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Inhibition of Inward Rectifying Tonoplast Channels by a Vacuolar Factor: Physiological and Kinetic Implications*

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Summary. Regulation of ion-channel activity must take place in order to regulate ion transport. In case of tonoplast ion channels, this is possible on both the cytoplasmic and the vacuolar side. Isolated vacuoles of young *Vigna unguiculata* seedlings show no or hardly any channel activity at tonoplast potentials >80 mV, in the vacuole-attached configuration. When the configuration is changed to an excised patch or whole vacuole, a fast (excised patch) or slow (whole vacuole) increase of inward rectifying channel activity is seen. This increase is accompanied by a shift in the voltage-dependent gating to less hyperpolarized potentials. In the whole vacuole configuration the level of inward current increases and also the activation kinetics changes. Induction of channel activity takes up to 20 min depending on the age of the plants used and the diameter of the vacuole. On the basis of the estimated diffusion velocities, it is hypothesized that a compound with a mol wt of 20,000 to 200,000 is present in vacuoles of young seedlings, which shifts the population of channels to a less voltage-sensitive state.

Key Words patch-clamp · plant vacuole · single-channel inhibition · kinetics

Introduction

Membranes form the boundaries between different compartments and are responsible for maintaining levels of ion, protein and metabolite concentrations in the various compartments. Ion channels play an important role in regulation and maintenance of these levels.

In vacuoles of higher plants at least three classes of K^+ conducting channels are present (Hedrich, Fluegge & Fernandez, 1986; Hedrich et al., 1988; Hedrich & Schroeder, 1989; Maathuis & Prins, 1990). Transport of several ions is mediated through these channels. In most vacuoles inward rectifying channels (IRC) have been observed that conduct a large range of cations and anions. Besides IRC with a conductance of 60 to 250 pS, sometimes an additional K^+ conducting channel is recorded with a lower conductance. This channel is either inward rectifying (Maathuis & Prins, 1990) or shows no rectification (Hedrich & Neher, 1987). Outward rectifying channels (ORC) have been shown to exist in several higher plant vacuoles and are responsible for K^+ and Na^+ (and probably other cations) transport from vacuole to cytoplasm (Maathuis & Prins, 1989).

The first group of IRC is the best characterized at the present. Its gating is voltage dependent (at positive tonoplast potentials) and a relatively high cytoplasmic Ca^{2+} concentration is necessary to induce maximal open probability (Hedrich & Neher, 1987; Hedrich & Schroeder, 1989; Maathuis & Prins, 1990). In the whole vacuole configuration currents through IRC reach their steady-state level after 300 to 1000 msec and are therefore referred to as slowly activated vacuolar (SV) type channels. The selectivity is low ($P_{K^+}/P_{Cl^-} \approx 4$–6, $P_{K^+}/P_{Na^+} \approx 1$), and these channels are permeable, even to relatively large organic anions like malate (Hedrich et al., 1986). Several physiological functions have been ascribed to this channel such as turgor regulation, transport of photosynthetic products and charge exchange. The open probability of IRC steeply increases with positive tonoplast potentials, though sometimes gating occurs at physiological tonoplast potentials (10 to 30 mV). The voltage range where channel opening occurs is not strictly determined and may depend on several factors, for instance the ion concentrations adjacent to the channel, Ca^{2+} concentrations, etc.

Apart from the tonoplast potential, at the present, cytoplasmic Ca^{2+} concentration is the only known short time-scale regulating factor of the open probability of IRC. Long term regulation may involve mechanisms like phosphorylation (Levitan, 1985; Walsh & Kass, 1988), G-proteins (Schubert et

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al., 1989), inositol phosphates (Alexandre, Lassalles & Kado, 1990), etc., but are still largely unknown regarding tonoplast ion channels.

In young seedlings of *V. unguiculata* cultivars, a factor is present inside the vacuoles that strongly inhibits channel activity. In the vacuole attached configuration hardly any channel activity can be recorded. If the vacuole attached configuration is changed to the whole vacuole configuration, channel activity gradually increases.

In this study we will show results concerning this 'vacuolar inhibiting factor'. Efforts are made to include the results in a kinetic model describing the gating of inward rectifying channels in tonoplast membranes.

Materials and Methods

Plant Material

Seeds of *Vigna unguiculata* L. cv.'s TN-8863, KN-1 and Sativa were germinated and grown on vermiculite. Plants were grown in the greenhouse with the following regime: 50% RH, day and night temperature of 20 and 18°C, respectively.

Isolation of Vacuoles

Stems of *V. unguiculata* cultivars were cut in thin slices with a sharp razor blade, while immersed in the experimental solution. Vacuoles, ranging from 5 to 50 μm in diameter, were spontaneously freed from the tissue and transferred to the experimental chamber.

Experimental Solutions

In most experiments the bath and pipette solution had the following composition (in mM): 10 MES/Tris at pH 7.5, 0.05 $CaCl_2$, 100 KCl or KNO_3, 1 $MgCl_2$. The high cytoplasmic Ca^{2+} concentration ensured good sealing of the tonoplast and maximal channel activity. The osmolarity was adjusted with sorbitol at 500 to 550 mOsmol.

Electrophysiology

Electrodes were prepared from soft glass capillaries (Kimble, Kimax 52); they were pulled on a two-stage puller, coated with Sylgard and heat polished. Electrode resistances varied between 3 and 10 MΩ, depending on the pipette geometry and the solution used. Gigaohm seals between electrode and tonoplast were obtained by gentle suction and usually appeared within a second. The whole vacuole configuration was made with 5 msec pulses of 600 to 1000 mV. Voltage-clamp and current-clamp measurements were done with a List Electronics EPC 7 amplifier. Capacity compensation and series resistance compensation (whole vacuole configuration) were done with circuitry on the amplifier. Pulses and data were transferred via a CED 1401 A/D converter, under control of 'Patch clamp' software (CED, Cambridge, UK). Single-channel data were filtered at 100 to 1000 Hz (8-pole Butterworth characteristics) and whole vacuole measurements at 1 to 100 Hz. Data were sampled at approximately eight times the filter frequencies. Analysis for open/closed time and amplitude distributions were done with the above-mentioned software.

Results

Inhibition of IRC Activity

In Fig. 1 traces are shown of single-channel data in the vacuole attached configuration. In this configuration, *V. unguiculata* vacuoles of young seedlings (3 to 6 days) needed pipette potentials of 90 to 110 mV to induce any channel opening, and no more than three channels were recorded in the patch studied. When the vacuole attached configuration was changed to a whole vacuole configuration, inward currents initially were no more than 25 pA at -50 mV pipette potential (Fig. 2). In time, currents increased to 2800 pA, 10 min after formation of the whole vacuole configuration (Fig. 2, bottom trace). An outside-out excised patch of the same vacuole (which should have an area similar to the vacuole attached patch) showed 8 to 10 active channels at -50 mV pipette potential (Fig. 3). Current-voltage relations, in both the vacuole attached and the excised patch configuration, showed a single-channel conductance of 102 $\pm$ 4 pS. Apparently one or more compounds are present in the vacuoles, which drastically decreased the open probability of ion channels. Alternatively, a factor, essential for gating, is missing and is supplied by the pipette solution. Regardless of which possibility is correct, exchange of the pipette solution for the vacuolar contents caused an increase of channel conducted currents (Fig. 2).

Figure 4 shows the time-dependent increase of inward currents. The sigmoidal shape of the curve can be explained as the result of diffusional exchange. The half time of 'stimulation'—i.e., the time to reach half the maximum current—was about 12 min. Not only are currents increasing (which essentially means that the number of open channels increases), but also the kinetics of opening changed in the sense that the τ of activation becomes smaller after formation of the whole vacuole configuration (Fig. 5), τ being calculated from fitting a single exponential (1-$\exp^{(-t/\tau)}$) to the experimental data. This can also be seen from Fig. 2, by comparing upper and lower traces. Both effects were most significant in young seedlings and tended to disappear in older plants. In Fig. 6 the half time of maximal currents in the whole cell configuration of 14 experiments is

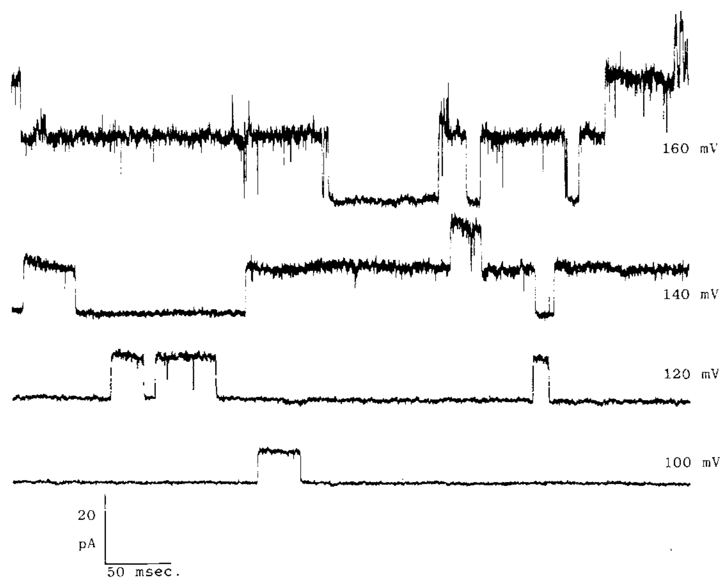

Fig. 1. Single-channel data of inward rectifying channels present in a vacuole-attached patch: Vacuole of a 5 day old *V. unguiculata* cv. Sativa seedling. The single-channel conductance is 102 ± 4 pS. Pipette and bath solution (in mM) 100 KCl, 10 MES/Tris at pH 7.5, 0.05 $CaCl_2$, and 1 $MgCl_2$

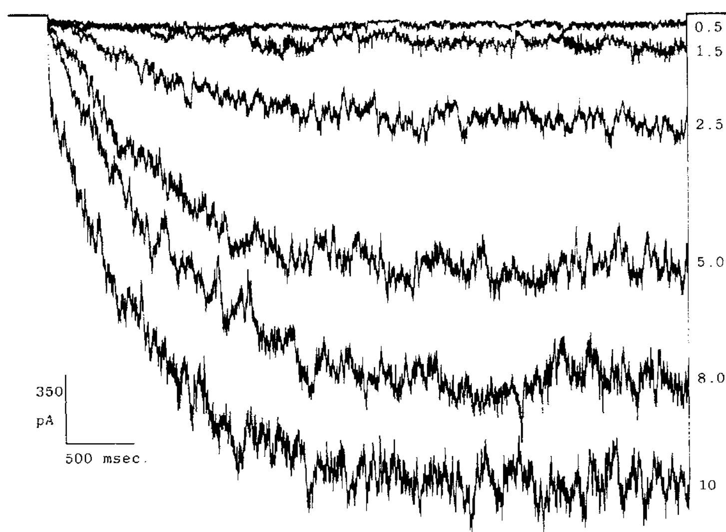

Fig. 2. Whole vacuole inward currents; different time spans after formation of the whole vacuole configuration. A pulse of −50 mV, 4 sec was applied, 0.5 to 10 min after formation of the whole vacuole configuration. The membrane was clamped at 0 mV between subsequent pulses. Solutions were as in Fig. 1

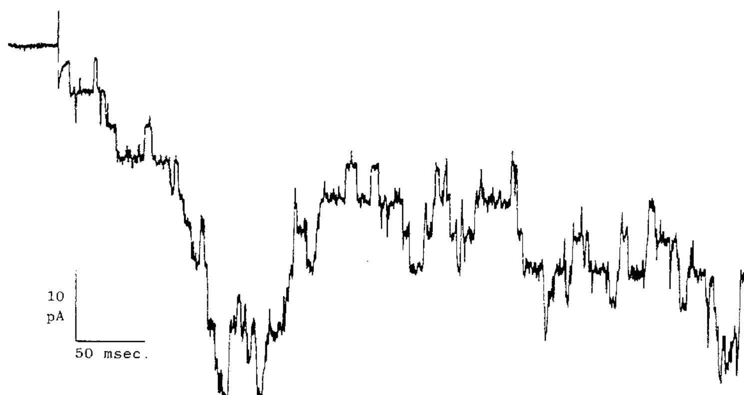

Fig. 3. Single-channel data of an outside-out excised patch. A pipette potential of −50 mV (300 msec) was used to invoke opening of inward rectifying channels. The outside-out patch was excised from the vacuole of Figs. 1 and 2. Solutions were as in Fig. 1

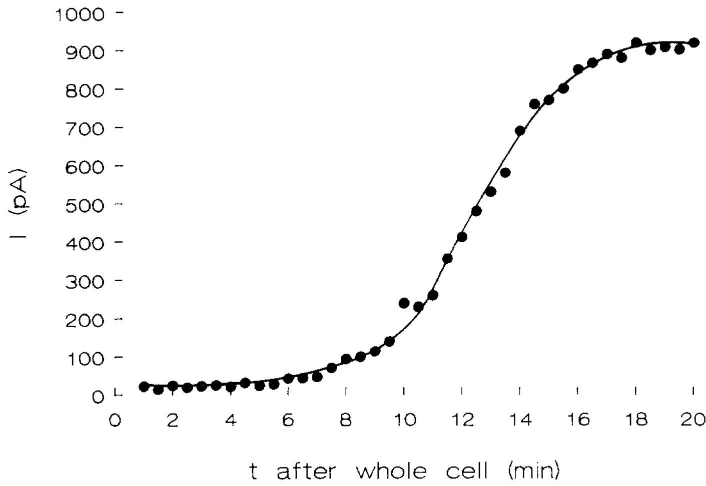

Fig. 4. Time course of inward currents in the whole vacuole configuration. After formation of the whole vacuole configuration every 30 sec a −50 mV, 5 sec, pulse was applied and the inward currents were determined. The half time of stimulation of inward current is approximately 12 min. Similar sigmoidal graphs were obtained from other vacuoles, though the half times of stimulation varied (*see* text). Vacuole diameter was 28 μm

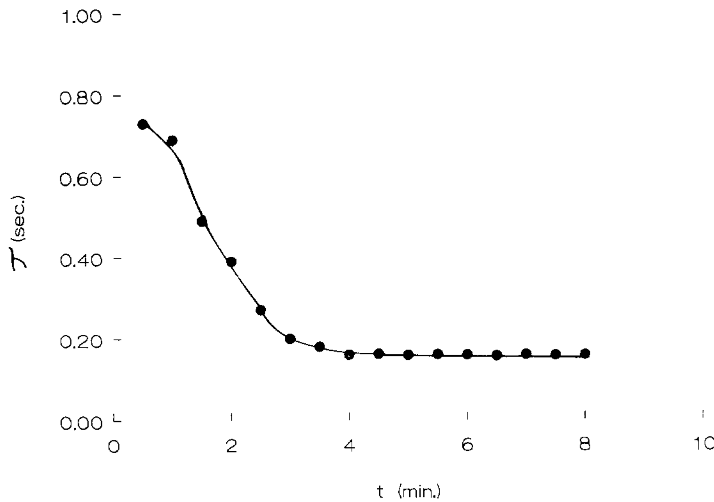

Fig. 5. Decrease of the τ of activation for inward currents, after formation of the whole vacuole configuration. Wash out of the vacuolar contents causes more channels to open, but also a change in activation kinetics. The time constant τ of activation decreased. The same vacuole was used as in Fig. 4

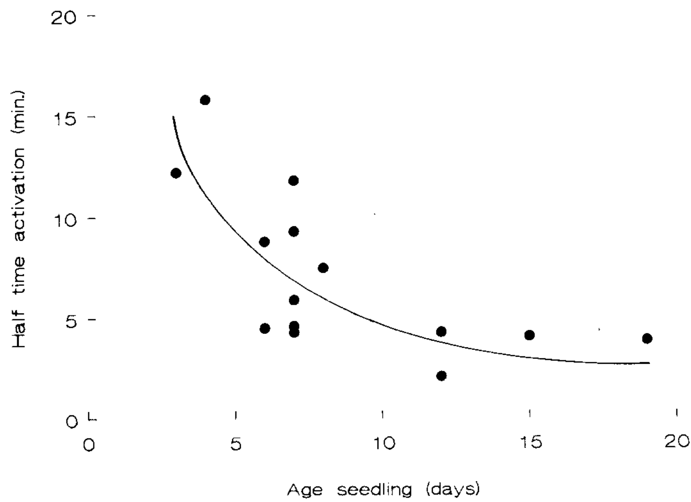

Fig. 6. Vacuolar inhibition in relation to growth of *V. unguiculata* seedlings. Vacuoles isolated from plants of different age were used to determine the half times of stimulation of inward currents. Vacuoles with comparable diameters were used since differences in diameter might affect diffusion times

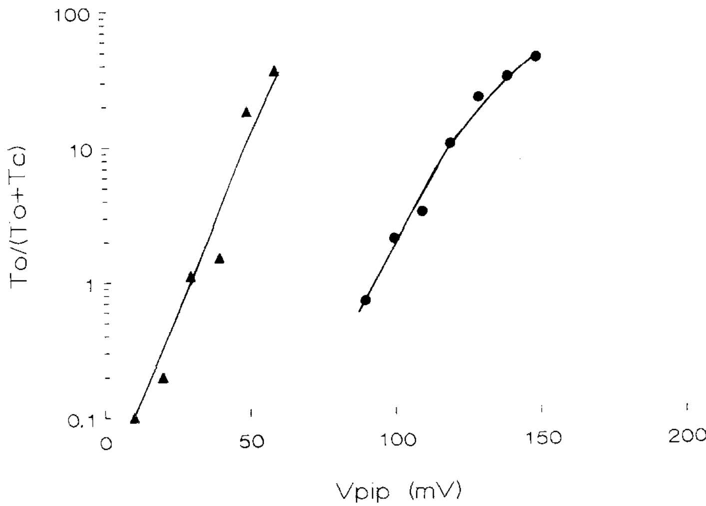

Fig. 7. Voltage dependence of gating probability. Patches of *V. unguiculata* tonoplasts were analyzed for the open time of a single channel, relative to the total sample time (*see* text). (●) *V. unguiculata* vacuole attached patch; about 27 mV change in potential causes a 10-fold increase in channel open time. (▲) *V. unguiculata* inside-out patch; the steepness of the voltage dependence is similar (20 mV per decade), but much smaller pipette potentials are needed to cause gating

plotted *vs.* the plant age. Plants of approximately 20 days and older never showed inhibition of vacuolar IRC. However, the number of successful experiments with these plants was low, since it was very difficult to get good seals in older plants.

Voltage Dependence of Gating and Gating Charge

Vacuoles of young seedlings in the vacuole attached configuration showed hardly any gating; large positive pipette potentials were necessary to invoke channel opening (Fig. 1). Figure 7 shows the relation between potential and open probability for a vacuole attached patch and an inside-out patch configuration of the same vacuole. The open probability is expressed as the fraction open time (%) of the channel, relative to the total sampled time

$$T_o/(T_o + T_c) \cdot 100 \quad (1)$$

in which T_o = total open time and T_c = total closed time. The steepness of the linear part of the curve can be taken as a measure of the gating charge and the voltage dependence of the gating (Hille, 1984). The fraction open time can be expressed according to the Boltzmann equation

$$\frac{T_o}{(T_o + T_c)} = \frac{1}{1 + \exp[+(w - zeE)/kT]} \quad (2)$$

in which w = the change in energy for a conformational change of the protein in the absence of a membrane potential, z = equivalent gating charge, e = elementary charge, E = the electrical potential difference in which the charges move (usually the membrane potential) and k and T have their usual meaning. Since kT/e is approximately 24 mV, a shift in pipette potential (E) of 24 mV, would cause an e-fold (i.e., 2.71) shift in $T_o/(T_o + T_c)$, provided z is 1. In this way, an estimate of the gating charge (z) can be calculated by substituting in Eq. (2) the change in pipette potential, necessary to cause an e-fold change in the fraction open time. In Fig. 7 an e-fold increase in $T_o/(T_o + T_c)$ requires about 6.6 mV change in pipette potential (cell attached patch configuration), and 6.0 mV in the inside-out patch configuration. This gives a minimal value for z of 24/6.6 = 3.6 and 24/6.0 = 4, (the value of z becomes larger if the gating charges don't move through the entire membrane). In both configurations a comparable charge is moved through the electrical field over the membrane every time a channel opens. This indicates that changing the vacuole attached configuration to an excised patch configuration does not change the voltage dependence of the gating but merely causes a large shift in the 'threshold potential,' i.e., the potential at which the first gating appears. Comparing the data for the vacuole attached and the inside-out patch, Fig. 7 shows that the threshold potential shifts towards a more physiological tonoplast potential, analogous to a decrease in w (Eq. (2)).

An alternative way of estimating the number of gating charges comes from the Hodgkin-Huxley theory (Hille, 1984; Hodgkin & Huxley, 1952; Schroeder, 1989). This theory shows that 'n' moving gating charges can be represented by an equivalent reaction scheme with 'n + 1' states. For instance, if n = 3

$$C \underset{a_{21}}{\overset{a_{12}}{\rightleftharpoons}} C \underset{b_{21}}{\overset{b_{12}}{\rightleftharpoons}} C \underset{c_{21}}{\overset{c_{12}}{\rightleftharpoons}} O \quad (3)$$

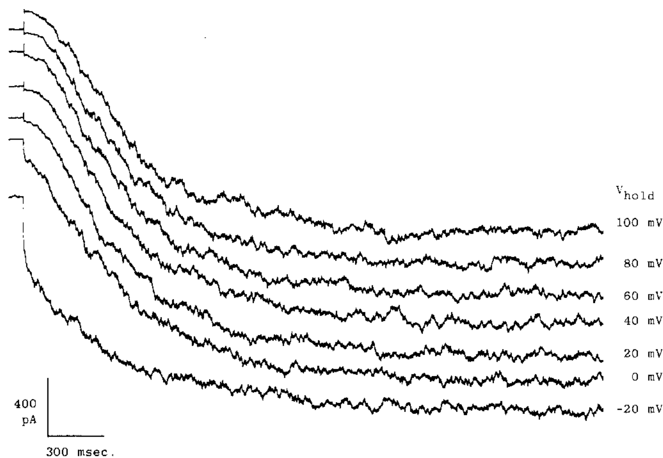

Fig. 8. Inward current activation at different holding potentials. A vacuole of *V. unguiculata*, in the whole vacuole configuration, was clamped at holding potentials ranging from 100 to −20 mV, and subsequently clamped at −50 mV for 4 sec to open channels. Graphs were fitted with a higher order exponential equation (*see* text) in order to determine the number of gating charges (Table)

Table. Order of the Hodgkin Huxley equation at different holding potentials[a]

V_{hold} (mV)	Order of exponential '*n*'
100	3.14
80	2.85
60	2.34
40	2.40
20	2.06
0	1.35
−20	1.08

[a] Whole vacuoles in symmetrical 100 mM KCl solutions were clamped at different holding potentials. The current traces (Fig. 8) were fitted by an exponential equation (*see* text).

where *C* is a closed state, *O* is the open state and *a*, *b* and *c* are (voltage dependent) rate constants of the transitions. If the subsequent transitions are 1st order processes, the reaction scheme may be expressed mathematically by a higher order exponential equation

$$I_t = I_l + I_{max}(1 - \exp(-t/\tau))^n \quad (4)$$

where I_t = current at time t, I_l = 'leak' current, I_{max} = current at $t = \infty$, τ is the time constant of activation and n is the number of gating charges.

Fitting of the experimental data will reveal a number of gating charges depending on the state of the kinetic scheme the channel population is in. Figure 8 shows whole vacuole recordings which were fitted with the above equation. Double pulse protocols were used with the first pulse (holding potentials) in the range of 100 to −20 mV (pipette potential); the second pulse was −50 mV in all traces. Clearly the sigmoidal shape of the curve disappears at more negative pipette potentials, leaving only a single relaxation time if the holding potential becomes negative. The Table shows the values of the power '*n*' derived from fitting the whole vacuole recordings in Fig. 8, with Eq. (4). The value of '*n*' becomes no larger than 3.14. This value was lower than that calculated from the Boltzmann equation, but it still remained in the same range.

Discussion

In intact stem vacuoles of *V. unguiculata,* the inward rectifying channel activity is very low. This closure of tonoplast channels, is most significant in young plants (3 to 7 days), but never complete since it can be overcome by high pipette potentials. In the whole vacuole configuration inhibition slowly disappears with half times up to 20 min. Half times were correlated to the vacuole volume as well as to the age of the used seedling (Fig. 6).

The finding that after formation of a whole vacuole configuration inhibition of channel activity is lost can be explained in two ways: (i) a compound in the vacuole which causes closure is washed out, or (ii) a factor in the vacuole necessary for activation is lacking, which is supplied by the pipette solution. In the pipette solution, only KCl, $CaCl_2$ and $MgCl_2$ are present that could play a role in stimulation of channel activity, assuming sorbitol and the Tris/MES buffer are inert. Experiments with lower KCl (1 mM) or different Mg^{2+} and Ca^{2+} concentrations, showed essentially the same inhibition (results *not shown*), as did lower pH values of the pipette solution. Above that, diffusion of these ions from pipette to vacuole would be a matter of (milli)seconds. Our conclusion, therefore, is that some regulating factor is present on the vacuolar side, which is washed out after formation of the whole vacuole configuration or lost after excision of a membrane patch.

By using Fick's laws of diffusion and the Stokes equation for migration of spherical particles, a rough estimate of the molecular weight can be made for

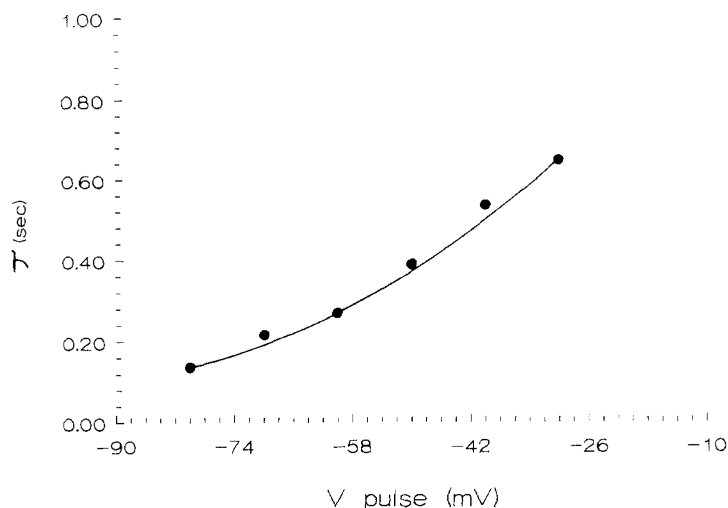

Fig. 9. Voltage dependence of the activation time constant. The τ of activation of inward rectifying channels was determined by application of a 10 mV holding potential (channels closed) and subsequent negative pulses to cause channel opening

the inhibiting factor (Hille, 1984). Diffusion coefficients of 10^{-7} to 10^{-8} m/sec have to be assumed to account for half times of 10 to 15 min over the distances involved here (10 to 100 μm). This corresponds with mol wt of 20,000 to 200,000, for a spherical particle, assuming linear diffusion and no interference from factors like cell shape, series resistance, etc.

The effect of the inhibiting factor is a decreased number of channels opening at a certain pipette potential and a change in τ of activation (Fig. 5). The number of gating charges and the voltage dependence, i.e., the change in $T_o/(T_o + T_c)$ per mV, are not affected. This means that a, b and c in Eq. (3) remain constant, but an additional, voltage-independent rate constant is changed. If an additional state is assumed with a voltage-independent equilibrium constant $K = k_{12}/k_{21}$,

$$C \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C \underset{a_{21}}{\overset{a_{12}}{\rightleftharpoons}} C \underset{b_{21}}{\overset{b_{12}}{\rightleftharpoons}} C \underset{c_{21}}{\overset{c_{12}}{\rightleftharpoons}} O \tag{5}$$

changes in K would cause a shift of the channel population to the left side of the reaction mechanism (inhibition) or the right side (activation). Both observed effects of the inhibiting factor— a decreased number of channels opening and increased τ of activation —can be explained by the above reaction mechanism.

Mechanistically this can be imagined by assuming that gating charges are more sensitive to the membrane electrical field, in case the inhibiting factor is absent; in that case limited membrane potentials cause gating of the channel. In the presence of the inhibitor, a— voltage independent —protein transition, which is catalyzed by the inhibiting factor, causes a decreased voltage sensitivity of the gating charges. In that case, gating charges would 'sense' the same electrical field (so the voltage dependence of the gating remains the same), but since w (Eq. (2)) is much larger, higher potentials will be needed to cause gating events. The τ of activation is also voltage dependent (Fig. 9), hence it will change as well as K (Eq. (5)) changes.

Since the kind of tonoplast channels involved here are usually thought to play a role in turgor regulation (Hedrich & Schroeder, 1989), inhibition of these channels in vacuoles of young *V. unguiculata* plants may reflect physiological aspects of young cells such as uptake of large amounts of ions to build up turgor and cause cell stretching. On the other hand, in older tissue redistribution of ions via inward rectifying tonoplast channels may become necessary and activation of these channels takes place. Thus the inhibition described here might play a role in the long-term regulation of channel activity and therefore regulate ionic fluxes between vacuole and cytoplasm. If this is true, these fluxes are varied, not by changing the membrane potential, but rather by changing the voltage sensitivity of the ion channel. This may be envisioned by assuming modification of the ion channel 'voltage sensor' (Catterall, 1988; Hille, 1984) to a less sensitive (inhibition) or more sensitive (activation) state. This modification involves the (voltage independent) conformational change of the channel protein.

The inhibition of IRC channel activity was not restricted to *V. unguiculata* cultivars as it was also sometimes recorded in experiments using vacuoles isolated from *Allium cepa* bulb scales.

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