dependence response of the membrane potential of protoplasmic droplets of *Chara* clamped at different values of the current density. The values to the right of each curve indicates the magnitude of the current density in A m^{-2}.

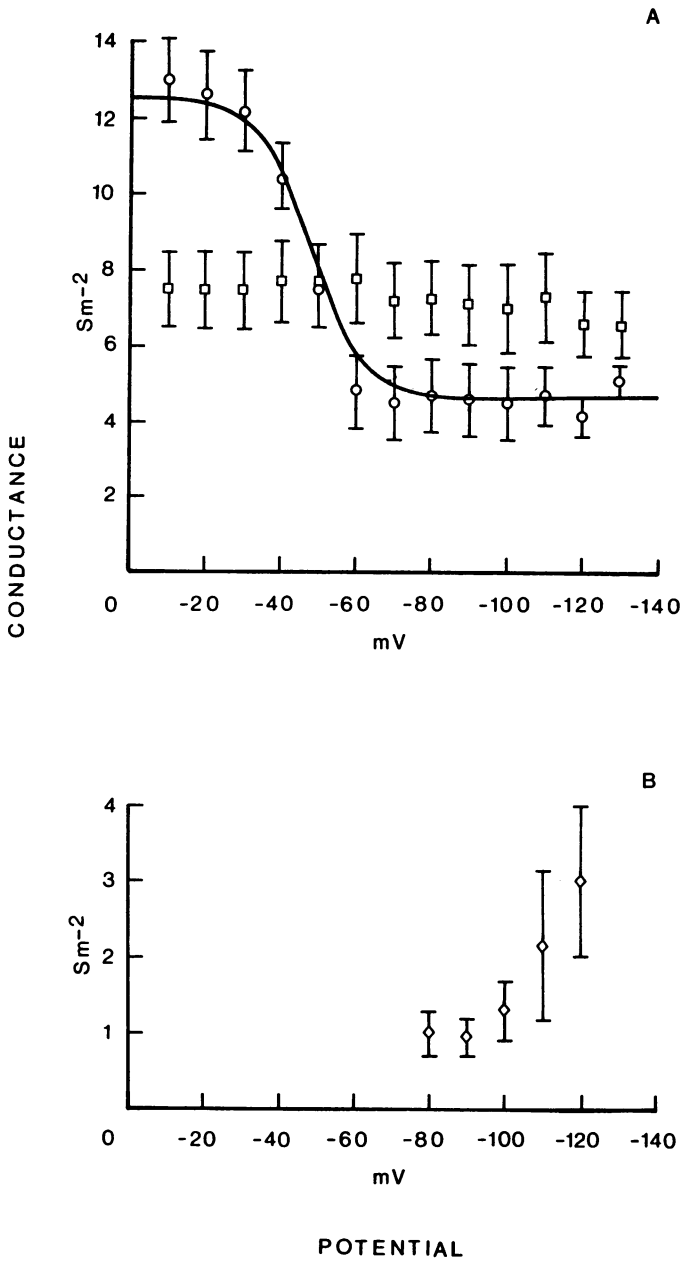

FIG. 4. Voltage-dependent conductance (A) of the early inward K^+-current in the absence (O) or presence of TEA (□) and (B) of the delayed inward K^+-current in the absence of TEA. The continuous line drawn in (A) has been calculated assuming a two state, constant dipole model (see text).

dependent conductance. However, in this case no saturation of the voltage-conductance curve was obtained because the limit to which the membrane could be clamped was −130 mV. Above this value the tight-seal usually broke.

The minimum membrane conductance of the early inward current is significantly lower than the membrane conductance measured when K^+-channels are blocked by TEA which suggests that TEA does not fully block the K^+-channels.

A K^+-Channel Model. It has been shown above that there are time- and voltage-dependent K^+-channels in the membrane of protoplasmic droplets of *Chara*. Thus each channel must have a voltage-sensitive sensor which controls the opening and closing of the channel; the process by which a channel opens and closes is called the gating process. From the results obtained on animal cells it is generally accepted that the voltage sensor consists of charges or dipoles that move under the influence of the electrical field applied across the membrane (*e.g.* 1).

In order to derive a mathematical expression which describes quantitatively the voltage-dependent conductance of K^+-channels associated with the early inward current it will be assumed that each K^+-channel is independent and can only exist in two states: open and closed. The equilibrium distribution of K^+-channels between these two states is given by:

$$\text{Open} \leftrightharpoons \text{closed}. \tag{1}$$

If each channel behaves like a dipole which extends over the width of the membrane then the energy of each dipole in the state i (W_i) is given by:

$$W_i = -qdE \cos \phi_i, \tag{2}$$

where E is the electrical field across the membrane, d is membrane thickness, q the charge on the dipole, and ϕ_i the angle between the direction of the dipole i and that of the electrical field. Thus the change in dipole energy (ΔW_i) when a channel goes from the closed to the open state is given by:

$$\Delta W_i = -qdE(\cos \phi_{\text{open}} - \cos \phi_{\text{closed}}). \tag{3}$$

The total change in energy (ΔW) which occurs during a channel change of state is the change in dipole energy (ΔW_i) plus any change of conformational energy of the channel (ΔW_0), which is independent of the electrical field:

$$\Delta W = \Delta W_i + \Delta W_0 \tag{4}$$

If each channel is independent, *i.e.* if no cooperative effects occur, then the distribution of channels between the open and

the closed state is given by the Boltzmann equation:

$$\frac{N_{open}}{N_{closed}} = \exp - (\Delta W/kT), \quad (5)$$

where N_{open} and N_{closed} are the numbers of open and closed channels respectively, k is the Boltzmann constant and T is the absolute temperature. Combining Equations 3, 4, and 5 gives:

$$\frac{N_{open}}{N_{closed}} = \exp - (qdE(\cos \phi_{closed} - \cos \phi_{open}) + \Delta W_0)/kT. \quad (6)$$

Writing $E = V_m/d$, where V_m is the membrane potential, Equation 6 leads to an expression for the ratio of the fraction of open and closed K^+-channels (θ_{open} and θ_{closed}, respectively):

$$\frac{\theta_{open}}{\theta_{closed}} = \exp - (\alpha V_m + (\Delta W_0/kT)) \quad (7)$$

where α is written for $ze(\cos \phi_{closed} - \cos \phi_{open})/kT$ (e is the electrical charge, z is the number of electronic charges and $ze = q$).

When $\theta_{open} = \theta_{closed}$,

$$\alpha V_m + \Delta W_0/kT = \alpha V_h + \Delta W_0/kT = 0, \quad (8)$$

thus

$$\Delta W_0/kT = -\alpha V_h \quad (9)$$

where V_h is the potential at which half of the channels are open. Combining Equations 7 and 9 gives:

$$\frac{\theta_{open}}{\theta_{closed}} = \exp - (\alpha(V_m - V_h)). \quad (10)$$

Assuming that the total number of K^+-channels is constant ($\theta_{open} + \theta_{closed} = 1$), Equation 7 can be rewritten as:

$$\theta_{open} = \frac{\exp - \alpha (V_m - V_h)}{1 + \exp - \alpha (V_m - V_h)}. \quad (11)$$

The voltage-dependent conductance of the K^+-channels ($g_k(V)$) is related to the maximum conductance of the K^+-channels (G_k) by:

$$g_k(V) = G_k \theta_{open}. \quad (12)$$

The results of Figure 4A show that the membrane conductance (g_m) is the sum of the voltage-dependent K^+-conductance ($g_k(V)$) and of a voltage-independent leak conductance (g_l) which accounts for the membrane conductance when the channels are closed, thus:

$$g_m = g_k(V) + g_l. \quad (13)$$

Combining Equations 11 to 13 gives an analytical expression for the membrane conductance:

$$g_m = g_l + G_k \frac{\exp - \alpha(V_m - V_h)}{1 + \exp - \alpha(V_m - V_h)}. \quad (14)$$

As g_l, G_k and V_h can be determined experimentally, only α has to be adjusted to fit the experimental data. The continuous line drawn in Figure 4A has been calculated with $\alpha = 250$ volt^{-1}.

By recognizing that the maximum value of $\cos \phi_{open} - \cos \phi_{closed} = 1$ and that the maximum value of the dipole length is the membrane thickness, we can now estimate the minimum equivalent gating charge (z) involved in the gating process of the K^+-channels to be $kT\alpha/e = 6$ electronic charges which is similar to the equivalent gating charges estimated for the Na^+-channels in animal cells (1).

DISCUSSION

Is There a Membrane? The presence of a 'true' biological membrane around the protoplasmic droplet has been questioned (18). But it has been shown that protease and lipase alter the biophysical properties of the protoplasmic droplets of *Nitella* (14) and that ionic channels are present in the membrane of protoplasmic droplets of *C. corallina* (13). The present results show that the potassium channels of the protoplasmic droplets of *Chara* have the same macroscopic electrical properties as those of the plasmalemma of intact cells. This does not mean that the membrane surrounding the protoplasmic droplet is a plasmalemma. It could be a tonoplast or another membrane containing the same kind of K^+-channels as those present in the plasmalemma. The fact that K^+-channels of protoplasmic droplets and of the plasmalemma have similar properties suggest that the protoplasmic droplets are surrounded by a 'true' biological membrane.

Equilibrium Potential of K^+. Potassium ions are in electrochemical equilibrium across the membrane of protoplasmic droplets (21). Therefore if the current flowing through the membrane during a voltage pulse does not modify significantly the ion activity on both sides of the membrane then one must expect the current to return instantaneously to its original level after the voltage pulse because no net K^+-current is expected to flow in the resting state. This is what is observed in Figure 2 thus supporting the idea that potassium ions are in electrochemical equilibrium across the membrane.

Identification of the Two K^+-Currents. Voltage clamp experiments on intact and perfused cells have shown that a voltage-dependent K^+-conductance is present in the plasmalemma of *Chara* (2–4, 28). The results of Figure 1 show that two voltage-dependent conductances occur in the membrane of protoplasmic droplets when voltage-clamped at negative potentials. The fact that they are both blocked by TEA supports the idea that they are K^+-channels.

Three simple alternative models can be put forward to explain the presence of two K^+ voltage-dependent conductances.

The two different voltage-dependent conductances could arise from one kind of K^+-channel with at least three different configurations: at low membrane potentials (near zero) the channels are open, at about −60 mV the channels are closed and at potentials more negative than −80 mV the channels partially reopen. Under the voltage clamp mode the transition between the first and the second state will yield the early inward current and the delayed inward current will flow when the transition between the second and third state occurs.

Alternatively, the increase in delayed potassium current at high negative membrane potential could arise from an increase in the number of open voltage-independent K^+-channels. In this case the early inward current could be described by a two state (open-close) voltage-dependent channel. The leak conductance in *C. corallina* is mainly a voltage-independent K^+-conductance (2). But an increase in the leak current at high negative membrane potential could thus explain the delayed inward current.

Finally, there is always the possibility that two different voltage-dependent K^+-channels are present in the membrane when it is hyperpolarized.

Our results do not permit a distinction to be made between these three simple models. However, the large fluctuations in the delayed inward current suggest that this current is probably not simply associated with an increase in the opening probability of a K^+-channel because these macroscopic fluctuations are larger than the fluctuations due to the stochastic transport of ions through channels (5).

Relation between Voltage- and Current-Clamp Data. The time course of the voltage response to a current-clamp pulse agrees with the current transients recorded in voltage-clamp mode. The

spontaneous rise of the membrane potential is related to the decrease of the early inward current. After the peak of potential the slight decrease arises from the increasing delayed inward current.

It has been suggested by Coleman and Findlay (4) that the delayed inward current of *Chara inflata* could possibly be caused by the opening of Cl^--channels. The results reported here obtained on protoplasmic droplets of *C. corallina* do not support such a suggestion because this current is inhibited by TEA (Fig. 1). In the constant current experiments of Coleman and Findlay (4) it is clear that TEA reduced the late decrease in membrane potential (Figure 7 of Ref. 4), and since TEA also shifted the membrane potential toward more negative values, it would be interesting to repeat these experiments in the voltage-clamp mode. However, in *C. inflata* no large fluctuations were observed in the delayed inward current. Thus, it is possible that this apparently similar voltage response involves different kinds of channels in intact cells and in protoplasmic droplets.

Partial Inhibition by TEA. If we assume that in its closed state the K^+-channel is totally impermeable to potassium ions then the difference between the membrane conductance in the presence of TEA and that measured when K^+-channels are closed must be equal to the residual conductance of the partially inhibited K^+-channels. Using the data of Figure 4 it can be calculated that 20 mM blocks 65% of the conductance of the K^+-channel associated with the inward current. The fact that in the presence of TEA the membrane conductance is slightly larger than when K^+-channels are in their closed state (Figure 4A) suggests that TEA could also affect the gating properties of the channels.

It has been reported that 20 mM TEA blocks 90% of the single-channel conductance in *Chara* protoplasmic drops (13). However no data were obtained for the effect of TEA on the mean open and closing time of the channel. Both values are needed for a comparison of the present results to those obtained with the single-channel study (see Appendix in Ref. 13).

Minimum Equivalent Gating Charge. In order to account for the voltage-dependent conductance of the early inward current it has been assumed that K^+-channels can be described by a two-state, constant-dipole model and that each channel is independent of the others (no cooperative effects exist). A good fit of the experimental data was obtained with this simple model (Figure 4A).

The amount of gating charge calculated in this study reflects a minimum equivalent gating charge if each dipole extends over the thickness of the membrane and if the dipole moment is parallel to the electrical field in the open state and perpendicular to the electrical field in the closed state. If the dipole does not fully extend over the membrane thickness (d) but only over a distance δ, d in Equation 6 will be lower by a factor of δ/d and thus the value of gating charge will be greater. A higher value of equivalent gating charge will also be found if in either state the dipoles make an angle between 0 and $\pi/2$ radian because then $(\cos \theta_{closed} - \cos_{open})$ of Equation 6 will be lower than 1.

It is important to point out that these two assumptions are necessary only to calculate the minimum equivalent gating charge. As Equations 7 to 14 are written in parametric form, the value of α and the fitting of the curve of Figure 4A are independent of these hypotheses.

CONCLUSION

The present work shows that there are two voltage-dependent K^+-conductances in the membrane of protoplasmic droplets of *C. corallina*. The channels were tentatively identified by the effect of TEA on the transient membrane currents recorded in the voltage-clamp mode. In the resting state the time- and voltage-dependent K^+-channels are open and account for at least 50% of the resting membrane conductance of the droplets (Table I).

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