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Temperature Sensitivity of the Tonoplast Ca^{2+}-Activated K^+ Channel in *Chara:* The Influence of Reversing the Sign of Membrane Potential

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Abstract. A detailed temperature dependence study of a well-defined plant ion channel, the Ca^{2+}-activated K^+ channel of *Chara corallina,* was performed over the temperature range of their habitats, 5–36°C, at 1°C resolution. The temperature dependence of the channel unitary conductance at 50 mV shows discontinuities at 15 and 30°C. These temperatures limit the range within which ion diffusion is characterized by the lowest activation energy (E_a = 8.0 ± 1.6 kJ/mol) as compared to the regions below 15°C and above 30°C. Upon reversing membrane voltage polarity from 50 to −50 mV the pattern of temperature dependence switched from discontinuous to linear with E_a = 13.6 ± 0.5 kJ/mol. The temperature dependence of the effective number of open channels at 50 mV showed a decrease with increasing temperature, with a local minimum at 28°C. The mean open time exhibited a similar behavior. Changing the sign of membrane potential from 50 to −50 mV abolished the minima in both temperature dependencies. These data are discussed in the light of higher order phase transitions of the Characean membrane lipids and corresponding change in the lipid-protein interaction, and their modulation by transmembrane voltage.

Key words: Ca^{2+}-sensitive K^+ channel — Temperature — Thermodynamics — *Chara* — Voltage sensitivity — Lipid phase transition

Introduction

Many membrane processes exhibit discontinuous behavior with a change in temperature. It is intriguing that these discontinuities occur at approximately the same temperatures (around 15 and 30°C) regardless whether one observes short-term or long-term phenomena. Both macroscopic phenomena such as a growth rate of a mung bean hypocotyl (Raison & Chapman, 1976) and metabolic heat rate of tomato cells (Hansen et al., 1994) and short-term reactions characteristic for the functioning of a membrane, such as various oxidative activity of mitochondria (Raison & Chapman, 1976; Raison, Chapman & White, 1977), β-galactoside and β-glucoside transport in *E. coli* (Thilo, Träuble & Overath, 1977), the reflection coefficient of chloroplast membrane permeability to different alcohols (Nobel, 1974), or the bioelectric potential of *Valonia* (Thorhaug, 1971) exhibit discontinuities in temperature dependence. Several authors have correlated these critical temperatures to the structural reorganization of membrane lipids (Raison & Chapman, 1976; Thilo et al., 1977; Hansen et al., 1994).

Though temperature is an important factor for plant growth (Simon, 1981), only few studies have been focused on the effect of temperature on gating and conductance processes of the plant membrane single ion channels (Tyerman, Terry & Findlay, 1992; Colombo & Cerana, 1993; Zanello & Barrantes, 1994; Ilan, Moran & Schwartz, 1995). Animal cells have been more frequently studied (Grygorczyk, 1987; Correa, Bezanilla & Latorre, 1992; McLarnon, Hamman & Tibbits, 1993; Rodriguez & Bezanilla, 1996), but discontinuities in temperature dependence of either conductance or kinetics have not been reported, except at 20°C for the mean open time of ATP-dependent potassium channels in cardiac myocytes (McLarnon et al., 1993). In contrast, a multiphase behavior of ion-channel current with a change of temperature has been demonstrated in a whole-cell patch-clamp study on *Arabidopsis thaliana* (Colombo & Cerana, 1993). It has been shown that the critical temperature range for the activity of three different K^+ channels lay between 15 and 20°C and phase

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transitions of membrane lipids were implicated. Unfortunately, the study was performed in a rather limited temperature range (11–22°C). In a patch-clamp study on guard-cell protoplasts of *Vicia faba* (Ilan et al., 1995) a wider temperature range was examined (13–36°C), but with a rather crude temperature resolution (7–8°C). Two types of K^+ channel behaved differently, one showing a monotonic dependence on temperature while the temperature function of the other channel showed a break at 20°C. The temperature sensitivity of the Ca^{2+}-activated K^+ channel in Characean tonoplast also has been investigated (Tyerman et al., 1992; Zanello & Barrantes, 1994). A thermodynamic analysis of the activity of this channel has been performed, but again with a relatively low temperature resolution (5°C) and, due to a limited ability to maintain the giga-seal at higher temperatures, in a somewhat limited temperature range, 3–25°C (Zanello & Barrantes, 1994). An increase in temperature resulted in a decrease of the probability of the channel opening. Further thermodynamic analysis revealed that the channel closure appears to have the highest energetic requirements and that the closing rate exhibits a less negative entropic change as compared to the more ordered open state. Similar findings have been reported for the Na^+ channel in the squid giant axon (Correa et al., 1992) or the *Shaker* K^+ channel (Rodriguez & Bezanilla, 1996). Tyerman and coworkers (1992) showed that temperature induces a change in the occupancy of different conductance substates of the Ca^{2+}-dependent K^+ channel in *Chara.* In neither of these two reports breaks in temperature functions of any single-channel parameter have been recorded. In contrast, a study on the temperature dependence of the unitary current of the K^+ channel in the cytoplasmic droplet of a charophyte alga, *Nitellopsis obtusa*, revealed multiphasic behavior with breaks at 10 and 27°C (Pottosin, 1990).

The lack of discontinuities may simply arise from an inadequate temperature resolution employed in aforementioned studies and/or particular experimental conditions (e.g., Zanello & Barrantes, 1994, have used negative potentials to scan single-channel activities at different temperatures, whereas preliminary data on *Nitella* by Pottosin (1990) that showed breaks in temperature dependence of the channel current at around 15 and 30°C have been measured at positive potentials). Apart from the question of adequate temperature resolution, some discrepancies may also arise as a consequence of the rate of temperature change. For example, measurements of the metabolic heat rate (Hansen et al., 1994) showed that the breaks can be observed only when experiments were performed under conditions of thermodynamic equilibrium, i.e., using a rather slow temperature scan rate (2°C/hr). On the other hand, most of the experiments in reports cited above have been performed using a relatively fast rate of temperature change, i.e., under nonequilibrium conditions. Nonetheless, discontinuities have been observed. In other words, observation of certain discontinuities in the reaction rate of membrane functions may depend on the relative ratio of the reaction rate of particular physiological parameter *vs.* the rate of temperature change. In our experiments both high temperature resolution and isothermal conditions were used. It therefore appears that temperature induces discontinuities in both short-term and long-term events of membrane functioning; hence their origin should be sought at the level of the structure-function relationship between membrane proteins and their lipid environment.

We studied the Ca^{2+}-activated K^+ channel in Characean tonoplast over a temperature range that encompasses the environmental temperature range of the Characean habitat (5–36°C), with resolution of 1°C per datum, to establish whether discontinuities in temperature behavior of single-channel parameters exist. Further, we tried to explain the existence of the breaks in the temperature dependence of the single-channel conductance by employing a combination of diffusion and chemical reaction processes. We also tried to correlate all the single-channel parameters measured with those previously reported for water transport in *Chara* (Andjus et al., 1987, 1999) or other membrane phenomena in plants (Thorhaug, 1971; Raison & Chapman, 1976; Thilo et al., 1977; Hansen et al., 1994) through the influence of the structural organization of membrane lipids. The influence of the sign of the membrane potential on the overall characteristics of temperature dependence of single-channel conductance and kinetics was investigated.

Materials and Methods

Experimental Object

The object of investigation was the tonoplast membrane of the freshwater alga *Chara corallina.* The alga was grown on sterilized sand in aquariums filled with dechlorinated tap water. Water temperature was maintained at 22 ± 2°C. The light regime was 12:12 hr and the light intensity was 2 Wm^{-2}.

The preparation of the cytoplasmic droplets (presumably bordered by vacuolar membrane only) was performed according to Bertl (1989). In short, both ends of the internodal cell were cut and perfused with an iso-osmotic experimental solution (in mM): 150 KCl, 0.9 EGTA, 0.5 citric acid, 1.3 $CaCl_2$ −0.15 free Ca^{2+}, 20 HEPES-KOH, pH 7.2). The cytoplasmic droplets, 30–60 μm in diameter, were isolated almost instantaneously.

Electrophysiology

Single Ca^{2+}-activated K^+ channel activity was measured in inside-out patches. This configuration is convenient for scanning of the temperature dependence since it is mechanically more stable and persists for a longer time period than other single-channel measuring configurations.

The micropipettes for patch-clamp recordings were made from thick wall borosillicate glass capillaries (0.86 mm i.d.; Clark Electro-

medical Instruments, UK). After filling the micropipettes with the internal solution (in mM): 150 KCl, 0.9 EGTA, 0.5 citric acid, 1.1 $CaCl_2$ −0.05 free Ca^{2+}, 20 HEPES-KOH, pH 7.2 the resistance of microelectrodes was 10–20 MΩ.

The channel activity was monitored in the temperature region 5–36°C at membrane potentials, V_m, of 50 and −50 mV. Single-channel currents were measured by means of an EPC9 patch-clamp amplifier (HEKA Elektronik, GmbH, Germany) controlled by the EPC9 SCREEN (HEKA) acquisition program installed on an Atari Mega ST-4 minicomputer. Acquisition was performed at a frequency of 2 kHz. Additional filtering was not employed because the observed signal-to-noise ratio (SNR) was sufficient for reliable discrimination of short events (~1 msec) from noise. Analysis was performed on 20 sec records usually containing about 2000 events. Single channel recordings were analyzed by the half-threshold technique (Colquhoun & Sigworth, 1983), implemented in the TAC software on the Atari computer (Instrutech, NY).

The sign of the membrane potential corresponds to the convention: luminal side (pipette in inside-out patches) is zero.

Temperature Control

The temperature in the experimental chamber was controlled by means of a system consisting of a Peltier element with a temperature control unit and two temperature sensors (Luigs & Neumann, Germany), one placed in the experimental bath and the other in the chamber holder. Starting from 20°C (the temperature at which all patches were isolated), the temperature was changed in 5°C steps in either heating or cooling direction. Prior to the data acquisition, the isolated patches were kept at the selected temperature for 15 min. Whenever the patch persisted, after reaching the outer limits of experimental temperatures (i.e., 5 or 36°C), a thermal scan in the opposite direction was performed in the same manner, but temperature points at which measurements were performed were shifted by 2–3°C, hence additional temperatures were covered in the same thermal region. The temperature intervals were also shifted in the same manner for different patch samples. Consequently, pooling these data yielded a temperature resolution of 1°C.

Pooling together data points obtained from cooling and heating cycles appears justified since hysteresis was not observed. Values for single-channel currents at 50 mV (mean ± SD), at 15 and 29°C (temperature of breaks) were: heating *vs.* cooling = 7.07 ± 0.23 *vs.* 7.09 ± 0.33 pA (15°C), and 9.4 ± 1.1 *vs.* 8.2 ± 0.9 pA (29°C) (n = 2–4).

Data Analysis

Activation energies (E_a) were obtained from Arrhenius plots by fitting the data to the equation:

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

where k is the rate constant (or a related measured parameter), A is the Arrhenius constant, R is the gas constant, and T is the absolute temperature.

Enthalpy (ΔH_a) and entropy (ΔS_a) of activation were calculated from the Eyring's transition states theory (Eyring, 1935) by fitting the data to the equation:

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

where k_b is the Boltzmann constant and h is the Plank constant. The temperature coefficient, Q_{10}, of single-channel parameters was calculated as a ratio of the values of a quantity over a 10°C change in temperature.

The data are presented as mean ± SE with the number of individual patches in brackets. The values obtained by fitting are presented with the standard error of the fit.

The significance ($P < 0.05$) of discontinuities was assessed by an analysis of variance (Anova) test, which tested the hypothesis that means from two groups of samples (data points at the minimum *vs.* data points limiting the region of the minimum) are equal.

Results

Single Channel Characteristics

The following results show that the studied channel is the previously well-described Ca^{2+}-activated K^+ channel. Current recordings from multichannel patches at membrane voltages, V_m, in the range −100 to 100 mV (Fig. 1*A*) showed that the channel was more active at hyperpolarizing potentials. Since multichannel patches are usually the most abundant even when high-resistance microelectrodes are used (Pottosin et al., 1993), the channel activity was analyzed as Np_o, where N is the number of channels in the patch and p_o is the single-channel open probability. Np_o values were averaged over a number of patches (n = 3–5) and plotted against voltage. The solid curve in Fig. 1*B* is the prediction of the effective number of open channels obtained using the Boltzmann distribution:

$$Np_o(V_m) = Np_{o(max)}(\exp[z\delta F(V_{1/2} - V_m)/RT]/(1 + \exp[z\delta F(V_{1/2} - V_m)/RT])) \quad (3)$$

where $Np_o(V_m)$ is channel activity at membrane voltage V_m, $Np_{o(max)}$ is the maximal value of Np_o, $z\delta$ is the effective valence of the gating charge, $V_{1/2}$ is the value of the voltage where half-maximal activation of the channel occurred, and other parameters have their usual meaning. The best fit was obtained for the following values: $V_{1/2}$ = −40.0 ± 12.9 mV, $z\delta$ = 0.32 ± 0.07 and $Np_{o(max)}$ = 1.7 ± 1.6.

The single-channel conductance, G_K, calculated from the slope of the *I/V* curve (Fig. 2*B*), at 20°C was 148 ± 2 pS. Both activation at hyperpolarization (Fig. 1) and large single-channel conductance are characteristic features of the Ca^{2+}-activated K^+ channel in the droplet membrane of *Chara* (Lühring, 1986; Laver & Walker, 1987; Laver, Fairly & Walker, 1989; Laver & Walker, 1991, Pottosin et al., 1993). Values for $V_{1/2}$ and $z\delta$ also correspond well with the values obtained by other authors using a similar preparation (Laver, 1990; Pottosin et al., 1993).

Time histogram analysis revealed the presence of at least one open state (mean open time constant, τ_o, of 8.3 ± 0.9 msec) and three closed states (Fig. 3) in accordance with previous studies on the Characean Ca^{2+}-activated K^+ channel where results were mainly discussed in terms

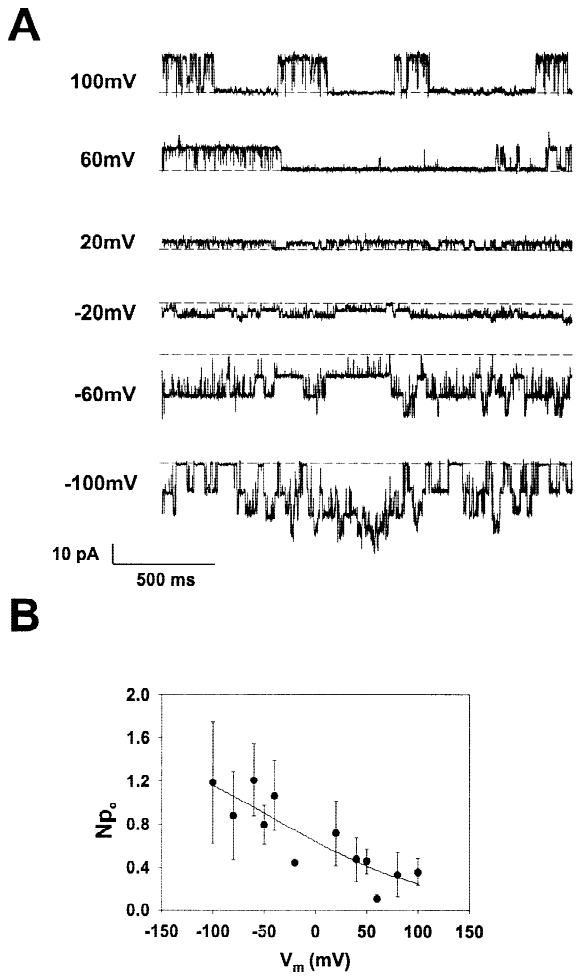

Fig. 1. Voltage dependence of the Ca^{2+}-activated K^+ channel activity at 20°C. (*A*) Original current recordings at different holding potentials (V_m) from a multichannel inside-out patch detached from the droplet membrane of *Chara corallina*. Dashed lines indicate closed channel levels. (*B*) Channel open probability, Np_o (N = number of channels in the patch; p_o = open channel probability) *vs.* voltage. Experimental points are mean values obtained from 3–5 independent inside-out patches. The solid curve is the best fit of the data to the Boltzmann distribution (*see* Results). Values of the half-activation potential, effective gating charge, and maximal number of open channels are $V_{1/2}$ = −40.0 ± 12.9 mV, $z\delta$ = 0.32 ± 0.07, and $Np_{o(max)}$ = 1.7 ± 1.6.

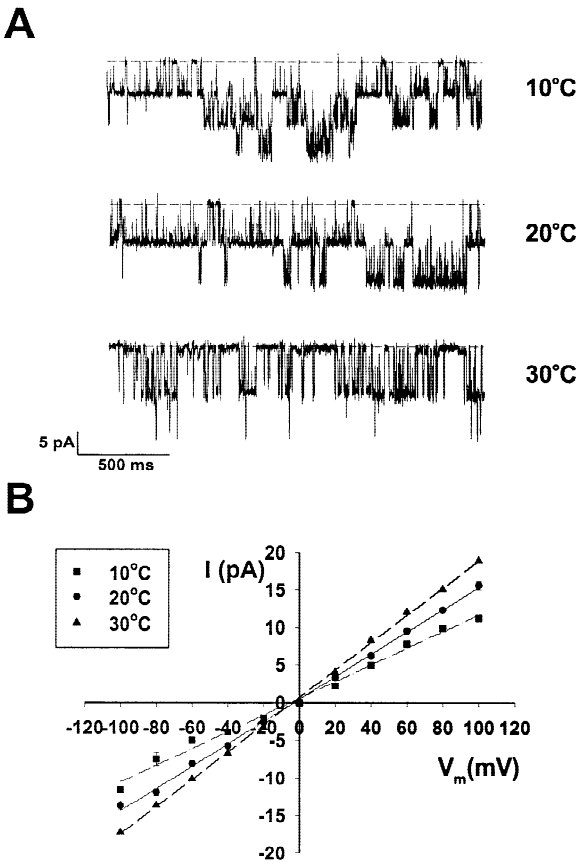

Fig. 2. (*A*) Original single channel recordings from an inside-out patch at 10, 20 and 30°C (as indicated for each trace on the right). Holding potential was V_m = −50 mV. Dashed lines denote the current baseline. (*B*) Voltage dependence of the unitary current of the Ca^{2+}-activated K^+ channel at different temperatures. Experimental points are the mean values obtained at different holding voltages for 3–5 independent inside-out patches. The slope of linear fit of each *I/V* curve gave the single-channel conductance, G_K, of 111 ± 3 pS (r = 0.995; 10°C), 148 ± 2 pS (r = 0.999; 20°C) and 181 ± 1 pS (r = 0.999; 30°C).

of one open state and several closed states (Laver & Walker, 1987). In a number of patches the mean open time distribution could be fitted with two components (1.9 ± 0.3 msec and 12.0 ± 2.1 msec), a possibility also considered by Laver (1990). Nevertheless, the use of the single component fit appears to be more justified both because of the small difference between two mean open time constants and because the relative contribution of these two components to the dwell-time histogram did not change with temperature (50 ± 14% for both components at all temperatures).

We conclude that the single-channel characteristics obtained from experiments in the present study are similar to or identical with the previously described characteristics of Ca^{2+}-dependent K^+ channel in the membrane of Characean cytoplasmic droplets.

Temperature Dependence of Channel Conductance

The rise in temperature induced an increase in single-channel current, *I*, and a change in channel kinetics (Fig. 2*A*). The *I/V* dependence was linear at all temperatures in the range of V_ms from −100 to 100 mV (*see* examples for three temperatures in Fig. 2*B*).

When values of G_K obtained at V_m = 50 mV were plotted against temperature in Arrhenius-type plot, beside the expected increase of G_K with temperature, two breaks appeared at 15 and 29°C (Fig. 4*A*). The region

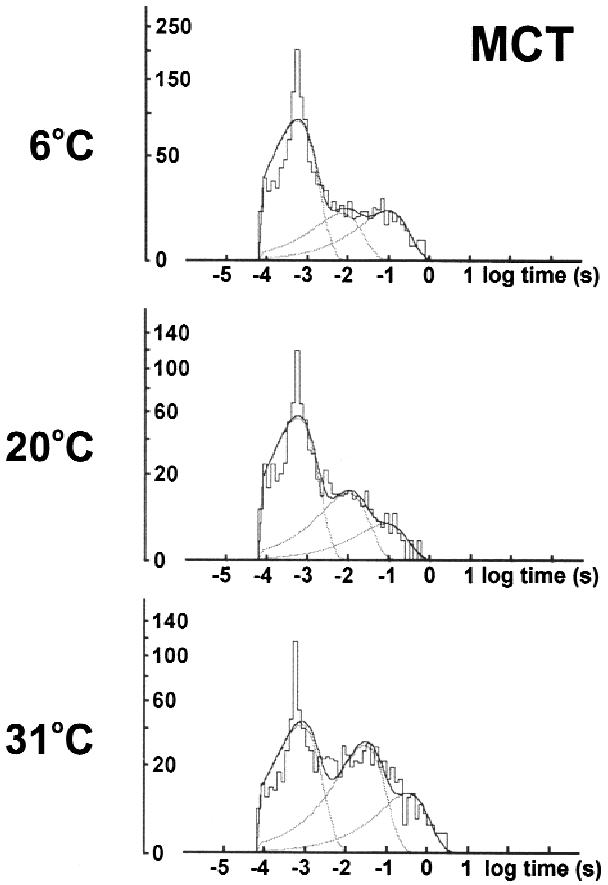

Fig. 3. An example of log-log histograms of channel mean closed dwell times (MCT) from the same patch at $V_m = -50$ mV at three different temperatures. The fitted pdf (probability density function) components are shown by thin lines under the thick line of the total fit. At 20°C MCT constants were 0.7 ± 0.06 msec, 8.7 ± 1.6 msec, and 96.0 ± 25.2 msec ($n = 10$).

between these breaks showed the lowest activation energy (Table 1) when compared to the values of E_a below 15°C and above 30°C. Upon reversing the sign of the membrane potential ($V_m = -50$ mV), the discontinuous temperature dependence was not observed and the temperature dependence of G_K in the entire temperature region is characterized with a single E_a (Fig. 4*B*, Table 1). It should be noted, that the use of Eqs. (1) and (2) to calculate thermodynamic parameters of transport is strictly valid only under equilibrium conditions, i.e., low heating rate or isothermal measurements (Hansen et al., 1994). The rate of change of temperature in our experiment (0.2°C/min), and time of equilibration (15 min) should be sufficient for attaining equilibrium conditions during measurement.

A substate of smaller conductance was often observed at all temperatures, but the frequency of its occurrence was higher at temperatures below 10°C, which is congruent with the observation of Tyerman et al.

Table 1. Thermodynamic parameters of Ca^{2+}-dependent K^+ channel conductance (G_K) at V_m of 50 or −50 mV

		Q_{10}	E_a (kJ/mol)
50 mV	5–14°C	1.28 (5–15°C)	16.5 ± 3.5
	15–28°C	1.12 (15–25°C)	8.0 ± 1.6
	29–36°C	1.31 (30–40°C)	21.5 ± 8.6
−50 mV	5–36°C	1.21 (15–25°C)	13.6 ± 0.5

Q_{10}—temperature coefficient, E_a—activation energy.

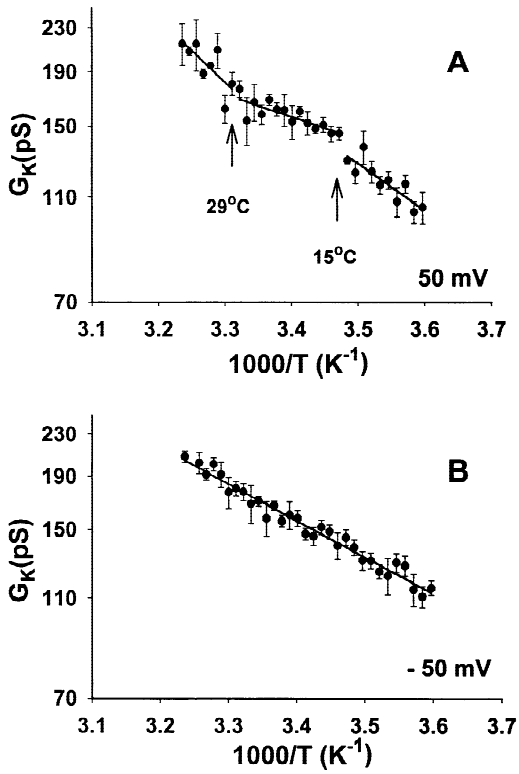

Fig. 4. Arrhenius plots of the unitary channel conductance, G_K, of the Ca^{2+}-activated K^+ channel at two opposite membrane potentials. (*A*) $V_m = 50$ mV; two breaks at 15 and 29°C (arrows) delimit temperature regions with different activation energies (*see* Table 1). (*B*) $V_m = -50$ mV; all data can be fitted with a single E_a of 13.6 ± 0.5 kJ/mol. Data points are mean values (with standard error bars) obtained from 3–15 measurements at each temperature.

(1992). Of 22 patches in the 5–10°C region, in 11 (50%) there was an obvious subconductance activity. The contribution of substate events to the total amplitude histogram was 15.7 ± 8.3% for temperatures from 5 to 10°C. In patches where substate activity was recorded, only the full-level data were used for the Arrhenius plots in Fig. 4.

Temperature Dependence of Channel Kinetics and Open Probability

Current recordings (Fig. 2*A*) indicate prolonged dwelling of the channel in closed states with the increase of tem-

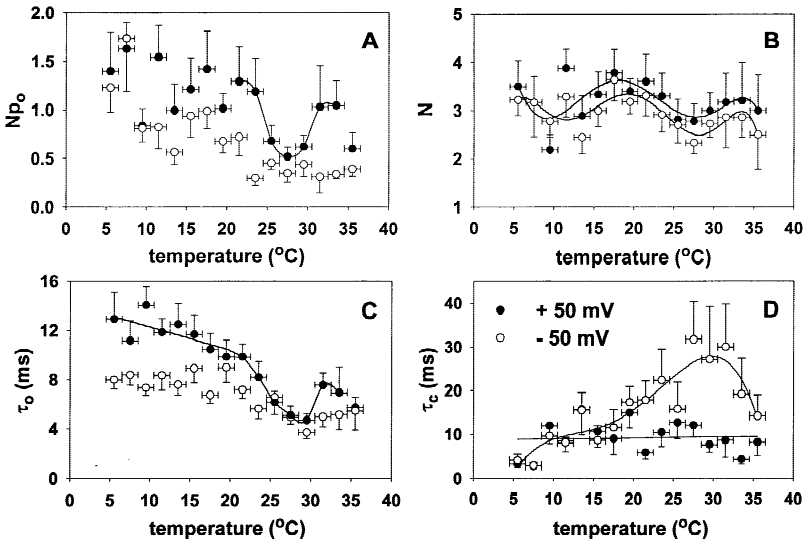

Fig. 5. Temperature dependencies of channel open probability, number of open channels and related dwell times at $V_m = 50$ and −50 mV. (*A*) Temperature dependence of effective number of open channels (Np_o) (calculated as Σ (dwell time × level no.)/total time); (*B*) temperature dependence of the number of open channels in the patch as estimated by the method suggested by Horn (1991). To guide the eye, data points were fit with a 6th order polynomial; (*C*) temperature dependence of mean open time (histograms were calculated as Σ (open dwell time × level no.)/number of opening transitions, where level no. was 1, and fitted by binomial distribution); (*D*) temperature dependence of mean closed time (histogram calculated as Σ (closed dwell time × level no.)/number of closing transitions, where level no. was 1, and fit with the multiple binomial distribution). To guide the eye, data points were fit with a 4th order polynomial. Data points are mean values with vertical standard error bars. Since data points are obtained by averaging data points 1°C apart over 2°C intervals (as indicated by horizontal bars) they are plotted in the middle of the temperature interval. Data points at the minimum are located at 27.5°C (*A*) and 29.5°C (*C*), and at the maximum at 29.5°C (*D*).

perature. Inspection of closed-time histograms (Fig. 3) shows that the increase of mean closed time (MCT) is dominated by the increase of the relative contribution of the intermediate closed-time component.

Figure 5 shows changes of the main kinetic parameters of single Ca^{2+}-dependent K^+ channel with temperature. The plot of channel mean open probability, Np_o, at −50 mV (Fig. 5*A*) showed a monotonic decrease with temperature over the full range of experimental temperatures (5–36°C). The Np_o at $V_m = 50$ mV showed a local minimum between 23.5 and 31.5°C. However, this Np_o minimum was not statistically significant ($P > 0.05$).

The number of open channels in the patch, N, was assessed by counting of the simultaneously open channels, which for $N \leq 4$ is the best statistical test for assessing N (Horn, 1991). Figure 5*B* appears to have two shallow minima around 10–12°C and 28°C for both membrane polarities, however, neither were statistically significant.

The mean open time constant, τ_o, showed the same general change with temperature as Np_o, i.e., a decrease with the increase of temperature and a local minimum between 23.5 and 31.5°C (Fig. 5*C*). At −50 mV the minimum was not statistically significant ($P > 0.05$) as in the case of Np_o, whereas at 50 mV it was ($P < 0.05$). Bellow 23°C, the temperature dependence of τ_o was more pronounced at 50 mV than at −50 mV. The only rate constant that can be inferred directly from these experiments is $\alpha = 1/\tau_o$, assuming that open state is the terminal state in the kinetic scheme (Laver & Walker, 1987; Zanello & Barrantes, 1994). Thermodynamic parameters of α calculated for the region 5–23°C, using Eqs. (1) and (2), show that channel closure entails a large positive enthalpic change (Table 2).

The temperature dependence of channel mean closed time, τ_c, at 50 mV is monotonic over the whole temperature region scanned (Fig. 5*D*). On the other hand, at −50 mV, τ_c exhibits a maximum at around 30°C ($P > 0.05$). Since $p_o = \tau_o/(\tau_o + \tau_c)$, the large scattering of τ_c data is a likely explanation for the negative result of Anova test for the Np_o minimum.

The temperature of the minimum of Np_o and τ_o (28.5°C) coincides well with the high temperature break (29°C) in the Arrhenius plot of G_K (Fig. 4*A*). Also, the disappearance of minima (maxima) at −50 mV is the same kind of behavior that G_K exhibits with the change in polarity of membrane voltage.

Discussion

Temperature Dependence of Channel Conductance

The temperature dependence of G_K (Fig. 4) can be summarized around three aspects: (i) the temperature dependence of G_K at 50 mV has breaks at 15 and 28°C (Fig. 4*A*) but, (ii) temperature dependence of G_K at −50 mV follows a simple Arrhenius type dependence (Fig. 4*B*), and (iii) the calculated activation energies and/or corresponding Q_{10} values (Table 1) are very low over the entire range of experimental temperatures.

The features in Fig. 4*A* can be explained using a single-site model and Michaelis-Menten formalism, assuming that discontinuities in the temperature dependence of G_K are related to phase transitions of the membrane. The kinetic equation that encompasses the entire temperature range can be written as a combination of the diffusion and chemical reaction processes:

$$K^+_{bulk} \xrightarrow{k_d} IonCh + K^+_{en} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} IonChK^+ \xrightarrow{k_2} IonCh + K^+_{ex} \qquad (4)$$

where *IonCh* represents the ion channel as an enzyme, K_{en}^+ and K_{ex}^+ are potassium ions that are entering and exiting the channel, respectively, *IonCh*K^+ is the complex of the channel and bound potassium, k_d is the rate constant for the diffusion, k_1 and k_{-1} are rate constants of binding and unbinding of K^+ ion to the channel protein before crossing the selectivity filter, a step which imposes a single energy barrier (Miller, Stahl & Barrol, 1988), and k_2 is the rate constant of potassium exiting the channel. The latter step is not given as a reversible one, because it can be safely assumed that ions can move in only one direction once passing the selectivity filter. The three regions in Fig. 4*A* then can be explained as follows:

Low temperature region (t < 15°C). The overall conductance is most probably rate-controlled by the diffusion of K^+ ions to the "reaction site," i.e., by k_d. The value of E_a = 16.5 kJ/mol (i.e., Q_{10} = 1.28, Table 1) is almost equal to the activation energy for the aqueous diffusion of small cations (Andersen, 1983; Hille, 1984).

Intermediate temperature region (15°C < t < 28°C). Temperature-induced changes of membrane organization (i.e., phase transition from gel to liquid state) at around 10°C may then lead to the condition where diffusion is supplying a sufficient amount of the substrate [K_{en}^+] so that the "chemical reaction" within the channel becomes the rate-limiting step (a situation similar to the kinetics of heterogeneous catalysis; Laidler, 1989). The conductance G_K in this region is then determined by the rate constants governing a catalytic reaction in Michaelis-Menten fashion (*see also* Gradmann, Kleiber & Hansen, 1987, for treating *I/V* curves of plant K^+ channels with Michaelis-Menten kinetics):

$$G_K = G_{K(max)}[K^+_{en}]/(K_m + [K^+_{en}])$$

and

$$K_m = (k_2 + k_{-1})/k_1 \qquad (5)$$

where $G_{K(max)} = k_2$ [*IonCh*] is the maximum conductance determining the conductivity at very high ion activity (the rate of exit from the channel), while $G_{K(max)}/K_m$ governs the conductance under conditions of low ion activity and can be defined as the rate of entry to the channel (Lattore & Miller, 1983). K_m is the half-saturation activity. Assuming that $k_2 > k_1 > k_{-1}$ (i.e., $k_2 >> k_{-1}$) and that [K_{en}^+] << K_m, G_K is determined by the rate of binding of K^+ ion to the channel protein, i.e., by the single rate constant k_1. The calculated E_a = 8 kJ/mol is then a genuine thermodynamic parameter describing the property of permeation mechanism in the region "between the phase transitions."

High temperature region (t > 28°C). An increase of the value of E_a in this region might be most plausibly explained by a no longer negligible contribution of k_{-1} (possibly due to increased thermal motion of both protein and ion). The higher value of E_a (21.5 kJ/mol) than in the intermediate region then can be attributed to the k_{-1} having a steeper temperature dependence.

Table 2. Thermodynamic parameters of α, rate constant of channel closure, at V_m of 50 or −50 mV

	E_a (kJ/mol)	Q_{10}	ΔH_a (kJ/mol)	ΔS_a (J/molK)
50 mV	21.8 ± 3.5	1.37 (10–20°C)	20.6 ± 3.5	−193 ± 12
−50 mV	15.1 ± 3.2	1.24 (10–20°C)	12.7 ± 3.2	−217 ± 11

Q_{10}—temperature coefficient, E_a—activation energy, ΔH_a—enthalpy of activation, ΔS_a—entropy of activation. E_a, ΔH_a and ΔS_a were obtained by fitting data to Arrhenius and Eyring equations.

A multibarrier single-file pore model proposed by Tester (1988) for the Ca^{2+}-dependent K^+ channel in *Chara*, as opposed to a single-barrier model we used, would require a more complicated discussion. Moreover, the multibarrier single-file model is based on the experiments on voltage-dependent blockade of K^+ channel by Cs^+ and anomalous mole fraction effect, which can be adequately explained by a single-barrier enzyme kinetic model with lazy state (Draber, Schultze & Hansen, 1991). Also, it has been postulated that the reason for the anomalous mole fraction effect is unresolved fast gating that reduces the apparent single channel current in the presence of Tl^+, which has been demonstrated by measuring single-channel currents at a sampling rate of 200 kHz (Farokhi, Keunecke, Hansen, 2000).

The temperature dependence of G_K does not behave symmetrically around zero voltage (Fig. 4*A* and *B*), i.e., no breaks were observed at −50 mV and conduction can be described by a single E_a (Table 1). A similar value for the activation energy of conductance at negative potentials (E_a = 12.3 kJ/mol) as well as a single slope has been obtained for the same channel in a study on *Chara contraria* (Zanello & Barrantes, 1994). This implies that, if the structural changes in the membrane are the origin of the discontinuities, then they do not influence the functioning of the Ca^{2+}-dependent K^+ channel identically at two membrane potentials of the opposite polarity. One should have in mind that the intracellular and extracellular (intra- and extravesicular) part of the structure of the Ca^{2+}-dependent K^+ channel protein is not symmetrical (the same applies to all K^+ channel types; *see* Jan & Jan, 1992; Breitwieser, 1996; Doyle et al., 1998). It is conceivable that structurally different parts of the channel would respond differently to bulk changes of the membrane lipid structure. Consequently, changes of all rate constants that compensate each other and yield a single slope cannot be excluded. On the other hand, the conductance of an open channel has a low Q_{10}

throughout the thermal region scanned (Table 1, *see also* Hille, 1984), which is similar to that for aqueous diffusion of ions, hence it might be difficult to experimentally observe switching between different rate-controlling steps.

The Effect of Temperature on the Kinetics of Channel Closure

The decrease of open channel probability with an increase of temperature (Fig. 5*A*) is a common behavior of ionic channels (Grygorczyk, 1987; McLarnon et al., 1993; Zanello & Barrantes, 1994), with one exception (Haverkampf, Benz & Kohlhard, 1995). Lowering of the open channel probability, p_o, is also described for the Ca^{2+}-dependent K^+ channel in the tonoplast of *Chara contraria* (Zanello & Barrantes, 1994). Temperature dependence of Np_o (Fig. 5*A*) cannot be analyzed in a simple Arrhenius fashion because Eq. (3) predicts a more complicated temperature function. Qualitatively, the minimum at 28°C in the Np_o *vs. T* (Fig. 5*A*) is coincident with the minimum in the dependence of τ_o *vs. T.* Since $p_o = \tau_o/(\tau_o + \tau_c)$, it is conceivable that any change in the behavior of τ_o with temperature will be reflected in the p_o *vs. T.* In addition, dependence of N on temperature also exhibits minima between 10 and 15°C and between 25 and 30°C. Though not significant, these minima will affect the form of the plot of Np_o *vs. T.*

The rate constant of opening and rate constants of leaving farther, longer, closed states cannot be discerned from these experiments because of the multichannel patches. Only the rate constant of channel closure (obtained as the reciprocal of the τ_o) can be calculated. As the open state is short-lived, the channel closure should be fast i.e., its temperature dependence should be small. Q_{10} values for α listed in Table 2 show that α is almost temperature independent. Such low values of Q_{10} for the α rate constant have been reported by Zanello and Barrantes (1994) in a study on *Chara contraria.* The rationale of low energy involvement during channel closure (and probably channel opening) may lay in the fact that the channel is active in "bursts." Fast switching between short open and short closed state cannot involve large changes of channel conformation, because these would have higher energy demands (making and breaking of bonds and interactions). Only the changes of conformation that are happening on the similar time scale as the diffusion can exhibit as a low temperature dependence as the diffusion process. Based on the energetic considerations alone, a change in the permeation energy barrier of 7–17 kJ/mol would be enough to functionally close the ion channel (Sigworth, 1994), which could be achieved with small perturbations in the structure of the selectivity filter. It is possible that even much larger conformational changes of the channel pore helices (Perozo, Cortes & Cuello, 1999) might accommodate for an energy barrier of about 70 kJ/mol (calculated from Table 2 using $\Delta G = \Delta H - T\Delta S$) needed for fast switching during "bursts" of the Ca^{2+}-dependent K^+ channel.

The thermodynamics of channel closure depends on the sign of membrane potential. As can be inferred from Table 2, at −50 mV a smaller energy (enthalpy) change is needed for the closing of the channel, which is accompanied by the more negative change of entropy, as compared to 50 mV. These values imply that the shortest closed state of the channel is more ordered at −50 mV than at 50 mV. If one assumes that the same applies for channel opening (otherwise longer bursts would not be frequent, if possible at all), the thermodynamic parameters are congruent with the channel being more active during hyperpolarization.

The mean closed time, τ_c (which a reflects higher temperature dependence of longer closed states, *see* Results), increases with temperature (Fig. 5) which is in accordance with the previous report on this channel in *Chara* (Zannelo & Barrantes, 1994), and the Ca^{2+}-dependent K^+ channel in human erythrocytes (Grygorczyk, 1987). Correspondingly, τ_o decreases with temperature, as well as Np_o. The rise in the contribution of the middle component in the τ_c histogram (Fig. 3) adds to the overall increase of mean closed time component, τ_c, with temperature. The changing of the contribution of different kinetic components with temperature (*Chara,* Zanello & Barrantes, 1994) is also described for both the *Shaker* potassium channel, with an increase in the intermediate component with the rise in temperature (Rodriguez & Bezanilla, 1996), and for the Ca^{2+}-dependent K^+ channel in human erythrocytes (Grygorczyk, 1987).

The influence of temperature on the single-channel kinetics is opposite at potentials with opposite sign (Fig. 5*C* and *D*). At 50 mV τ_o decreases by a factor of two with a rise in temperature and exhibits a minima at about 30°C, while τ_c is almost independent of temperature. At −50 mV τ_o changes less with temperature than τ_c, which exhibits maximum at around 30°C. This can be explained by a different effect of temperature on the occupancies of open and closed states of the channel (i.e., rate constants that lead to or away from the state) at opposite membrane potentials. The increase of τ_o at 50 mV with lowering the temperature could be attributed to the increased rate constant of opening. But, since the occupancy of the shortest closed state is highest at low temperatures (Fig. 3), the more probable explanation would be that the frequency of bursts increased by increasing the forward rate constant between intermediate and short closed states. At negative potentials the temperature increases the occupancy of intermediate closed state (Figs. 3 and 5*D*) which means that the ratio of rate constants that lead toward the state and rate constants

that lead away from it is increased, while rate constants that affect open state are mostly unchanged.

Reports on the existence of discontinuities in the temperature dependence of channel kinetic parameters in plant cells are scarce. In *Vicia faba* guard cell protoplasts, a drop in the number of both hyperpolarization-activated and depolarization-activated K^+ channels occurs around 20°C (Ilan et al., 1995), resembling a beginning of the minimum in the temperature dependence of Np_o and N around 23°C (Fig. 5*A* and *B*). It is difficult to state whether these discontinuities are analogous with the minima obtained in this report because of the low temperature resolution used in that work.

Nonlinearities in Ion-Channel Transport Characteristics

It has been shown using various techniques that the temperatures of the discontinuities (or breaks) in the reaction rate of different membrane functions coincide with the temperatures of the beginning and the end of the phase transition of membrane lipids. Such a wide transition range (from 15 to 30°C) found in biological systems (Raison & Chapman, 1976; Raison et al., 1977; Thilo et al., 1977; Hansen et al., 1994), corresponding to the data shown in Fig. 4*A*, has been explained by the phase separation and the existence of mixed, fluid + gel, domains within this temperature region. In this region ion-channel conductance exhibited the lowest E_a (Fig. 4*A* and Table 1), possibly due to the "optimal" interaction of the membrane lipids with proteins, which is essential for channel functioning in cells that were grown under conditions "within the phase transition" (i.e., around 22°C). The existence of two thermal lipid phase transitions located around 15–18°C and 30–35°C has been shown by differential scanning calorimetry of plasma membranes isolated from *Chara* grown at 25°C (Beljanski et al., 1997). Also, in algae grown at 10°C and/or 30°C (M.R. Djurišić and P.R. Andjus, *in preparation*), the boundaries of the transition range with the lowest E_a were shifted to encompass the ±10°C range around the new temperature of acclimation.

It has been also shown that a change of electrostatic environment may exert an effect on lipid phase transitions (Träuble & Eibl, 1974). Therefore, the change of the sign of applied potential might alter the structure of the lipid moiety and hence the lipid-protein interactions, and/or directly affect the functioning of channel proteins and consequently their transport properties. However, since discontinuities in transport properties of the *Chara* membrane have also been observed when studying water transport (Andjus et al., 1999), which is supposed to be independent of the changes of membrane potential, it is more likely that differences in transport characteristics at −50 *vs.* +50 mV arise from the intrinsic differences in the structure of the pore mouths on the opposite sides of the membrane (Doyle et al., 1998) and/or their reaction to the bulk change of the membrane fluidity with temperature, rather than the direct effect of applied potential difference.

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