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Permeation of Ca^{2+} through K^+ Channels in the Plasma Membrane of *Vicia faba* Guard Cells

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Summary. The whole-cell patch-clamp method has been used to measure Ca^{2+} influx through otherwise K^+-selective channels in the plasma membrane surrounding protoplasts from guard cells of *Vicia faba*. These channels are activated by membrane hyperpolarization. The resulting K^+ influx contributes to the increase in guard cell turgor which causes stomatal opening during the regulation of leaf-air gas exchange. We find that after opening the K^+ channels by hyperpolarization, depolarization of the membrane results in tail current at voltages where there is no electrochemical force to drive K^+ inward through the channels. Tail current remains when the reversal potential for permeant ions other than Ca^{2+} is more negative than or equal to the K^+ equilibrium potential (−47 mV), indicating that the current is due to Ca^{2+} influx through the K^+ channels prior to their closure. Decreasing internal $[Ca^{2+}]$ (Ca_i) from 200 to 2 nM or increasing the external $[Ca^{2+}]$ (Ca_o) from 1 to 10 mM increases the amplitude of tail current and shifts the observed reversal potential to more positive values. Such increases in the electrochemical force driving Ca^{2+} influx also decrease the amplitude of time-activated current, indicating that Ca^{2+} permeation is slower than K^+ permeation, and so causes a partial block. Increasing Ca_o also (i) causes a positive shift in the voltage dependence of current, presumably by decreasing the membrane surface potential, and (ii) results in a U-shaped current-voltage relationship with peak inward current *ca.* −160 mV, indicating that the Ca^{2+} block is voltage dependent and suggesting that the cation binding site is within the electric field of the membrane. K^+ channels in *Zea mays* guard cells also appear to have a Ca_i- and Ca_o-dependent ability to mediate Ca^{2+} influx. We suggest that the inwardly rectifying K^+ channels are part of a regulatory mechanism for Ca_i. Changes in Ca_o and (associated) changes in Ca_i regulate a variety of intracellular processes and ion fluxes, including the K^+ and anion fluxes associated with stomatal aperture change.

Key Words: K^+ channel · Ca^{2+} channel · selectivity · permeation · plant · *Vicia faba*

Introduction

The concentration of free calcium ions in the cytoplasm of plant cells (Ca_i) is known to vary up to 10-fold during cell function (e.g., Miller & Sanders, 1987). Variations in Ca_i regulate intracellular processes such as protein phosphorylation (Blowers & Trewavas, 1989), calcium-binding proteins (Marme, 1989) and gene expression (Guilfoyle, 1989; Braam & Davis, 1990). In guard cells, variations in Ca_i are thought to transduce extracellular signals into the ion fluxes that regulate cell turgor and hence stomatal aperture. For example, abscisic acid, a plant hormone released under water stress, can induce a transient rise in Ca_i in guard cells (McAinsh, Brownlee & Hetherington, 1990; Schroeder & Hagiwara, 1990*a*; but *see* Gilroy et al., 1991) and induces stomatal closure. Elevation of the external Ca^{2+} concentration (Ca_o) also increases Ca_i (Gilroy et al., 1991) and induces stomatal closure or reduces stomatal opening in response to light or K^+ (De Silva et al., 1985; Schwartz, 1985; Inoue & Katoh, 1987; Schwartz, Ilan & Grantz, 1988). Electrophysiological data show that an increase in Ca_i can decrease channel-mediated K^+ influx (required for stomatal opening; Schroeder & Hagiwara, 1989) and open channels mediating anion efflux (required for stomatal closure; Keller, Hedrich & Raschke, 1989; Hedrich, Busch & Raschke, 1990; Schroeder & Hagiwara, 1990*b*).

Measurements using fluorescent indicators show that 'resting' levels for Ca_i in guard cells vary between 70 and 250 nM in *Commelina* (McAinsh et al., 1990; Gilroy et al., 1991), where stomatal closure is triggered if Ca_i exceeds 600 nM (Gilroy, Read & Trewavas, 1990; Gilroy et al., 1991). Ca_i may increase by release from intracellular stores (Hepler & Wayne, 1985; *see also* Gilroy et al., 1990) and/or by influx across the plasma membrane (Schroeder & Hagiwara, 1990*a*). Even if Ca_i regulation during

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stomatal function depends wholly on the release and sequestration of Ca^{2+} by internal stores, Ca^{2+} influx across the plasma membrane is required at some point to maintain Ca^{2+} supply, as outwardly directed Ca^{2+}-ATPases also operate in this membrane (Rasi-Caldogno, Pugliarello & de Michelis, 1987; Rasi-Caldogno, Olivari & de Michelis, 1989; *see also* Briskin, Gidensoph & Basu, 1990). Ca^{2+} influx need only be small to alter Ca_i, given the relatively slow rate of extrusion by the ATPase and the low concentration of Ca^{2+} in the cytoplasm. Given millimolar concentrations of extracellular Ca^{2+}, this low internal concentration creates a large electrochemical force to drive Ca^{2+} inward, and so Ca^{2+} influx depends only on the availability of a pathway for traversing the membrane.

In animal cells, several types of calcium-selective channels in the plasma membrane have been well characterized according to their tissue and species specificity, kinetics and pharmacology (e.g., Fox, Nowycky & Tsien, 1987; *see also* Hille, 1984). In contrast, very little is known about the *mechanism(s)* by which Ca^{2+} enters plant cells and in particular guard cells. In the Charophyte, *Nitellopsis*, it is known that channel-mediated Ca^{2+} influx precedes action potentials (Lunevsky et al., 1983; Shiina & Tazawa, 1987; *see also* review by Tester, 1990). Further, channels of unknown ion selectivity but bearing some resemblance in kinetics and voltage dependence to 'L-type' Ca^{2+} channels in animal cells have infrequently been observed in the plant plasma membrane (in *Zea* shoot cells (Fairley, Laver & Walker, 1991) and in *Samanea* pulvinar cells (Moran, 1990)). Verapamil, which blocks L-type Ca^{2+} channels in animal cells (Fox et al., 1987), reduces Ca^{2+}-dependent K^+ flux in *Zea* shoot and root cells (Fairley et al., 1991; Ketchum & Poole, 1991) and reduces $^{45}Ca^{2+}$ uptake in carrot protoplasts (Graziana et al., 1988). To our knowledge, there has been no conclusive, direct evidence for a membrane-transport mechanism for Ca^{2+} entry into higher plant cells, however.

We show here that Ca^{2+} can enter *Vicia* guard cells throught otherwise K^+-selective, inwardly rectifying channels in the plasma membrane. Inward current is directly measured using the patch-clamp method (Hamill et al., 1981) in whole-cell mode. Evidence is provided to show that Ca^{2+} can also enter *Zea mays* guard cells through inwardly rectifying K^+ channels. These inwardly rectifying channels have previously been identified and characterized in *Vicia* (Schroeder, Raschke & Neher, 1987; Schroeder, 1988) and in *Zea* (Fairley-Grenot & Assmann, 1992). They are activated by hyperpolarization of the membrane potential and, by allowing K^+ influx, are believed to contribute to the increase in guard cell turgor which causes stomatal opening during the regulation of leaf-air gas exchange (*see* Zeiger, Farquhar & Cowan, 1987).

Materials and Methods

Isolation of Guard Cell Protoplasts from *Vicia faba*

Guard cell protoplasts were isolated from the epidermis of the youngest expanded leaves of 3–4-week-old plants of *Vicia* according to the procedure of Kruse, Tallman and Zeiger (1989). Plants were grown under 10 hr daylight (100 μmol m^{-2} sec^{-1}) at 21°C (day)/20°C (night). Leaves were harvested at the end of the dark period.

Isolation of Guard Cell Protoplasts from *Zea mays*

Guard cell protoplasts were isolated from the epidermis of the earliest emerging leaf of seven-day *Zea* seedlings (cultivar Wf9 × M017). Seedlings were grown at 32°C (day)/28°C (night) under 16 hr daylight of 160-μmol m^{-2} sec^{-1} intensity. The method for protoplast isolation (Fairley-Grenot & Assmann, 1992) resembles that used for *Vicia*. Protoplasts from both *Vicia* and *Zea* were kept on ice in the dark for at least 1 hr before being used in patch-clamp experiments.

Current-Voltage Recording and Analysis

Protoplasts were allowed to settle in a bath that was kept at ground potential by an electrode connected to ground via the headstage of a patch-clamp amplifier (Axopatch 1B, Axon Instruments, Burlingame, CA). Protoplasts were approached with pipettes made from borosilicate glass capillaries (Kimax-51, VWR, Boston, MA) using a vertical two-stage puller (Narashige PP-83, Tokyo). Prior to use, pipettes were fire polished using a fire-polishing device (Narashige MF-83, Tokyo). Final pipette resistances were between 30 and 130 MΩ, typically 50 MΩ in K^+-containing solutions and 120 MΩ in K^+-free solutions (solutions are described below). All membrane potentials reported have been corrected for liquid junction potentials (Barry & Lynch, 1991); these were measured using 3-M KCl agar bridges as described by Fenwick, Marty and Neher (1982).

Patch clamping was performed at 20 ± 2°C under green light of 6 μmol m^{-2} sec^{-1} intensity obtained by placing a Roscolenex (Woburn, MA) #874 filter (peak transmitted wavelength: 520 nm, half-bandwidth: 31 nm) in the light path. When whole-cell seals were obtained the membrane potential was measured and then held at −60 mV using the data acquisition program, Q (obtained from Drs. T. Hoshi and R. Aldrich and improved in our laboratory by Ms. A. Gershenson) executed on a microcomputer (INDEC 11-73, Indec, Sunnyvale, CA). The microcomputer was interfaced to a multipurpose I/O device (INDEC LDS). After allowing time for the establishment of diffusional equilibrium between the protoplast cytoplasm and the pipette solution, this program was then used to subject the membrane to a series of voltage protocols as described in Results. Whole-cell current was measured during each protocol, filtered at 2 kHz, and digi-

Table 1. Pipette solutions (in mM)

	K^+	Na^+	Mg^{2+}	Cl^-	Ca^{2+}	ATP	EGTA	HEPES	Glu[a]
*P*1:	107	—	4	8	1	2	2	10	98
*P*1$^-$:	107	—	4	6	0	2	2	10	98
*P*2:	5	104	4	6	1	2	2	10	98

[a] Glutamate$^-$

Table 2. Bath solutions (in mM)

	K^+	Na^+	Mg^{2+}	Cl^-	Ca^{2+}	HEPES	Glu[a]
*B*1:	13.7	—	4	10	1	10	10
*B*2:	—	13.2	4	10	1	10	10

[a] Glutamate$^-$

tized and displayed on the screen function of the INDEC LDS. Current records were then recorded to disk. Current was later redisplayed and analyzed using the program, A (source same as for Q). Positive current is defined as the movement of positive charge out of the cell. Time-activated current was measured as the mean of 40 sample points taken after 1.85 sec at a given membrane potential, when a steady state had been attained, minus instantaneous current, measured after *ca.* 5 msec at the same given membrane potential.

Patch-Clamp Solutions

Pipette solutions (Table 1) were adjusted to 500 mosmol kg^{-1} using mannitol and to pH 7.2 using KOH (*P*1, *P*1$^-$) or NaOH (*P*2). Final K^+ and Na^+ concentrations are given. MgATP was prepared as a frozen stock solution (500 mM in 1 M Tris) and added on the day of an experiment. The [free Ca^{2+}] = 200 nM (*P*1, *P*2) and 2 nM (*P*1$^-$); [free Mg^{2+}] = 3.8 mM (*P*1), 3.6 mM (*P*1$^-$), and 1.7 (*P*2). Free divalent cation concentrations were calculated using the program BUFFA (Dr. R.G. Ryall, Flinders Medical Centre, Australia).

Bath solutions (Table 2) were adjusted to 450 mosmol kg^{-1} using mannitol and to pH 7.2 using KOH (*B*1) or NaOH (*B*2). Again, final K^+ and Na^+ concentrations are given.

Equilibrium potentials for the sets of solutions used are as follows (in mV): *P*1/*B*1: $E_K = -47$, $E_{Cl} = -10$, $E_{Mg} = -15$, $E_{Ca} >> 100$ (=229); *P*1$^-$/*B*1: $E_K = -47$, $E_{Cl} = -17$, $E_{Mg} = -17$, $E_{Ca} >> 100$ (=345); *P*2/*B*2: $E_{Na} = -48$, $E_{Cl} = -17$, $E_{Mg} = -36$, $E_{Ca} >> 100$ (=229).

These values were calculated using activity coefficients derived using the Debye-Huckel equation (*see* Margolis, 1966). Ionic radii were estimated (in nm) as: 0.133 (K^+), 0.181 (Cl^-), 0.33 (Ca^{2+}, Mg^{2+}).

Results

Time-Activated Current

Figure 1 shows that hyperpolarization of the membrane potential (V_m) from a holding potential of −60 mV to values more negative than −108 mV resulted in the activation of inward current (time-activated current). Repolarization of the membrane to the holding potential resulted in small, rapidly inactivating inward tail current (Fig. 1*A*, arrow). Tail current was also consistently observed when the holding potential was set to E_K (−47 mV; $n = 11$). The tail current reversal potential was *ca.* −33 mV (Fig. 1*B*; *see also* Fig. 3*C*). This was determined by activating the channels with a hyperpolarizing pulse (−188 mV), stepping to a variety of less negative membrane potentials (Fig. 1, upper right), plotting resultant tail current *vs.* membrane potential, and then interpolating to obtain the reversal potential. The tail current reversal potential was independent of holding potential over the range from −8 to −128 mV (*data not shown*). In only one protoplast out of those observed ($n > 70$), there was no tail current after inward current (Fig. 1*C* and *D*). The traces shown in Fig. 1 are from protoplasts from the same preparation assayed within 30 min of each other under identical conditions.

Tail current at E_K remained when the composition of pipette and bath solution was modified as little as possible (and not at all with respect to K^+ or Ca^{2+}) but so that the equilibrium potential for either Cl^-, glutamate$^-$ or Mg^{2+} was set at a potential more negative than or equal to the holding potential (*data not shown*). For example, in three separate sets of experiments, where calculated E_{Cl} was −97 mV, or E_{Mg} was −47 mV, or E_{glu} was −59 mV, tail current reversal potentials always remained >−40 mV.

In protoplasts showing tail current the magnitude of the tails varied (i) with small variations in the amplitude of time-activated current in different protoplasts, and (ii) in fixed proportion with variations over time in time-activated current measured from any one protoplast. Further, tail-current amplitude showed the same voltage dependence as the time-activated current.

Figure 2 shows that replacing K^+ with Na^+ eliminated both time-activated and tail current. In these experiments E_{Na} was set at −48 mV and all other ionic conditions were unchanged. It is known that Na^+ has limited permeability in the inwardly rectifying K^+ channels of *Vicia* guard cells:

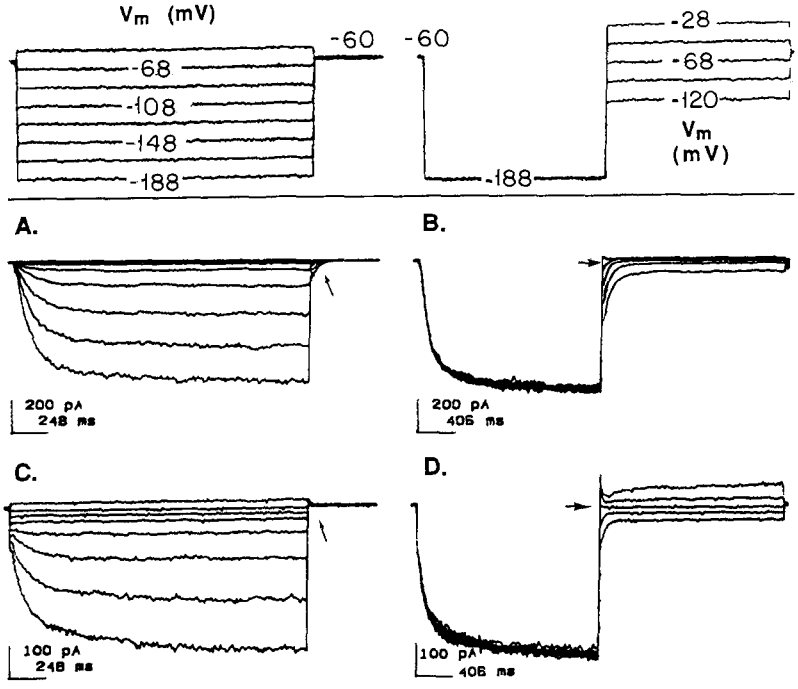

Fig. 1. Whole-cell current time courses measured during excursions of the membrane potential (V_m) from a holding potential of −60 mV according to the voltage protocols shown in the upper panel. Traces at left measure the current-voltage characteristic of the membrane. The arrows indicate the presence (*A*) or absence (*C*) of detectable tail current upon repolarization of the membrane. Traces at right measure the time-activated current reversal potential indicated by the arrows (*B* and *D*). Current records at different membrane potentials are superimposed. Each pair of traces (*A* and *B*, *C* and *D*) is from the same protoplast. Seal resistances are (in GΩ): 2.0 (*A* and *B*), 0.9 (*C* and *D*). In this experiment Ca_i = 2 nM, Ca_o = 1 mM.

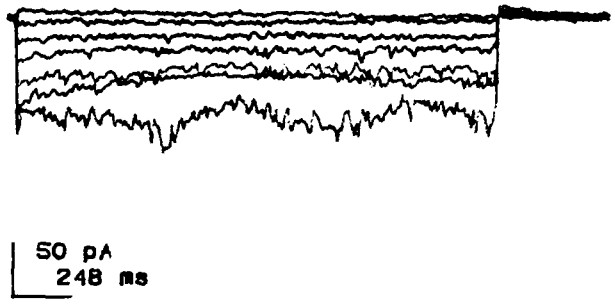

Fig. 2. Whole-cell current time courses measured during application of the voltage protocol shown in Fig. 1 (upper panel, left) to a protoplast when K^+ has been replaced by Na^+ in the pipette and bathing solutions. The absence of an ionic pathway to quickly dissipate the imposed voltage gradients under these conditions resulted in seal instability at extreme membrane potentials. For this reason the current time course at V_m = −188 mV is omitted. Seal resistance = 1.5 GΩ. In this experiment Ca_i = 200 nM, Ca_o = 1 mM.

P_{Na}/P_K = 0.06 (Schroeder et al., 1987; Schroeder, 1988). The absence of time-activated current (Fig. 2) indicates that in the absence of K_o^+ (and with only 5 mM K_i^+) either the inwardly rectifying K^+ channels do not open or they are fully blocked by Na^+.

Since the experimental tests described above eliminated the possibility that Cl^-, glu^- or Mg^{2+} were the ions responsible for the tail current, the possibility that the tail current is due to Ca^{2+} influx was tested by imposing changes in Ca_o and/or Ca_i sufficient to significantly alter the electrochemical force driving Ca^{2+} entry. The magnitude of these changes was subject to two experimental limitations: increasing Ca_i above micromolar values blocks all current through the channels, while decreasing Ca_o destabilizes the membrane, making it impossible to obtain the high resistance seals required for whole-cell current measurement. Within these limitations, Fig. 3 illustrates that increasing Ca_o from 1 to 10 mM (n = 4) or decreasing Ca_i from 200 to 2 nM (n = 6) shifted the reversal potential from −38 mV to −32 or −28 mV, respectively. Average and range of reversal potential values for the conditions of Fig. 3 were −43 ± 5 mV (200 nM Ca_o/1 mM Ca_o), −37 ± 5 mV (200 nM Ca_o/10 mM Ca_o), and −33 ± 5 mV (2 nM Ca_o/1 mM Ca_o). Increasing Ca_o also slowed channel deactivation.

Figure 4 shows that decreasing Ca_i from 200 to 2 nM also increased the ratio of tail current to time-activated current from 0.10 ± 0.01 (n = 6) to 0.23 ± 0.03 (n = 9; Fig. 4*A*). This increase is in addition to an associated increase in the amplitude of time-activated current (Fig. 4*B*). This ratio was calculated by dividing the tail current, measured *ca.* 5 msec after repolarization of the membrane to −60 mV from V_m = −188 mV, by the steady-state time-activated current (after instantaneous current subtraction) at V_m = −188 mV.

Figure 5 shows that increasing Ca_o from 1 to 10 mM also increased the ratio of tail current to time-activated current from 0.11 ± 0.01 to 0.55 ± 0.14

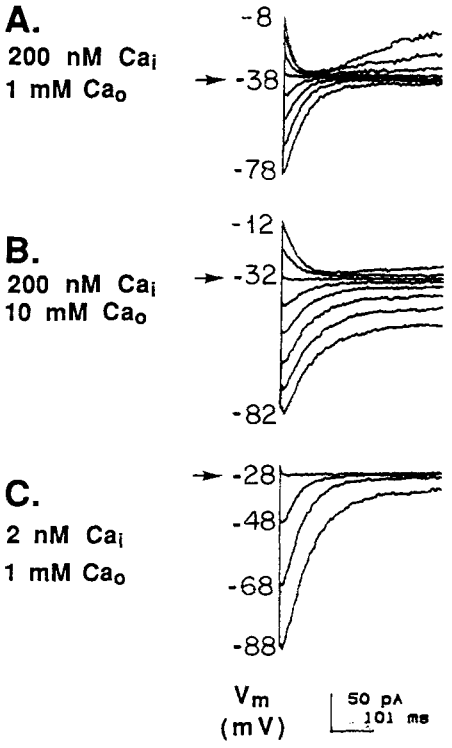

Fig. 3. Effect of increasing external $[Ca^{2+}]$ (Ca_o) or decreasing internal $[Ca^{2+}]$ (Ca_i) on current reversal potential. Each set of traces corresponds to tail current recorded upon repolarization of the membrane to a series of potentials (V_m) after hyperpolarization (protocol analogous to that shown in Fig. 1, upper panel, right). In (*A* and *B*) the repolarization potentials change in 10-mV increments. In (*C*) the repolarization potentials change in 20-mV increments. $Ca_{i/o}$ conditions were as indicated. (*A* and *B*) Data obtained from the same protoplast before and after increasing Ca_o (seal resistance: 2.3 GΩ (*A*), 3.2 GΩ (*B*)). (*C*) Data obtained from a different protoplast (seal resistance: 4.8 GΩ). The arrows indicate current reversal potential in each case.

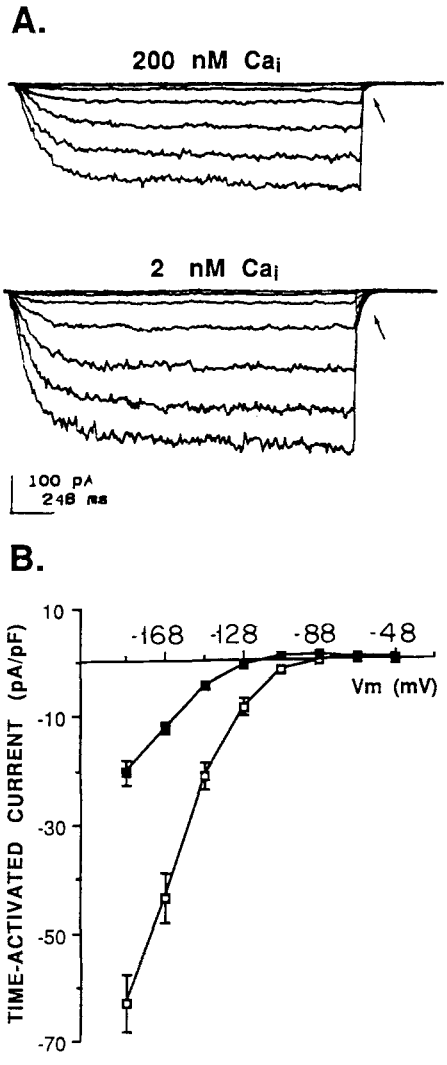

Fig. 4. Effect of decreasing internal $[Ca^{2+}]$ (Ca_i) on (*A*) tail current and (*B*) time-activated current. (*A*) Whole-cell current time courses measured during application of the voltage protocol shown in Fig. 1 (upper panel, left) using 2 or 200 nM Ca_i. The two sets of traces are from different protoplasts. Seal resistances: 8.2 GΩ (upper), 3.5 GΩ (lower). The arrows indicate tail current. (*B*) Time-activated current (per unit whole-cell capacitance) as a function of membrane potential (V_m) using 2 (□) or 200 (■) nM Ca_i. Values represent the mean ± SE of seven (□) and five (■) protoplasts, where the mean (±SE) seal resistance was (in GΩ): 1.5 ± 0.4 (□), 1.0 ± 0.3 (■). In these experiments Ca_o = 1 mM.

(n = 4; Fig. 5*A*). This was especially marked as increasing Ca_o also caused a decrease in time-activated current (Fig. 5*A* and *B*). Measurements of current at 1 and 10 mM Ca_o were made from the same protoplast (total n = 6) before and after the addition of $CaCl_2$ to the bath. The associated increase in external Cl^- from 10 to 28 mM shifted calculated E_{Cl} from −10 to −36 mV. This shift is in the opposite direction to the observed shift in tail current reversal potential and so cannot account for the observed effects on tail-current amplitude.

Figure 5 shows that increasing Ca_o from 1 to 10 mM not only altered the tail current but also had two marked effects on the time-activated current-voltage relationship: (i) increasing Ca_o caused a +20-mV shift in the current-voltage curve; (ii) increasing Ca_o caused the amplitude of time-activated current to saturate at a membrane potential that varied between −152 and −182 mV in different protoplasts and to be reduced at still more negative membrane potentials. The values given are from observations made 5 min after the increase in Ca_o. Intermediate effects were observed at 2–4 min after Ca_o increase (*data not shown*).

Changing Ca_i or Ca_o (Figs. 4 and 5) had no significant effect on the half-time for K^+channel activation ($P > 0.05$, ANOVA).

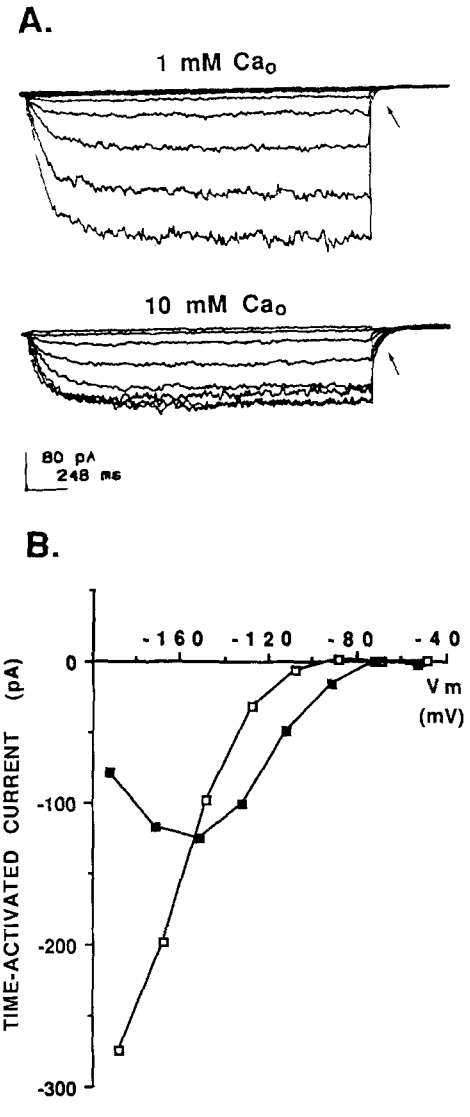

Fig. 5. Effect of increasing external $[Ca^{2+}]$ (Ca_o) on (*A*) tail current and (*B*) time-activated current. (*A*) Whole-cell current time courses measured during application of a voltage protocol analogous to that shown in Fig. 1 (upper panel, left) using 1 or 10 mM Ca_o. The two sets of traces are from the same protoplast. Seal resistances: 3.6 GΩ (upper), 2.9 GΩ (lower). The arrows indicate tail current. Note the change in voltage dependence of time-activated current at higher Ca_o: current responses to the four most negative voltage pulses overlap. This is more clearly represented in *B*: time-activated current from the protoplast used for *A* as a function of membrane potential (V_m) using 1 (□) or 10 (■) mM Ca_o. Tail current magnitude showed the same voltage dependence as time-activated current at 10 mM Ca_o. In these experiments Ca_i = 200 nM.

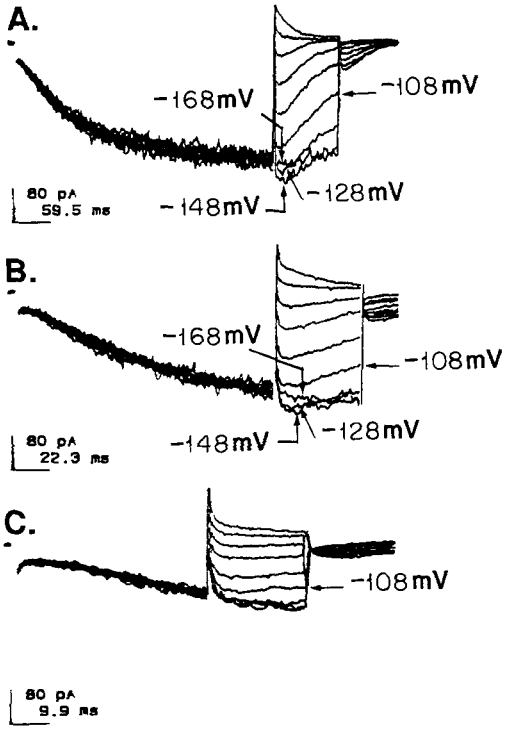

Fig. 6. Whole-cell current timecourses showing the effect of hyperpolarization time on 'hook' current. (*A*) Time course during 400-msec hyperpolarization of the membrane potential (V_m) from a holding potential of −60 mV to −188 mV followed by repolarization to a series of potentials ranging from V_m = −168 to −8 mV (20-mV increments: protocol similar to Fig. 1, upper panel, right), The figures appear complicated because of the U-shaped voltage dependence of tail current. (*B* and *C*) Current time courses during excursions of the membrane potential according to the same voltage protocol as in *A* but allowing only 150 msec (*B*) or 50 msec (*C*) at V_m = −188 mV. Note that 'hook' current does not occur in *C* even though there is a significant amount (−80 pA) of time-activated current. The three sets of traces are from the same protoplast, with seal resistance (in GΩ): 3.0 (*A*), 3.8 (*B*), 3.6 (*C*). In this experiment Ca_i = 2 nM, Ca_o = 1 mM.

In 4 of 13 protoplasts with 1 mM Ca_o and in all protoplasts with high Ca_o, where voltage-dependent time-activated current saturation was evident (*cf.* Fig. 5*B*), there was a 'hook' in the tail current, most obvious after repolarization of the membrane from V_m = −188 mV to V_m = −178 to −128 mV. Figure 6*A* shows an example of hooked tail current (*see* vertical and angled arrows). The hook results from an increase in current during the first 20 msec at the repolarizing voltage, before tail current is diminished due to channel inactivation. The value at which the hook was greatest varied between −130 and −180 mV in different protoplasts. Protoplasts exhibiting hook current only did so when the hyperpolarizing (channel-activating) pulse was applied for longer than 50 msec (Fig. 6*B cf. C*).

Similar experiments have been performed on guard cell protoplasts from *Zea*. A general description of time-activated, predominantly K^+ current in *Zea* has been given elsewhere (Fairley-Grenot & Assmann, 1992). In *Zea*, tail current does not occur

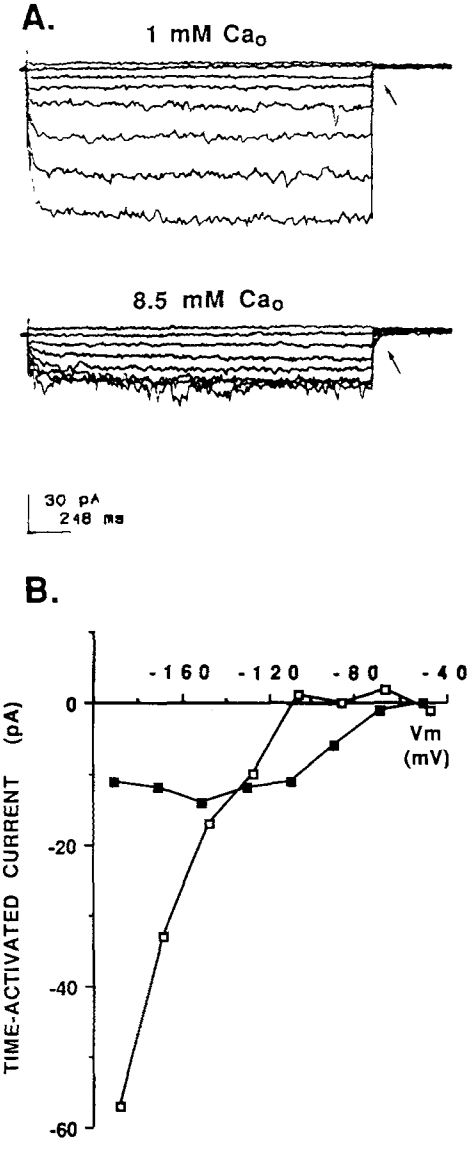

Fig. 7. Effect of increasing external $[Ca^{2+}]$ (Ca_o) on (*A*) tail current and (*B*) time-activated current in *Zea*. (*A*) Whole-cell current time courses measured using 1 or 8.5 mM Ca_o. The two sets of traces are from the same protoplast. Seal resistances: 3.6 GΩ (upper), 5.5 GΩ (lower). The arrows indicate tail current. Note the change in voltage dependence of current at higher Ca_o: current responses to the four most negative voltage pulses overlap. This is more clearly represented in (*B*): time-activated current from the protoplast used for *A* as a function of membrane potential (V_m) using 1 (□) or 8.5 (■) mM Ca_o. In this experiment Ca_i = 2 nM.

when Ca_i is 180 nM (*data not shown*) but does occur when Ca_i is 2 nM (Fig. 7). Figure 7 shows that an increase in Ca_o from 1 to 8.5 mM (2 nM Ca_i) increased the tail current/time-activated current ratio, decreased the magnitude of time-activated current and varied the current-voltage characteristic of these protoplasts, just as observed in *Vicia* (*cf.* Fig. 5).

Discussion

The Tail Current Is Mediated by Inwardly Rectifying K^+ Channels

The voltage dependence, kinetics and K^+ dependence of time-activated inward current (Figs. 1 and 2) indicate that it is conducted by a previously characterized, inwardly rectifying, K^+-selective channel population in the *Vicia* guard cell plasma membrane (Schroeder et al., 1987; Schroeder, 1988).

Three observations indicate that the tail current is mediated by these K^+ channels and not by a different channel population activated either during the time when the membrane is hyperpolarized or instantaneously when the membrane is repolarized: (i) the observation that the ratio of tail and time-activated current varies in fixed proportion within any one protoplast; (ii) the observation that tail and time-activated current show the same voltage dependence, including a U-shaped voltage dependence for both currents at high (10 mM) Ca_o (Figs. 5*A* and 7*A*); (iii) the most important observation, that in the absence of K^+ and presence of Na^+, when the K^+ channels do not conduct current, there is no tail current (Fig. 2). Despite measurable relative permeability of the channels to Na^+ under bi-ionic conditions (P_{Na}/P_K = 0.06; Schroeder, 1988) absolute Na^+ permeability may be so low that the channels are essentially Na^+ blocked under our conditions (*see* Eisenman & Horn, 1983; Hille, 1984; for discussions of relative *vs.* absolute permeability). Alternatively, the absence of measurable time-activated current in the absence of K_o^+ but in the presence of another permeant ion (Na_o^+) may occur because channel gating is K_o^+ dependent or because 13.2 mM Na_o is not sufficient to activate the channels. The dependence of gating of inwardly rectifying K^+ channels on K_o is well documented in animal cells (*see* discussion by Hille, 1984) where evidence suggests that the actual permeation of the channel pore by K^+ ions in some way interacts with the gating mechanism (Stanfield, Ashcroft & Plant, 1981; *see also* Leech & Stanfield, 1981).

The Tail Current Is Due to Ca^{2+} Influx

The ability of monovalent cations other than K^+ to move through these channels has previously been investigated (Schroeder, 1988). Apart from Rb^+ (P_{Rb}/P_K = 0.2), which has no known physiological

role in plant cells, the most permeant cation was Na^+. There has been no investigation of the permeability of divalent cations in these channels.

In the present work, K^+ is the only monovalent cation present. However, the presence of inward tail current at E_K and the parallel observation that the reversal potential for tail current is consistently more positive than E_K suggests the permeation of a second ionic species whose equilibrium potential is more positive than E_K. The reversal potential was more positive than E_K by a consistent and greater amount than could be explained, for example, by background drift or experimental inaccuracy in establishing E_K (K_o would need to be twice its stated value, or K_i half of its stated value, to account for the shift). It is not possible that the tail current is simply a diffusion-limitation effect due to localized depletion of K^+ at the outside channel face and/or to K^+ accumulation at the internal face as such an effect would tend to shift the reversal potential to more negative values.

In the most commonly used solutions ($P1/B1$ or $P1^-/B1$) Mg^{2+}, Cl^- and glutamate$^-$ had reversal potentials more positive than the holding potential (−60 mV). However, systematic manipulation of the pipette and bath solution compositions so that the reversal potential for these ions was more negative than −60 mV did not affect the tail current. Under these conditions it is physically impossible for Mg^{2+} to move passively inward or for Cl^- or glutamate$^-$ to move passively outward through the channels at $V_m = -60$ mV.

The only remaining ions with reversal potentials more positive than the holding potential are H^+ and Ca^{2+}. The ion is highly unlikely to be H^+ as, due to the low H^+ concentration at the pH used ($[H^+]_o = 0.7 \times 10^{-7}$ M), the H^+ permeability relative to K^+ would need to be unrealistically high ($P_H/P_K = 2 \times 10^5$; calculated from the Generalized Null Potential equation (Barry & Gage, 1984)) to cause the tail current reversal potential to be, e.g., 17 mV more positive than E_K.

We postulated, therefore, that the inward tail current is due to Ca^{2+} influx through the otherwise K^+-selective channels. This postulate was supported by the observation that either decreasing Ca_i or increasing Ca_o increased the amplitude of tail current and the ratio of tail- to time-activated current and shifted the observed reversal potential (Figs. 3–5). These three observations support the postulate that Ca^{2+} can permeate the K^+ channels. The ability to manipulate the reversal potential in this way is a necessary criterion for establishing that Ca^{2+} is the ion accompanying K^+ through the channels. The independence of the reversal potential from the holding potential suggests that this mechanism for Ca^{2+} entry operates over a wide voltage range.

Relative Permeability of K^+ and Ca^{2+}

The relative permeability of K^+ and Ca^{2+} in the channels under the three sets of conditions described has been calculated using the Constant Field Equation (Goldman, 1943) extended for application to channels which can conduct both monovalent and divalent species (*see* Spangler, 1972; Jan & Jan, 1976; Lewis, 1979). This equation was derived for channels which could simultaneously conduct several cations and can be simplified in the present system, where only K^+ and Ca^{2+} are considered, to:

$$E = (RT/F) \ln \{[-b + (b^2 - 4ac)^{1/2}]/2a\}$$

where $a = [K^+]_i + 4(P_{Ca}/P_K)[Ca^{2+}]_i$; $b = [K^+]_i - [K^+]_o$; and $c = -[K^+]_o - 4((P_{Ca}/P_K)[Ca^{2+}]_o)$.

When Ca_o is increased to a value (10 mM) near K_o (13.7 mM), P_{Ca}/P_K is 0.3 ± 0.2 (the error term results from calculations utilizing $E \pm 5$ mV), suggesting that, given similar availability at the channel mouth, Ca^{2+} is less permeable than K^+ at physiological Ca_i (200 nM). At lower Ca_o (1 mM), Ca^{2+} is more permeable (but less available) than K^+ (P_{Ca}/P_K = 1.8 ± 0.3). When Ca_i is reduced to very low levels (2 nM), Ca^{2+} permeability rises (P_{Ca}/P_K = 4.9 ± 2.3). The $Ca_{i/o}$ regulation of P_{Ca}/P_K suggests that the K^+ channels play a significant role in Ca_i homeostasis. The observation that P_{Ca}/P_K values vary with the ionic ratios when calculated using this equation suggests that the channels behave as multi-ion pores (*see* Eisenman & Horn, 1983). This is also implied by the 'knock-on' effects of voltage and K^+ (current hooks, Fig. 6).

The Constant Field Equation used to calculate P_{Ca}/P_K assumes (Goldman, 1943) that the membrane is homogeneous and that the intramembrane (channel) electric field is constant. It further assumes that there is no unbalanced membrane surface potential (Frankenhaeuser & Hodgkin, 1957). The +20-mV shift in the voltage dependence of time-activated current when Ca_o is increased in our system (Fig. 4*B*, *see also* Fig. 7*B*) indicates that surface potential effects are present. Interestingly, preliminary data indicate that increasing Mg_o from 0.4 to 4 mM also results in a *ca.* 20-mV positive shift in the current-voltage relationship (*data not shown*). Surface potential effects would cause the P_{Ca}/P_K values in these channels to be lower than those stated above by an amount which depends on the density and distribution of negative charge on the membrane surface (Lewis, 1979). The impor-

tance of surface potential effects in intact cells, where the membrane is adjacent to a cation-charged cell wall, is yet to be established.

Ca^{2+} Permeation Is Slower than K^+ Permeation

Figures 3 and 4 (and Fig. 7) show that increasing the driving force for Ca^{2+} influx decreases the magnitude of time-activated current even though tail current is enhanced. This indicates that when more Ca^{2+} ions are forced into the channels, positive charge (K^+ or Ca^{2+}) cannot permeate as quickly and so net current is reduced. This may be due to repulsion between the cations or to their competition for one or more binding sites in the channel. The saturation of current between $V_m = -152$ and -182 mV in high Ca_o (and the less negative slope (indicating lower conductance) at less negative potentials; Fig. 4*B*) indicates that the Ca^{2+} block is voltage dependent, becoming more apparent with more extreme hyperpolarization of the membrane, and therefore, that the cation binding site(s) is (are) within the channel pore, partway across the electric field of the membrane (*see* Woodhull, 1973; *see also* Hille, 1984).

The hook in tail current recorded in some protoplasts upon depolarization of the membrane (Fig. 6) is also a consequence of partial block of the channels by Ca^{2+} due to its slower permeation. Similar hook currents have been observed during the (blocking) interaction of Ba^{2+} with K^+ channels in squid giant axon (Armstrong & Taylor, 1980). The fact that inward (tail) current increases on repolarization implies that, although current appears saturated at -168 mV, there are in fact Ca^{2+}-blocked channels. Relief of the (voltage-dependent) block at the less negative repolarization potential transiently increases the current as Ca^{2+} leaves the channels. The current magnitude then decays as the channels close. The observation that hyperpolarization for longer than 50 msec is required before hook current occurs (Fig. 6*B cf. C*) results from the time-dependent frequency of channel opening and of Ca^{2+} occupation.

Comparison with Other Mechanisms of Ca^{2+} Entry

There is little precedent for the concept that Ca^{2+} and K^+ can enter higher plant cells through a common channel in the plasma membrane. Moran (1990) and Schroeder and Hagiwara (1990*a*) refer to cation-selective channels as pathways for Ca^{2+} permeation but do not identify the channels or relative permeabilities of the cationic species. Ca^{2+} permeates several types of Na^+-selective channels in animal membranes, including frog node and squid axon, where $P_{Ca}/P_{Na} = 0.1$ (Hille, 1972; Meves & Vogel, 1973). There are no known Na^+-selective channels in plant membranes, where the function of membrane depolarization is performed by K^+ channels. By corollary, the finite permeability of Ca^{2+} in K^+ channels seems less unusual. It has been shown that K^+ channels in squid axon can conduct Ca^{2+} (*see* Inoue, 1981). Further, there are several nonselective cation channels in animal systems that conduct (among other cations) both K^+ and Ca^{2+} (Adams, Smith & Thompson, 1980; *see also* Hille, 1984).

We suggest that inwardly rectifying K^+ channels provide one mechanism for Ca^{2+} entry into *Vicia* and *Zea* guard cells. The fact that one protoplast showed no Ca^{2+} current and that only 30% of protoplasts show hook current before elevation of Ca_o suggests that this mechanism may be subject to some physiological switch(es) and does not function simply as a passive (leakage) mechanism, restoring Ca_i when the Ca^{2+} gradient becomes too steep or preventing Ca^{2+} entry when Ca_i is elevated. When the switch is off (Fig. 1*C* and *D*), K^+ channels could remain open (on average) for extended periods of time (such as during stomatal opening) without causing Ca_i to rise. Conversely, stimuli inhibiting stomatal opening might increase the Ca^{2+} permeability of these channels. Sorbera and Morad (1990) show that the permeability of cardiac Na^+ channels to Ca^{2+} depends on activation by the hormone, atrionatriuretic acid, for example. Further, Hollmann, Hartley and Heinemann (1991) have recently shown that by providing the appropriate cRNA combination for kainic or proprionic acid receptor subunit expression, Na^+/K^+ channels from neurons can be made either Ca^{2+}-permeable or Ca^{2+}-impermeable when expressed in *Xenopus* oocytes.

Conclusions

Ca^{2+} can enter *Vicia* and *Zea* guard cells via otherwise K^+-selective channels in the plasma membrane. Ca^{2+} permeation can be manipulated by changing the electrochemical force driving Ca^{2+} entry but also appears subject to some biological switch. Ca^{2+} ions permeate K^+ channels more slowly than K^+, resulting in voltage-dependent block at high Ca_o. Increasing Ca_o inhibits stomatal opening (De Silva et al., 1985; Inoue & Katoh, 1987; Schwartz et al., 1988), consistent with our observation that increasing Ca_o, by partially blocking chan-

nels, decreases K^+ influx. Our data and those of Schroeder and Hagiwara (1989) suggest that increasing Ca_i also reduces K^+ influx and the magnitude of stomatal opening. Decreased K^+ influx following elevation of Ca_i by Ca^{2+} entry may additionally function as a negative feedback mechanism which regulates the rate of stomatal opening. We conclude that the inwardly rectifying K^+ channels are part of a regulatory mechanism for Ca_i, which in turn regulates a host of cellular processes.

The observation of this phenomenon in guard cells of the C_3 dicot, *Vicia*, and the C_4 grass, *Zea*, suggests that it may be a common mechanism of Ca^{2+} influx and Ca_i regulation in guard cells. Further research is needed to establish whether Ca^{2+} can permeate inwardly rectifying K^+ channels in other plant cell types. Further research is also needed to more rigorously assess the biophysical aspects of Ca^{2+} permeation of these channels, including the effects of membrane surface charge and divalent cation block.

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