Plant Cell Physiol. 35(2): 243–255 (1994)

Temperature Sensitivity of the K^+ Channel of *Chara*. A Thermodynamic Analysis

Laura Patricia Zanello and Francisco José Barrantes

Instituto de Investigaciones Bioquímicas, Consejo de Investigaciones Científicas y Técnicas and Universidad Nacional del Sur, C.C. 857, 8000 Bahía Blanca, Argentina

Temperature sensitivity of the K^+ channel of *Chara* cytoplasmic droplets has been characterized by means of the patch-clamp technique. The activity of the channel was recorded in inside-out patches over a range of temperatures (3°C to 25°C). An increment in the unitary channel conductance and a decrease in the probability of channel opening was found as temperature augmented. This could be explained by the combined effect of a reduction in the mean open duration and an increase in the closed times (Q_{10} values of 0.7 and 1.1–1.6 respectively). Eyring's transition state theory was applied to the thermodynamic analysis of conductance and kinetics of the K^+ channel. The values obtained for the activation enthalpy and entropy were compared with, and found to be similar to, those reported for voltage-dependent K^+ channels in animal cells. The relative insensitivity of channel conductance to temperature (activation enthalpy of 2.4 kcal mol^{-1}) suggests that ions traverse the pore by diffusion. Channel closure appears to have the highest energetic requirements (activation enthalpy of 6.4 kcal mol^{-1}). The channel closing rate, α, exhibits a less negative entropic change (-22.54 cal K^{-1} mol^{-1}), which would provide the driving force for stabilizing the closed configuration of the channel as the temperature increases.

Key words: Activation energy — *Chara contraria* — Cytoplasmic droplet — Fresh-water alga — Patch-clamp — Potassium channel.

Temperature influences the activity of ion channels by affecting their gating and conductance processes (Correa et al. 1991, 1992, Hodgkin et al. 1952, McLarnon and Wang 1991, Pahapill and Schlichter 1990, Urry et al. 1984). By means of the patch-clamp technique (see Neher 1992 and Sakmann 1992) it has been possible to measure these effects at the single-channel level in a few animal cells. In plant cells the only known publication on this specific topic consists of a description of subconductance states in the K^+ channel from *Chara corallina* at two different temperatures (Tyerman et al. 1992).

The giant cytoplasmic droplets mechanically obtained from internodal cells of charophytes (Kamiya and Kuroda 1957) have proved to be a convenient model system for the study of plant ionic channels, partly due to the fact that electrical recordings can be obtained easily and with high reproducibility from these droplets. This has resulted in the most complete characterization of a K^+ channel of plant origin to date (Draber et al. 1991, Katsuhara et al. 1989, Laver 1990, Laver and Walker 1987, 1991, Lühring 1986, Tyerman et al. 1992, Zanello and Barrantes 1992). The K^+ channel present in charophyte species is characterized by a high selectivity for K^+, a large conductance (about 100 pS in 100 mM KCl), activation by cytoplasmic Ca^{2+} and inhibition by specific K^+ channel blockers such as Cs^+ and TEA^+.

The present work investigates the direct effect of temperature on the K^+ channel present in the cytoplasmic droplet of the fresh-water alga *Chara contraria* at the level of single-channel recordings. By carrying out these recordings on excised patches of membrane it is possible to avert possible effects exerted by temperature on the electrical state of the cell membrane via metabolic processes such as photosynthesis (Fisahn and Hansen 1986). We find that temperature affects the duration of open and closed states to a larger extent than the amplitude of unitary K^+ currents. We derive values for the activation energy, enthalpy and entropy for the processes of K^+ conductance and for some aspects of channel kinetics. Our results provide the first demonstration of a key direct modulatory role of temperature in plant ionic channel function. A preliminary ac-

Abbreviations: V_m, membrane potential; P_o, open channel; E_a, activation energy.

count of this work has recently been presented in abstract form (Zanello et al. 1993).

Materials and Methods

Plant material—Chara contraria A. Braun ex Kutz oospores were cultivated in 10 cm-diameter glass flasks filled with double-distilled water on a 4 cm layer of heat-sterilized fine sand. The water-filled vessel was allowed to equilibrate for 48 h before introducing the *Chara* oospores. The algae grew steadily under natural illumination conditions and at a room temperature of about 20°C. Internodal cells about 1 cm in length and showing active cytoplasmic streaming as observed by light microscopy were chosen. Cytoplasmic droplets were obtained from internodal cells by the mechanical method developed by Kamiya and Kuroda (1957) as previously described (Zanello and Barrantes 1992). Briefly, cells were first allowed to lose turgor by transpiration; after cutting off one end, internodal cells were rapidly immersed in an isoosmotic solution containing 100 mM KCl, 5.5 mM $CaCl_2$, 5.5 mM $MgCl_2$, and 5 mM MES/KOH, pH 5.8. The osmolarity of the solution was 260 mOs, as measured with a Wescor 5500 vapour pressure osmometer (Wescor, Inc, Utah). Spherical droplets larger than 50 μm in diameter were preferred for patch-clamp recordings, and their single-channel activity was recorded within the first two hours after isolation as described previously (Zanello and Barrantes 1992).

Patch-clamp recordings—Patch electrodes were pulled from Kimax-51 capillary tubes (Kimble Products) by the two-stage pulling method (Hamill et al. 1981) using a vertical electrode puller (David Kopf model 700 C), and coated with Sylgard (Dow Corning Corp., Midland, MI). It was not necessary to fire-polish the pipette tips to obtain stable gigaohm seals. Pipettes were filled with the same solution as the bath medium. All solutions were filtered (Millipore 0.22 μm) prior to use. Pipettes with resistances of 5–10 MΩ, corresponding to a tip diameter of 1 μm or less (Hamill et al. 1981), were routinely used. Gigaohm seals were performed at an initial bath temperature of 10°C.

Experiments were carried out using the inside-out configuration (Hamill et al. 1981) at fixed pipette potentials ($-V_m$). Each patch of membrane exhibiting only one level of channel activity was successively subjected to increasing temperatures. In order to confirm the reversibility of temperature effects, long-living patches were afterwards subjected to decreasing temperatures. The bath temperature was changed using a Haake (Berlin, Germany) model D3 thermostated bath connected to the PCT recording chamber of a Luigs & Neumann (Ratingen, Germany) patch-clamp tower. The temperature limits for the maintenance of giga-seals were found to be 3°C and 25°C. The bath temperature in the recording chamber was maintained at ±0.2°C of the desired value and was allowed to stabilize for at least 2 min prior to each recording.

Patch-clamp recordings were obtained with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The signals were digitized and stored with 16-bit resolution using a video cassette recorder (Panasonic Corp.) and a modified pulse-code modulator (Sony model 701 PE) and subsequently transferred to a microprocessor after filtering at 2 kHz with an eight-pole lowpass Bessel filter (Frequency Devices, Inc., Haverhill, MA).

Data analysis—Single-channel events were analyzed using the programme TRANSIT (A.M.J. Van Dongen, 1989; Baylor College of Medicine), which uses an algorithm for the idealization of the single-channel recordings based on the calculation of the first derivative of the current amplitude at each sampled point. Transitions and levels between transitions are identified on the basis of minimum slope and current criteria set by the experimenter. Recordings of 120 s duration, containing about 5,000 opening events, were sampled at a frequency of 20 kHz. Mean lifetimes (τ) of open and closed states were calculated by fitting dwell-time histograms, constructed with a logarithmic time axis, with single or multiple exponential probability density functions (p.d.f.) by means of a fitting module included in the TRANSIT programme. For each p.d.f. the best fit was chosen according to the maximum likelihood criterion (Colquhoun and Sigworth 1983). Mean unitary currents were obtained from peak values of Gaussian functions fitted to single-channel amplitude histograms also generated by TRANSIT. Values for the open channel probability (P_o) in the analysed recordings are given automatically by TRANSIT.

Unless specifically stated otherwise in the text, the data points in the figures are the mean values±S.E. from at least 4 experiments.

Data simulation—In order to test the adequacy of the kinetic model proposed for the *Chara* K^+ channel (see Results), simulated single channel recordings were obtained by means of the programme CSIM (The Research Foundation, SUNY). Basically, CSIM requires the experimenter to provide the values of the transition rate constants associated to the postulated kinetic model, which must be introduced as a matrix transition. A matrix of 4×4 elements was used, in accordance with the four-state kinetic model proposed for this channel. Data were simulated under the same filtering and sampling frequency conditions used in the analysis of actual recordings. The simulated data were subsequently analyzed by means of the TRANSIT programme as described above, and compared with the experimental data.

Calculation of energy parameters—The Arrhenius equation (Arrhenius 1889) relates the values obtained for the rate constants of a process at different temperatures with the activation energy (E_a) of a transition complex that may exist between two states of a system during the pro-

cess. In its linearized form, the Arrhenius equation can be written as:

$$\ln k = \ln A - E_a/RT \quad (1)$$

where k is the rate constant of the process at different temperatures, A is the Arrhenius constant considered almost independent of temperature, R is the gas constant, and T is the absolute temperature. In our work, k was replaced by values experimentally obtained for the mean unitary current of the channel and for the channel transition rate constants at different temperatures, and the E_a for each process was calculated from the slope of the Arrhenius plots.

Eyring's transition state theory (Eyring 1935) was used for the calculation of the free energy (ΔG_a), enthalpy (ΔH_a) and entropy (ΔS_a) of activation of the channel conductance and kinetics. This theory relates a kinetic process, which is a phenomenon that evolves with time, with the energy fluxes associated with the state changes, i.e. the thermodynamics of the process, by means of the following equation:

$$\ln k = \ln (k_B T/h) - \Delta H_a/RT + \Delta S_a/R \quad (2)$$

where k_B and h are the Boltzmann and Planck constants respectively. ΔG_a for the channel conductance and gating processes was calculated from equation (2) as follows:

$$\Delta G_a = -RT \ln k + RT \ln k_B T/h \quad (3)$$

where k has the same meaning as in equation (1). Values for ΔH_a and ΔS_a associated with the processes were calculated from E_a according to the methods followed by McLarnon and Wang (1991), using the following relationships:

$$\Delta H_a = E_a - RT \quad (4)$$
$$\Delta S_a = -(\Delta G_a - \Delta H_a)/T \quad (5)$$

As a measure of the temperature dependence of ion conduction through the pore and on channel kinetics, Q_{10} values were determined from the ratios of the current and the kinetic constants at 10°C (T_1) and 20°C (T_2) according to the following equation:

$$Q_{10} = \exp [-E_a R (T_2^{-1} - T_1^{-1})] \quad (6)$$

Q_{10} values for the mean open and closed lifetimes of the channel were calculated directly from plots of τ vs. temperature, as the ratio of values obtained at 20°C and 10°C from a linear regression fitted to the experimental points.

Results

When cytoplasmic droplets obtained from the internodal cell of the charophyte *Chara contraria* are bathed in a solution containing a high K^+ concentration (100 mM), isoosmotic with respect to the cytoplasm (Okihara and Kiyosawa 1988), it is possible to record the activity of a voltage-dependent K^+ channel assumed to be of tonoplast origin (Lühring 1986, Sakano and Tazawa 1986). This is by far the most frequently found ion channel activity present in the droplet membrane, and has been the subject of exhaustive electrophysiological characterization during recent years (Katsuhara et al. 1989, Laver 1990, Laver and Walker 1987, 1991, Lühring 1986, Zanello and Barrantes 1992). Fig. 1 shows traces of activity of the K^+ channel in *C. contraria* at three different temperatures, in an excised inside-out patch of the droplet membrane, held at −80 mV and bathed with symmetric ionic solutions. Under these experimental conditions, when positive pipette potentials (negative V_m) are applied, potassium ions cross the channel from the vacuolar to the cytoplasmic side of the membrane (Katsuhara et al. 1989, Laver and Walker 1987, Lühring 1986). From visual inspection of the single-channel recordings (Fig. 1), it can be seen that a rise in temperature causes an increase in channel conductance and a decrease in the time the channel spends in the open state, qualitatively apparent in the prolonged closed intervals. This effect was fully reversible (data not shown).

Effect of temperature on the K^+ channel conductance —Fig. 2 shows three current amplitude histograms and their corresponding fitted Gaussian functions (see Materials and Methods) obtained from three recordings on the same patch of membrane subjected to different tempera-

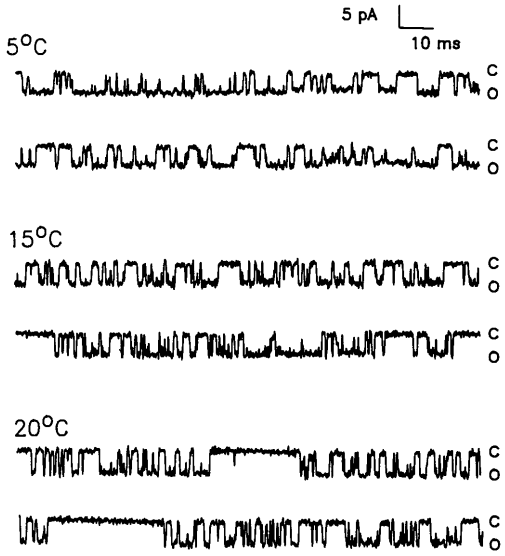


Fig. 1 Single-channel events recorded from a cytoplasmic droplet of *Chara contraria* using the inside-out patch configuration at a V_m of −80 mV and at the indicated temperature values. Low-pass filter: 2 kHz; sampling frequency: 20 kHz. The composition of pipette and bath solutions is given under Materials and Methods. o: open; c: closed channel.

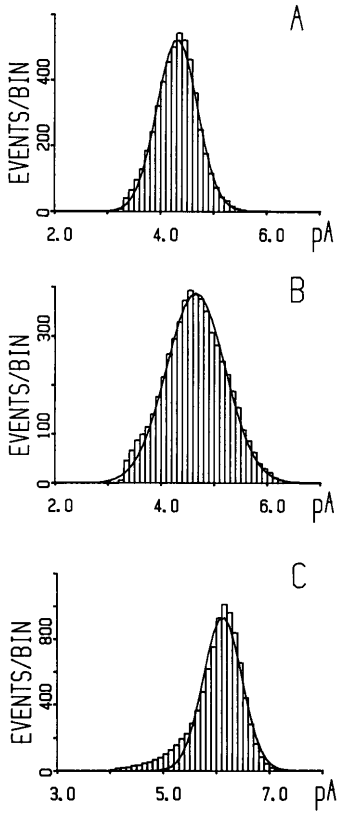


Fig. 2 Histograms for the distribution of single-channel current amplitudes and their corresponding p.d.f., at three different temperatures. $V_m = -80$ mV. A: 5°C, peak value=4.4±0.3 pA; B: 10°C, peak value=4.7±0.5 pA; C: 25°C, peak value=6.1±0.3 pA.

tures. Only one peak was detected in these histograms for the conductance state of the K^+ channel (filtering and sampling frequency specified in Materials and Methods). Subconductance states such as those reported by Tyerman et al. (1992) in the large K^+ channel of *C. corallina* under similar ionic conditions were not detected in the present analysis, even though the programme used is based on the same principles as those used by the latter authors. Recordings longer than 2 minutes and containing more than 10,000 opening events revealed the existence of a mid-conductance state with an open state probability of not more than 0.04 in the K^+ channel of *C. contraria* (data not shown). However, the noticeable increment in the permanence of the midstate with decreases in temperature, as predicted in the work of Tyerman et al. (1992), was not observed in *C. contraria*. For this reason we only analysed the sensitivity of the main conductance state to changes in temperature. As can be seen in Fig. 3, temperature exerted a reproducible (S.E. of the current averages <0.5 pA), progressive linear increase (r=0.981) in the mean unitary current of the channel, from 4.2±0.3 pA at 3°C to 6.2±0.3 pA at 25°C and at a V_m of −80 mV.

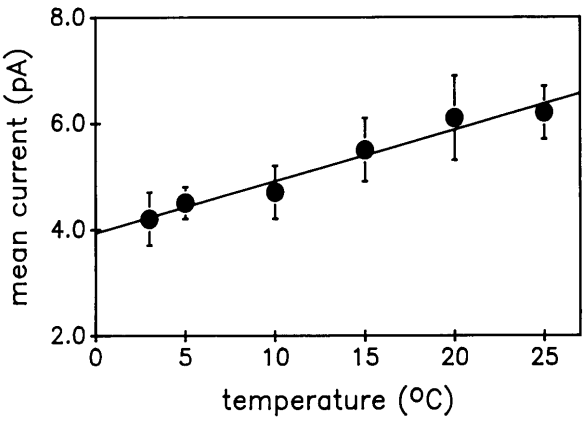


Fig. 3 Temperature dependence of single-channel current. Data correspond to the average values obtained from 4–6 experiments using the inside-out configuration at $V_m = -80$ mV.

Fig. 4 shows the current-to-voltage relationships for the K^+ channel activity in a patch of membrane held at different potentials, at two temperatures. Conductance values calculated from the points around 0 mV were 60 pS and 90 pS at 10°C and 20°C respectively. Both inward and outward K^+ currents showed saturating behaviour at the two different temperatures, in accordance with previous descriptions at a single temperature of about 20°C (Laver 1990, Laver and Walker 1987, 1991, Lühring 1986, Zanello and Barrantes 1992).

Effect of temperature on the probability of channel opening—The open channel probability, P_o, defined as the period of time spent in the open state relative to the total duration of the analysed recording, was also influenced by temperature (Fig. 5A, B). P_o markedly decreased from

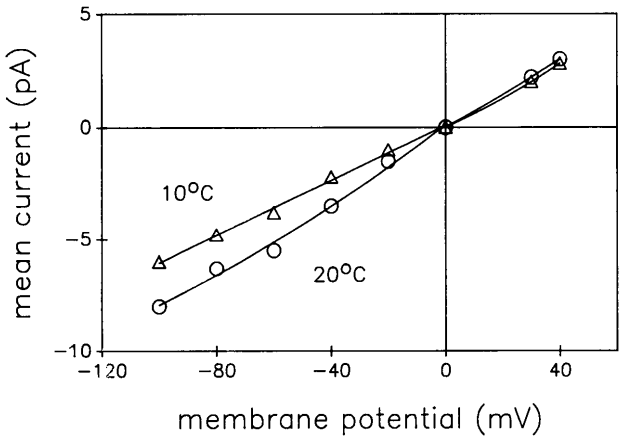


Fig. 4 Current-to-voltage relationships for the K^+ channel of *Chara* obtained at two different temperatures. A patch of membrane was exposed firstly to 10°C and then to 20°C, and the electrical activity of the channel recorded at the different clamped membrane potentials.

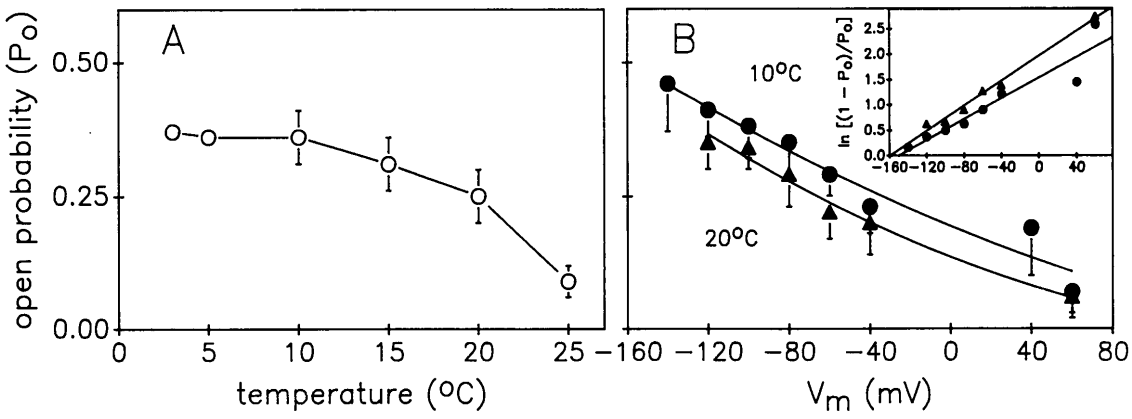


Fig. 5 A: Probability of channel opening (P_o) as a function of temperature. $V_m = -80$ mV. B: Voltage activation curves obtained at 10°C and 20°C. Inset: linearized form (eq. 7) of the Boltzmann distribution for P_o vs V_m at the two corresponding temperatures. Values obtained for V_0 and n are given in the text.

almost 0.4 to 0.1 as the temperature was raised from 3°C to 25°C, at a clamped membrane potential of −80 mV. Fig. 5B shows the open channel probability to vary slightly within the range of the applied potentials, from about 0.45 ($V_m = -140$ mV) to 0.07 ($V_m = 60$ mV) at 10°C, and from about 0.35 ($V_m = -120$ mV) to 0.06 ($V_m = 40$ mV) at 20°C. As can be seen from Fig. 5B, a 10°C increment in temperature caused the voltage activation curve to shift to more negative values along the voltage axis. The value of the applied voltage (V_0) at which P_o equals 0.5 was obtained from the linearized form of a Boltzmann distribution for P_o as a function of voltage (Fig. 5B, inset):

$$\ln\left(\frac{1-P_o}{P_o}\right)=\frac{nFV_0}{RT}-\frac{nFV}{RT} \qquad (7)$$

where V is the voltage applied to the membrane, F is the Faraday constant, R is the gas constant, T is the absolute temperature, and n is the effective charge that moves in the membrane during a transition between the closed and the open state of the channel.

As can be seen from Fig. 5B, the change in bath temperature from 10°C to 20°C displaced the channel voltage dependence by about 10 mV. The direction of the displacement would indicate that heating acts by stabilizing the closed configuration of the channel. Similar observations can be found in the literature for an Na^+ channel in the squid axon (Correa et al. 1992). The effective gating charge, n, was not significantly altered by temperature ($n = -0.25$ at 10°C; $n = -0.31$ at 20°C), as was also found by Correa and coworkers.

Effect of temperature on the duration of open and closed states—We investigated the effect of temperature on the mean open and closed channel dwell-times in order to obtain a better description of the diminution of P_o with heating. As reported previously (Laver and Walker 1987, Tyerman et al. 1992, Zanello and Barrantes 1992), the open time distributions of the K^+ channel in charophytes can be well described by a single-exponential function, whereas the closed transitions exhibit more complicated kinetics. Figs. 6A–F show the semilogarithmic histograms obtained for dwell-times of the open and closed states for the K^+ channel of *Chara* at three different temperatures and at a V_m of −80 mV. With the use of a logarithmic time axis instead of a linear one, multiple dwell-time distributions are compressed, and components that are poor in events can be more easily detected. P.d.f. of dwell-times were fitted to the data (see Materials and Methods) to obtain the time constants for the distribution of open and closed times. The values obtained from the fit to the experiments shown in Fig. 6 are listed in Table 1. At all the temperatures studied, open times could be described by a single-exponential func-

Table 1 Parameters fitted to the open and closed time histograms of Fig. 6

	Temperature		
	3°C	15°C	25°C
n	6,771	6,068	4,114
τ_o	2.756	2.009	0.979
τ_{c1}	0.167	0.208	0.217
x_1	0.632	0.557	0.351
τ_{c2}	1.156	1.736	1.550
x_2	0.259	0.420	0.562
τ_{c3}	5.843	24.002	21.886
x_3	0.099	0.019	0.069
τ_{c4}	200.50	567.83	289.71
x_4	0.010	0.003	0.018

n, total number of events per histogram; τ_o, mean open lifetime; $\tau_{c1...4}$, mean closed lifetimes (#1 is the briefest and #4 the longest); $x_{1...4}$, fraction of total fitted events of each individual component.

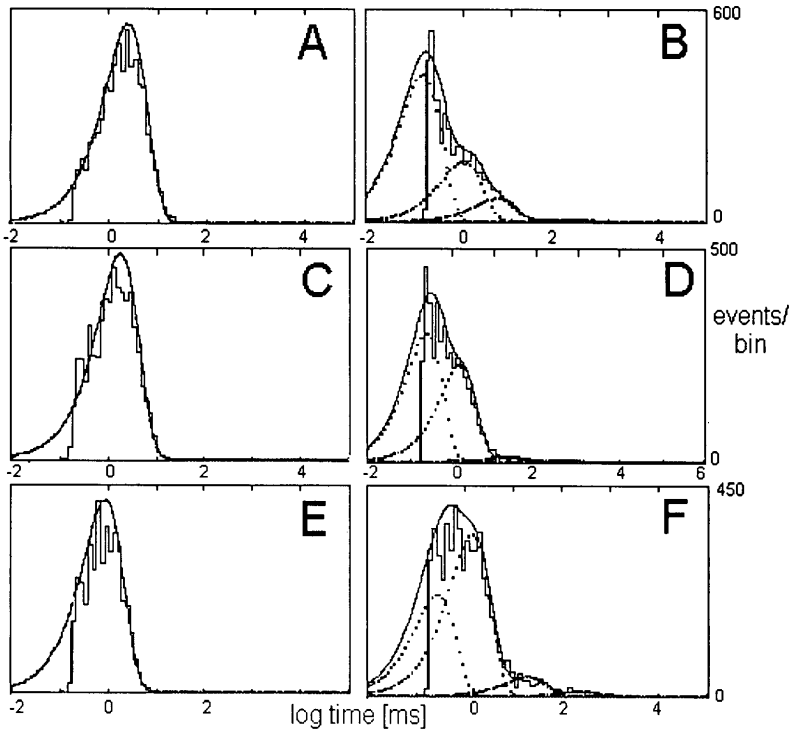


Fig. 6 Semilogarithmic histograms of open (left column) and closed (right column) state durations for the K^+ channel of *Chara* at three different temperatures: 3°C (A, B), 15°C (C, D), and 25°C (E, F). $V_m = -80$ mV. Parameters of the p.d.f. fitted to the histograms are shown in Table 1.

tion, while closed-time histograms were fitted by the sum of four exponentials, suggesting the presence of at least four closed states (Fig. 6). The curves (full lines) in closed duration histograms represent the overall fit (sum of four exponential functions), whereas the dotted lines depict the corresponding area of each component. The longest component of closed intervals ($\tau > 200$ ms) represented only a small fraction (<1%) of the total number of closed events,

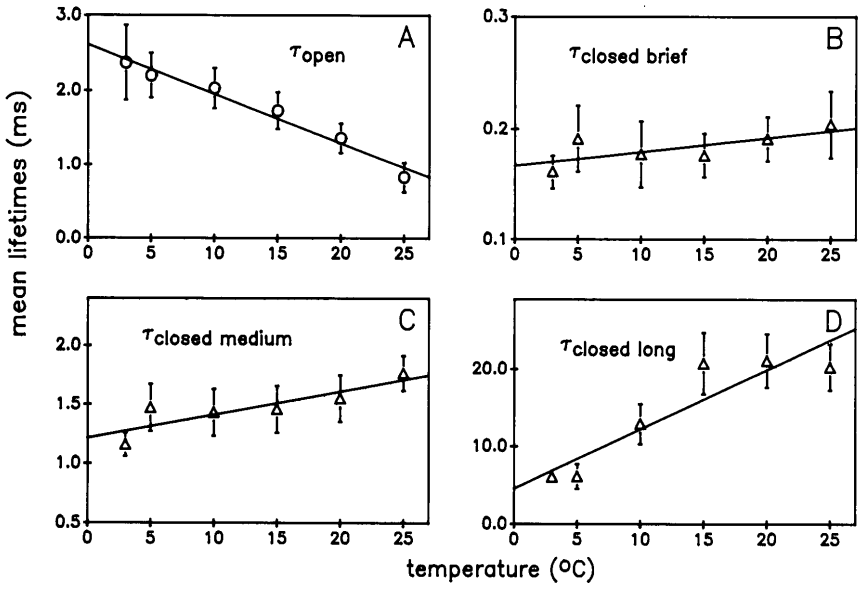


Fig. 7 Temperature dependence of the mean open (A) and closed (B–D) dwell times of the K^+ channel, at a membrane potential of −80 mV. Each point is the average ± S.E. of 4–6 experiments.

and constituted the most variable values of all the experiments (S.E. in the order of 50 ms). For this reason we only studied the effect of temperature on the mean lifetimes of three of the four detected exponential components, defined as the brief ($\tau<0.3$ ms), medium (τ between 1.0 and 1.8 ms) and long ($\tau>5.0$ ms) closed events.

Fig. 7A shows the variation of the mean open dwell-times of the K^+ channel with temperature. The mean open time diminished in a linear fashion from 2.4 ± 0.3 ms at 3°C to 0.8 ± 0.2 ms at 25°C ($r=0.985$). A Q_{10} value of 0.66 was calculated from the linear regression in Fig. 7A for the diminution of τ_o between 10°C and 20°C.

The duration of the detected channel closed states exhibited a general tendency to increase as a function of temperature, albeit with different sensitivities (Figs. 7B–D). Long-closed times (Fig. 7D) were the most clearly influenced by changes in temperature, with a Q_{10} of 1.6 calculated between 10°C and 20°C, whereas brief- and medium-closed durations (Figs. 7B and C) did not seem to contribute as significantly as increased long-closed periods and decreased open durations to the diminution of P_o with increments in temperature (Q_{10} of 1.10 and 1.14 respectively).

The relative magnitude of each of the three closed state components—expressed as a percentage of the total number of closed events—was compared for the different temperatures studied. As can be seen in Fig. 8A–C, the amplitude of the majority, brief-closed component showed a tendency to decrease as the temperature was raised, while the medium-closed component tended to increase. These results could reflect the tendency of the channel to remain predominantly in a closed configuration of intermediate duration which becomes prolonged with heating, while transitions to the briefer closed state become less frequent. The magnitude of the long-closed component, on the contrary, markedly decreased with higher temperatures (see Fig. 8C).

Thermodynamics of conductance and kinetics of the C. contraria K^+ channel—A thermodynamic analysis of the influence of temperature on K^+ conductance through the pore and on channel gating, based on Eyring's transition state theory (Eyring 1935), enabled us to evaluate the activation energy (E_a), enthalpy (ΔH_a) and entropy (ΔS_a) associated with the two processes. According to Eyring's theory, a system that undergoes a state change must overcome an initial energy barrier to reach the activated state. This would correspond to the activation energy empirically found by Arrhenius. The transition state theory conceives this activated complex as a molecular entity having defined thermodynamic properties (see equations 2 to 5). Fig. 9A shows the Arrhenius plot constructed for the single-channel unitary currents. A linear fit to the mean current values gave an E_a for the conduction process of 2.95 kcal mol^{-1} ($r=0.929$), and a Q_{10} value of 1.20 (see eqs. 1 and 6).

For the purposes of the thermodynamic analysis of the effect of temperature on the channel gating a kinetic model relating the detected states must be considered. Only one open state was detected for the *Chara* K^+ channel and, according to published kinetic studies (Laver 1990, Laver and Walker 1987), it can be linearly connected to a multiplicity of closed states, its number varying in accordance with the

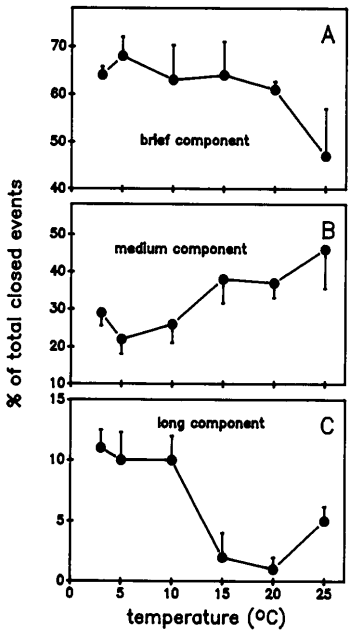


Fig. 8 Magnitude of the brief (A), medium (B) and long (C) component of closed events detected from closed-times histograms like those shown in Fig. 6.

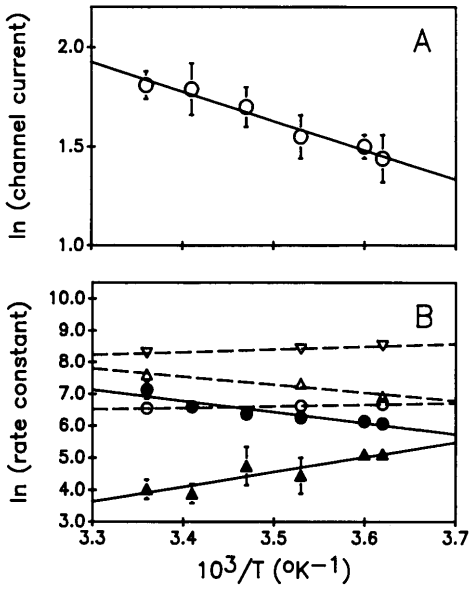


Fig. 9 Arrhenius plots for the unitary currents (A) and transition rate constants (B) defined in scheme (8). In B, filled circles: α; filled triangles: k_{12}; open circles: k_{23}; open triangles: k_{32}; open inverted triangles: β.

recording conditions (Laver 1990). In our case, we could characterize the effect of temperature on three of the four detected closed states (see Figs. 6 to 8), as explained above. The simplest mechanism connecting this unique open state to these three closed states is the linear scheme below:

$$C_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C_3 \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O \qquad (8)$$

where O, the open state, is connected to the brief closed state C_3, in agreement with Laver and Walker's model (1987). C_1 and C_2 are proposed to be the long- and medium-channel closures, respectively. This scheme (8) can be considered a simplified form of Laver and Walker's model which, according to these authors, can be applied to single-channel recordings obtained at a negative membrane potential and in the presence of a high concentration of external Ca^{2+} (Laver and Walker 1987, Laver 1990). Alfa and β are the channel closing and opening rates respectively, and $k_{i,j}$ are the rate constants for the transition from the closed state i to the closed state j. It was possible to calculate the transition rate constants associated with the processes of channel closure (α) and with the reaction step from the long (C_1)- to the medium (C_2)-closed states (k_{12}) directly as the reciprocal of the measured mean open and long-closed lifetimes, respectively. Thus:

$$\alpha = 1/\tau_o \qquad (9)$$

$$k_{12} = 1/\tau_{C1} \qquad (10)$$

Beta, k_{21}, k_{23}, k_{32} and k_{34} were related to the mean durations of the medium (C_2)- and brief (C_3)-closed states of the channel through the following expressions (Colquhoun and Hawkes 1983):

$$k_{21} + k_{23} = 1/\tau_{C2} \qquad (11)$$

$$k_{32} + \beta = 1/\tau_{C1} \qquad (12)$$

and could not be directly calculated from the reciprocal of τ values. Table 2 lists three groups of transition rate constants estimated for three different temperatures, and also the results obtained for the mean open and closed durations from the analysis of the simulated recordings. The agreement between simulated and experimental data (see Fig. 7) fully supports the kinetic model (8) proposed for this K^+ channel. The proportion of events in each closed state closely approximated that obtained from the analysis of real data, and showed a similar behaviour with the change in temperature, as can be seen in Fig. 8. The only exception was the percentage of simulated long (C_1)-closed events, which showed a tendency to increase with temperature. This can be accounted for if we consider a simplified version of scheme (8), in which the longest-closed state ($\tau >$ 200 ms, and frequency of occurrence $<1\%$) detected in dwell-time histograms (cf. Fig. 6), is not taken into account. Other models comprising one open and three closed states (Table 3) were tested for the *Chara* K^+ channel. Values obtained for the transition rate constants for each set of simulated data (Table 4) did not describe the experimental data as satisfactorily as kinetic scheme (8) (cf. Fig. 7).

Fig. 9B shows the Arrhenius plots constructed for the transition rate constants defined in scheme (8). In the case of the channel closure, an E_a value of 6.97 kcal mol^{-1} was obtained from the slope of the linear regression fitted to the

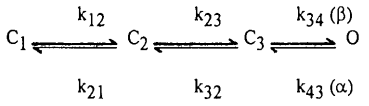
Table 2 Values for the transition rate constants and mean lifetimes obtained from simulated recordings of the *Chara* K^+ channel according to kinetic scheme (8)

Transition	State	Rate constant (s^{-1})			Mean time (ms)		
		3°C	10°C	25°C	3°C	10°C	25°C
k_{12}		170	100	50			
k_{21}		100	100	100			
k_{23}		800	750	700			
k_{32}		1,000	1,500	2,000			
β		5,000	4,500	4,000			
α		600	800	1,500			
	C_1				6.326 (3)	10.351 (5)	17.542 (6)
	C_2				1.251 (11)	1.574 (22)	2.054 (29)
	C_3				0.155 (86)	0.198 (73)	0.193 (65)
	O				2.690	2.255	1.202

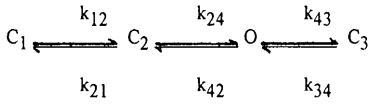
Transitions between one channel state and another and the states of the channel themselves are defined in the text (see model 8). Values between brackets correspond to the percentage of events in the corresponding closed state with respect to the total in the simulated recording.

Table 3 Four-state models tested for the *Chara* K^+ channel

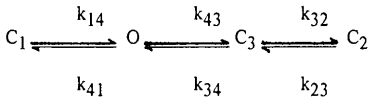
MODEL 1:



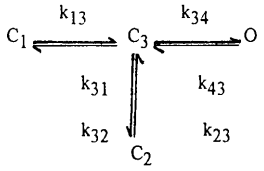
MODEL 2:



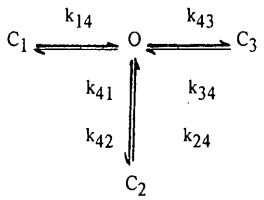
MODEL 3:



MODEL 4:



MODEL 5:



experimental points for a ($r=0.929$). The reaction step leading away from the long (C_1)-closed state of the channel, which was shown to be the most affected by temperature (see Fig. 7), yielded an E_a value of -9.13 kcal mol^{-1} ($r=0.905$) calculated from the corresponding plot for the empirical k_{12} (Fig. 9B). This negative value for E_a would be indicative of a more complicated mechanism connecting C_1 and C_2 than that proposed in scheme (8), probably arising from the existence of a reaction step connecting with a (longest)-closed state of the channel with $\tau > 200$ ms. One intermediate step in the C_1-C_2 pathway would imply a negative ΔG term that would contribute in turn to the negative apparent E_a measured for k_{12}. Values for Q_{10}, ΔH_a, ΔG_a and ΔS_a associated with K^+ conductance and channel kinetics are listed in Table 3.

Discussion

Temperature modifies the internal energy of macromolecules and its effect is evident in both the gating and ion permeation processes in ionic channels. Determination of temperature sensitivity is an essential component in establishing the kinetics of a given reaction mechanism. Here we have investigated the effects exerted by temperature on the K^+ channel of *Chara* cytoplasmic droplets and have found channel kinetics to be more strongly affected than ionic conductance properties. This is in agreement with the early observations of Hodgkin et al. (1952) on the squid axon Na^+ channel, showing that the rate of gating increases with temperature, with a Q_{10} of 2 to 4, while the conductance is relatively temperature-insensitive, with a Q_{10} of only 1.2 to 1.5.

From the present analysis of the *Chara* K^+ channel it appears that the environment within the pore of the channel is favourable to the diffusion of K^+ ions. The Q_{10} value of 1.2 (Table 5) for the conductance process is very similar to that obtained for the aqueous diffusion of K^+ (Q_{10} of 1.3; Hille 1984), as with other voltage-dependent channels (Correa et al. 1991, Estrada 1991, Horn et al. 1984). This can be related to the low activation energy found for the process of ion conductance through this channel (2.95 kcal mol^{-1}, Fig. 9A), which exhibits a similar value to that for cation flux through veratridine-activated Na^+ channels (Tanaka et al. 1983) and the Ca^{2+}-activated K^+ channel of skeletal muscle sarcoplasmic reticulum (4.14 kcal mol^{-1}; Estrada 1991). The enthalpic change for this process in the K^+ channel of *Chara* (2.37 kcal mol^{-1}, Table 5) is even lower than that reported for the enthalpic change of Na^+ conductance through the batrachotoxin-modified Na^+ channel in the squid giant axon (6.25 kcal mol^{-1}; Correa et al. 1991), indicating that ions traverse a low energy barrier when crossing the channel. Furthermore, the values for the entropy of activation of ion conductance through the pore of this channel (-46.6 cal mol^{-1} K^{-1}, Table 5) and the Ca^{2+}-activated K^+ channel from skeletal muscle sarcoplasmic reticulum (-33.7 cal mol^{-1} K^{-1}; Estrada 1991) indicate an increase in the order of the system during the development of the process.

The rate of conformational change in the channel proteins involved in gating processes is also modified by temperature (Hodgkin et al. 1952). In the case of the voltage-dependent K^+ channel of *C. contraria*, we found that a rise in temperature caused a marked decrease in the probability of channel opening, which is similar to the effect reported for an Na^+ channel of an animal cell (Correa et al. 1992). This was evidenced by a shorter open state (Q_{10} of 0.66) and longer closed periods (Q_{10} between 1.1 and 1.6) at increasingly higher temperatures at a fixed membrane potential, suggesting that heating favours the closed configuration of the channel molecule.

It still remained unclear which of the mechanisms leading to the stabilization of the closed state is most affected by temperature, and in particular whether temperature increase has a greater impact on the transition from the open to the closed state or vice versa. A plausible

Table 4 Transition rate constants and mean durations of open and closed states for data simulated according to models presented in Table 3

	MODEL 1	MODEL 2	MODEL 3	MODEL 4	MODEL 5
Rate constant (s^{-1}):					
k_{12}	170	180	—	—	—
k_{21}	100	100	—	—	—
k_{23}	800	—	780	850	—
k_{32}	1,000	—	1,000	900	—
k_{34}	5,000	6,800	5,500	5,000	6,500
k_{43}	600	400	550	600	400
k_{24}	—	800	—	—	850
k_{42}	—	200	—	—	180
k_{13}	—	—	—	170	—
k_{31}	—	—	—	100	—
k_{14}	—	—	140	—	170
k_{41}	—	—	10	—	10
Mean lifetime (ms):					
O	2.690	2.269	2.406	2.256	2.244
C_1	6.326 (3)	5.862 (6)	8.043 (2)	2.552 (16)	6.973 (1)
C_2	1.251 (11)	1.268 (28)	1.910 (17)	0.584 (1)	1.341 (29)
C_3	0.155 (86)	0.228 (66)	0.266 (81)	0.293 (83)	0.267 (70)

Values between brackets are percentages of events in the corresponding closed state. Data were simulated for a temperature of 3°C.

answer to this question is provided in the present work through comparison of the actual data with those of simulated data, from which most transition rate constants could be calculated (cf. Table 2 and Figs. 7 and 8).

Q_{10} values slightly larger than 1.0 have been reported for the temperature sensitivity of the channel closing rate α in the case of voltage-dependent K^+ channels from animal cells (McLarnon and Wang 1991); the value found in the present work for the K^+ channel of *Chara* was somewhat higher (1.52, cf. Table 5). The activation energy (6.97 kcal

Table 5 Channel conductance, kinetic constants and thermodynamic parameters for conductance and kinetic processes in the *Chara* K^+ channel

Process	Channel amplitude and rates	E_a	Q_{10}	Thermodynamic parameters		
				ΔH_a	ΔG_a	ΔS_a
K^+ conductance	5.5 pA	2.95	1.20	2.37	15.57	−46.65
Channel kinetics:						
α	578 s^{-1}	6.97	1.52	6.40	12.89	−22.54
k_{12}	115 s^{-1}	−9.13	0.58	−9.70	14.12	−49.06
k_{23}	750 s^{-1}	−0.97	0.94	−1.53	12.81	−50.67
k_{32}	1,500 s^{-1}	5.03	1.35	4.47	12.43	−28.13
β	4,500 s^{-1}	−1.71	0.90	−2.27	11.81	−49.75

Rate constants are defined according to kinetic scheme (8), and correspond to a membrane potential of −80 mV. α and k_{12} were calculated from the experimental data according to the expressions (9) and (10), at 288 K (15°C). k_{23}, k_{32} and β are the estimated values utilized for data simulation with CSIM as explained in the text (−80 mV and 283 K). E_a, ΔH_a and ΔG_a are in kcal mol^{-1}. ΔS_a is in cal mol^{-1} K^{-1}. Consequently, results for the estimated k_{21}, which was shown to be insensitive to temperature (Table 2), are not included in the table.

mol^{-1}, Fig. 9B) and entropy (-22.54 cal mol^{-1} K^{-1}, Table 5) found for the closing process of this K^+ channel were similar to those in animal cells: 11.82 kcal mol^{-1} and -10.96 cal mol^{-1} K^{-1} respectively for the Ca^{2+}-dependent K^+ channel of skeletal muscle sarcoplasmic reticulum (Estrada 1991) and 6.10 kcal mol^{-1} and -25.60 cal mol^{-1} K^{-1} for the unblocking rate constant in the $K^+(Ca^{2+})$-channel in hippocampal neurons in the presence of the drug RP-62719 (McLarnon and Wang 1991). Positive values for the activation enthalpy found for two consecutive steps in the reaction mechanism (scheme 8) leading away from the open configuration of the channel (6.40 kcal mol^{-1} and 4.47 kcal mol^{-1} for a and k_{32} respectively, Table 5) are indicative of endothermic processes associated to channel closure. The values found for the activation entropies during the transitions from the open to the closed states (-22.54 cal K^{-1} mol^{-1} -28.13 cal K^{-1} mol^{-1} for a and k_{32}, Table 5) are less negative than those of the inverse reactions, and presumably provide the driving force for stabilizing channel closed configurations as the temperature increases.

We can thus say that temperature increase has a greater impact on the process of closure, which has been demonstrated to have higher energetic requirements (higher values for E_a, see Table 3) than the opening process in the *Chara* K^+ channel. Although the absence of energy barriers (E_a near zero) and the exothermic terms associated with the reaction rates k_{23} and β that lead to the open configuration of the channel provide the necessary energetically favourable conditions for the molecule to adopt the open configuration, the highly negative values for the activation entropies during these steps favours the opposite behaviour. The energy provided by heating is thus invested mainly in increasing the order of the system, which could explain the stabilization of the closed configuration of the channel with the rise in temperature. As postulated by Correa et al. (1992), a series of chemical modifications such as the breakup and formation of salt bridges and hydrogen bonds could lead to a more ordered state of the system during the transition to the activated complex. This reorganization of the channel molecule would be accompanied in the case of the K^+ channel of *Chara* by a liberation of thermal energy, as indicated by the negative values for the activation enthalpies associated with channel opening.

It is also worth comparing the temperature sensitivity of voltage-gated to that of ligand-gated channels. The paradigm rapid ligand-gated channel, the nicotinic acetylcholine receptor (see review in Barrantes 1988), has a similar temperature sensitivity to that found here for the K^+ channel of *Chara* (Anderson and Stevens 1973, Dilger et al. 1991, Zanello et al. 1993), indicating that once opened, ion permeation through either ligand-gated or voltage-gated channels proceeds with the same temperature dependence as that operating in the case of ions in solution (Hille 1984).

The influence of temperature on the K^+ channel of a charophyte has been previously reported by Fisahn and Hansen (1986) on the basis of membrane potential and resistance measurements by conventional electrophysiological techniques (Fisahn and Hansen 1986). Variations in these electrical parameters are known to result from the compound effect of temperature on certain metabolic processes such as photosynthesis (Fisahn and Hansen 1986) and on several passive (Beilby and Coster 1973, 1976, 1979, Hogg et al. 1968) and active transporters (Spanswick 1972, Blatt 1974). Due to technical limitations, it was not possible in earlier studies to assign the measured activation energies to a specific membrane transporter. Application of the patch-clamp technique to excised patches of the algal membrane allowed us to dissect the effect of temperature on the conduction and gating processes of the *Chara* K^+ channel, and to obtain values for the activation energies and thermodynamic parameters for these processes at the single-channel level. We are able to conclude that small variations in temperature within the environmental range have a significant influence on the behaviour of channel molecules in plant cells, particularly during the transition to and from the open and closed states. In the light of the present study, rapid variations in the electrical parameters of algal membranes under raised temperature—such as those described by Fisahn and Hansen (1986) as not being under metabolic control—can now be re-interpreted as the result of direct modulation by temperature.

The authors wish to thank Mr. Mario Alcaraz for his help throughout the work. This work was supported by grants from the International Center for Genetic Engineering and Biotechnology (ICGEB, UNIDO) and the Red Latinoamericana de Botánica to F.J.B. *Chara* oospores were kindly provided by the Laboratory of Weed Control, Dept. of Agronomy, Universidad Nacional del Sur.

References

Anderson, C.R. and Stevens, C.R. (1973) Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junctions. *J. Physiol.* 235: 655–691.

Arrhenius, S.A. (1889) Uber die Reaktiongeschwindigkeit bei der Inversion von Rohrzucker durch Sauren. *Z. Phys. Chem.* 4: 226–248.

Barrantes, F.J. (1988) Muscle endplate cholinoreceptors. *Pharm. Therap.* 38: 331–385.

Beilby, M.J. and Coster, H.G.T. (1973) The action potential in *Chara corallina*: effect of temperature. *Aust. J. Plant Physiol.* 3: 275–289.

Beilby, M.J. and Coster, H.G.T. (1976) Effect of temperature on punchthrough in the electrical characteristics of the plasma-

lemma of *Chara corallina*. *Aust. J. Plant Physiol.* 3: 819–826.

Beilby, M.J. and Coster, H.G.T. (1979) The action potential in *Chara corallina*. IV. Activation enthalpies of the Hodgkin-Huxley gates. *Aust. J. Plant Physiol.* 6: 355–365.

Blatt, F.J. (1974) Temperature dependence of the action potential in *Nitella flexilis*. *Biochim. Biophys. Acta* 339: 382–389.

Colquhoun, D. and Hawkes, A.G. (1983) The principles of the stochastic interpretation of ion-channel mechanisms. *In* Single Channel Recording. Edited by Sakmann, B. and Neher, E. pp. 135–175. Plenum, New York.

Colquhoun, D. and Sigworth, F.J. (1983) Fitting and statistical analysis of single channel records. *In* Single Channel Recording. Edited by Sakmann, B. and Neher, E. pp. 191–264. Plenum, New York.

Correa, A.M., Bezanilla, F. and Latorre, R. (1992) Gating kinetics of batrachotoxin-modified Na^+ channels in the squid giant axon. Voltage and temperature effects. *Biophys. J.* 61: 1332–1352.

Correa, A.M., Latorre, R. and Bezanilla, F. (1991) Ion permeation in normal and batrachotoxin-modified Na^+ channels in the squid giant axon. *J. Gen. Physiol.* 97: 605–625.

Dilger, J.P., Brett, R.S., Poppers, D.M. and Liu, Y. (1991) The temperature dependence of some kinetic and conductance properties of acetylcholine receptor channels. *Biochim. Biophys. Acta* 1063: 253–258.

Draber, S., Schultze, R. and Hansen, U.-P. (1991) Patch-clamp studies on the anomalous mole fraction effect of the K^+ channel in cytoplasmic droplets of *Nitella*: an attempt to distinguish between a multi-ion single-file pore and an enzyme kinetic model with lazy state. *J. Membr. Biol.* 123: 183–190.

Estrada, E. (1991) Efecto de la temperatura en la actividad del canal de potasio activado por calcio del músculo esquelético de la rata. Ph.D. Thesis, Facultad de Ciencias, Universidad de Chile.

Eyring, H. (1935) The activated complex in chemical reactions. *J. Chem. Physics* 3: 107–115.

Fisahn, J. and Hansen, U.-P. (1986) The influence of temperature on a K^+ channel and on a carrier-type transporter in *Nitella*. *J. Exp. Bot.* 37: 440–460.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Improved patch-clamp techniques for high resolution current recording from cells and from cell-free membrane patches. *Pflügers Arch.* 381: 85–100.

Hille, B. (1984) *Ionic Channels of Excitable Membranes*. Sinauer Associates Inc. Sunderland, MA, U.S.A.

Hodgkin, A.L., Huxley, A.F. and Katz, B. (1952) Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol.* 116: 424–448.

Hogg, J., Williams, E.J. and Johnston, R.J. (1968) The temperature dependence of the membrane potential and resistance in *Nitella translucens*. *Biochim. Biophys. Acta* 150: 640–648.

Horn, R., Vanderberg, C.A. and Lange, K. (1984) Statistical analysis of single sodium channels. Effects of N-bromoacetamide. *Biophys. J.* 45: 323–335.

Kamiya, N. and Kuroda, K. (1957) Cell operation in *Nitella*. I. Cell amputation and effusion of the endoplasm. *Proc. Japanese Acad.* 33: 149–152.

Katsuhara, M., Mimura, T. and Tazawa, M. (1989) Patch-clamp study on a Ca^{2+}-regulated K^+ channel in the tonoplast of the brackish Characeae *Lamprothamnium succinctum*. *Plant Cell Physiol.* 30: 549–555.

Laver, D.R. (1990) Coupling of K^+-gating and permeation with Ca^{2+} block in the Ca^{2+}-activated K^+ channel of *Chara australis*. *J. Membr. Biol.* 118: 55–67.

Laver, D.R. and Walker, N.A. (1987) Steady-state voltage-dependent gating and conduction kinetics of single K^+ channels in the membrane of cytoplasmic drops of *Chara australis*. *J. Membr. Biol.* 100: 31–42.

Laver, D.R. and Walker, N.A. (1991) Activation by Ca^{2+} and block by divalent ions of the K^+ channel in the membrane of cytoplasmic drops from *Chara australis*. *J. Membr. Biol.* 120: 131–139.

Lühring, H. (1986) Recording of single K^+ channels in the membrane of cytoplasmic drops of *Chara australis*. *Protoplasma* 133: 19–28.

McLarnon, J.G. and Wang, X.P. (1991) Temperature dependence of drug blockade of a calcium-dependent potassium channel in cultured hippocampal neurons. *Biophys. J.* 60: 1278–1287.

Neher, E. (1992) Ion channels for communication between and within cells. *Science* 256: 498–502.

Okihara, K. and Kiyosawa, K. (1988) Ion composition of the *Chara* internode. *Plant Cell Physiol.* 29: 21–25.

Pahapill, P. and Schlichter, L. (1990) Modulation of potassium channels in human T lymphocytes: effects of temperature. *J. Physiol.* 422: 103–126.

Sakano, K. and Tazawa, M. (1986) Tonoplast origin of the envelope membrane of cytoplasmic droplets prepared from *Chara* internodal cells. *Protoplasma* 131: 247–249.

Sakmann, B. (1992) Elementary steps in synaptic transmission revealed by currents through single ion channels. *Science* 256: 503–512.

Spanswik, R.M. (1972) Evidence for an electrogenic pump in *Nitella translucens*. I. The effects of pH, K^+, Na^+, light and temperature on the membrane potential and resistance. *Biochim. Biophys. Acta* 288: 73–89.

Tanaka, J.C., Eccleston, J.F. and Barchi, R.L. (1983) Cation selectivity characteristics of the reconstituted voltage-dependent sodium channel purified from rat skeletal muscle sarcolemma. *J. Biol. Chem.* 258: 7519–7526.

Tyerman, S.D., Terry, B.R. and Findlay, G.P. (1992) Multiple conductances in the large K^+ channel from *Chara corallina* shown by a transient analysis method. *Biophys. J.* 61: 736–749.

Urry, D.W., Alonso-Romanowski, S., Venkatachalam, C.M., Bradley, R.J. and Harris, R.D. (1984) Temperature dependence of single channel currents and peptide liberation mechanism for ion transport through the gramicidin A transmembrane channel. *J. Membr. Biol.* 81: 205–217.

Zanello, L.P., Aztiria, E. and Barrantes, F.J. (1993) Temperature sensitivity of a plant voltage-gated channel and a ligand-gated neurotransmitter receptor channel. *Biophys. J.* 64: A328.

Zanello, L.P. and Barrantes, F.J. (1992) Blockade of the K^+ channel of *Chara contraria* by Cs^+ and tetraethylammonium resembles that of K^+ channels in animal cells. *Plant Sci.* 86: 49–58.

(Received September 16, 1993; Accepted December 3, 1993)