Glutamate-Gated Calcium Fluxes in Arabidopsis[1]

Kirsten L. Dennison and Edgar P. Spalding*

Department of Botany, University of Wisconsin, 430 Lincoln Drive, Madison, Wisconsin 53706

It is well accepted that endogenous and environmental signals can influence cellular activities by changing $[Ca^{2+}]_{cyt}$ (Malhó et al., 1998; Sanders et al., 1999). Despite the importance of this mechanism for coupling stimuli to responses (Malhó et al., 1998), the molecules responsible for generating increases in $[Ca^{2+}]_{cyt}$ during cell signaling in plants are not known at the genetic level. The results presented here raise the possibility that ligand-gated ion channels in plants such as those predicted by the discovery of ionotropic glutamate receptor (iGluR)-like genes in Arabidopsis (Lam et al., 1998) are key components of a Ca^{2+} influx mechanism important to signal transduction.

In animal brains iGluR channels mediate fast chemical transmission across synapses by increasing the permeability of the post-synaptic cell membrane to K^+, Na^+, and Ca^{2+} after binding Glu released by the presynaptic cell (Hille, 1992; Hollmann and Heinemann, 1994; Dingledine et al., 1999). The resulting Ca^{2+} entry in particular has been associated with long-term potentiation of the synapse, a physiological adaptation important to the learning process (Baudry and Lynch, 1993; Bliss and Collinridge, 1993). The Glu receptor homologs recently identified in plants (Lam et al., 1998) are too divergent from animal iGluRs to know with any certainty what ligand(s) gate them, what ions are conducted in the open state, and in which membrane(s) of the cell they function (Chiu et al., 1999). Thus the identification of iGluR sequences in the Arabidopsis genome raises intriguing questions about the physiological functions of neurotransmitter-gated channels in plant cells.

The possibility that Glu gates Ca^{2+}-permeable channels at the plasma membrane of plant cells was explored by measuring $[Ca^{2+}]_{cyt}$ in transgenic seedlings expressing aequorin, a Ca^{2+}-sensitive luminescent protein (Knight et al., 1991). As shown in Figure 1A, Glu application immediately triggered a very large, transient spike in $[Ca^{2+}]_{cyt}$. In separate experiments the effect of Glu on membrane potential (V_m) was measured with intracellular microelectrodes inserted into root apices of intact seedlings. Figure 1A also shows that switching the bathing medium from 1 mM KCl to 1 mM K-Glu induced a large and rapid depolarization of the membrane, as would be expected if the abrupt increase in $[Ca^{2+}]_{cyt}$ was due to Glu opening Ca^{2+}-permeable channels at the plasma membrane. The average peak change in V_m induced by 1 mM Glu was 55 ± 7 mV ($n = 6$ seedlings). This positive shift in V_m, though consistent with Glu gating an inward electrogenic Ca^{2+} current across the plasma membrane, may also be due to secondary effects of the increased $[Ca^{2+}]_{cyt}$ on other ion transporters. Another scenario to consider is that Glu directly gates channels permeable to ions such as Cl^- (Cully et al., 1994) in addition to Ca^{2+}-permeable channels to cause the depolarization. And last, an electrogenic Glu-uptake mechanism (Boorer et al., 1996) may also contribute to the electrical response. Because these and perhaps other scenarios are not mutually exclusive, more electrophysiological studies of the connection between the large Glu-gated changes in $[Ca^{2+}]_{cyt}$ and the effect on V_m are warranted.

Figure 1B shows that the magnitude and time course of the rapid increase in $[Ca^{2+}]_{cyt}$ was similar to the well-studied response to cold shock, i.e. in the micromolar concentration range and completed within several seconds (Knight et al., 1991, 1996; Lewis et al., 1997). Treatment with 1 mM Glu induced a response that was typically hundreds of fold higher than the control injection of equimolar KCl, which produced a touch response that may reflect Ca^{2+} entering the cytoplasm from internal stores (Haley et al., 1995; Legué et al., 1997). The post-peak shoulder apparent in the selected response to Glu was often, but not always observed. Activation of iGluRs by Glu causes very similar Ca^{2+} changes in cells of the mammalian nervous system (Kirischuk et al., 1999; Obrietan and van den Pol, 1999).

If the increase in $[Ca^{2+}]_{cyt}$ triggered by Glu resulted at least in part from flux across the plasma membrane from the apoplasm, impermeant channel blockers and external chelators of Ca^{2+} should reduce the response. The results in Figure 2A demonstrate that pretreatment with La^{3+}, a frequently used blocker of plasma membrane Ca^{2+} channels, inhibited the Ca^{2+} spike to the low level induced by the control treatment. Chelating extracellular Ca^{2+} by pre-treating seedlings with EGTA was similarly inhibitory (Fig. 2B). The combined evidence support our suggestion that Glu triggers an influx of Ca^{2+} across the plasma membrane and this leads to a dramatic change in $[Ca^{2+}]_{cyt}$. Calcium-induced Ca^{2+}-release from inter-

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* Corresponding author; e-mail spalding@facstaff.wisc.edu; fax 608–262–7509.

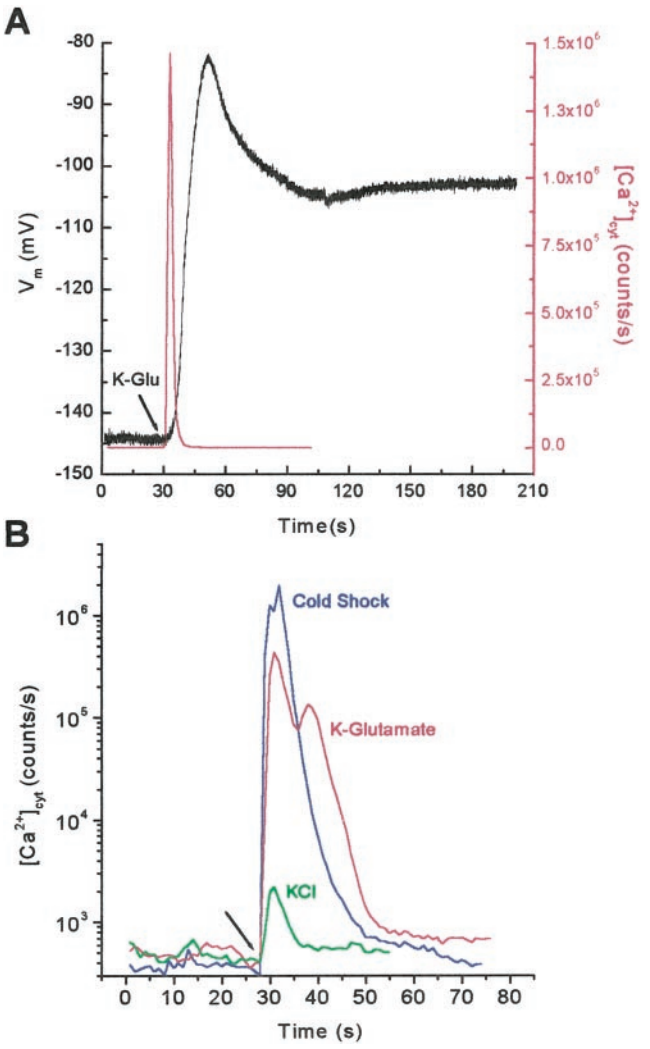

Figure 1. Glu triggers a large transient increase in $[Ca^{2+}]_{cyt}$ and an accompanying membrane depolarization. A, The red trace shows Ca^{2+}-dependent luminescence from whole aequorin-expressing Arabidopsis seedlings (5- to 8-d-old) measured with a luminometer as described previously (Lewis et al., 1997). The black trace shows the response of V_m measured by impaling a cell near the root apex with an intracellular microelectrode as previously described (Spalding et al., 1999). Intact seedlings between 7- and 14-d-old were used for the V_m measurements. Glu at a final concentration of 1 mM was delivered as the K^+ salt. The pH was buffered at 5.7 with 2.3 mM MES [2-(*N*-morpholino)-ethanesulfonic acid]. B, The kinetics and magnitude of the change in $[Ca^{2+}]_{cyt}$ induced by Glu resembles the response to cold shock, but is much larger than the touch response induced by the control treatment. Cold shock was achieved by injecting 0°C 1 mM KCl into the luminometer cuvette, whereas the control treatment was room temperature 1 mM KCl. Glu was delivered as 1 mM K-Glu and all solutions were buffered at pH 5.7. Arrow indicates the time of treatment.

nal stores such as the vacuole may also contribute (Allen et al., 1995).

La^{3+} also blocked the depolarization triggered by Glu without affecting the resting V_m (Fig. 2C), indicating that the depolarization is a consequence of the inward Ca^{2+} movement. However, La^{3+} is not a specific Ca^{2+}-channel blocker (Lewis and Spalding, 1998) and it may prevent the depolarization by blocking a separate Glu-gated conductance in addition to the Ca^{2+} pathway. Thus despite the fact that La^{3+} blocks the Ca^{2+} flux and the membrane depolarization, the exact relationship between the two Glu-

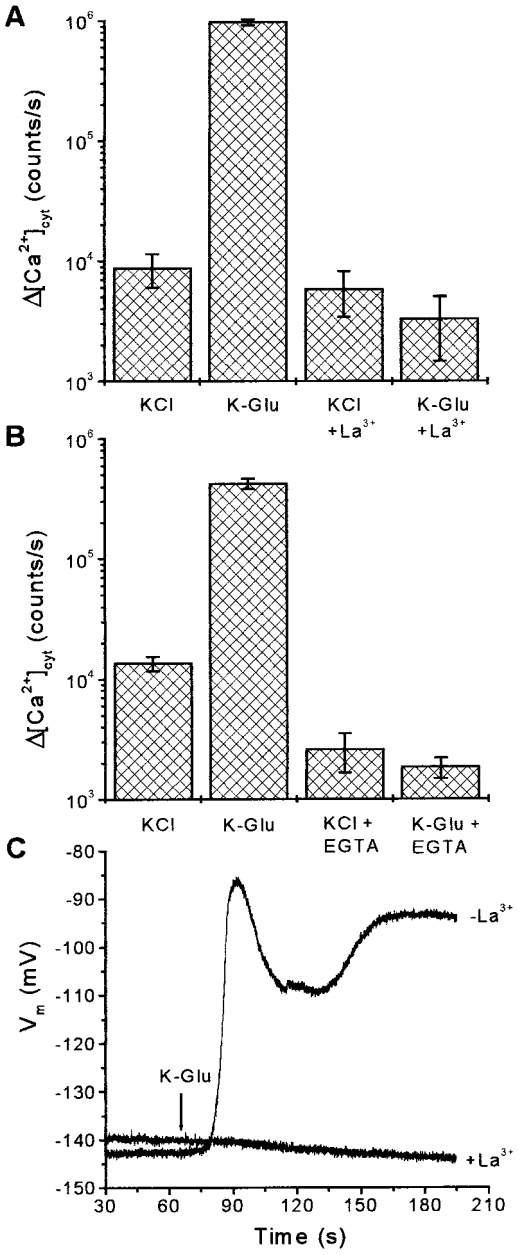

Figure 2. Inhibitory effects of La^{3+} and EGTA. A, Pretreatment of seedlings with 5 mM La^{3+}, an extracellular Ca^{2+}-channel blocker, inhibited the change in $[Ca^{2+}]_{cyt}$ induced by Glu, but had a much lesser effect on the 100-fold smaller response to the control treatment. B, Chelating extracellular Ca^{2+} by pretreatment with EGTA inhibited the Glu-induced Ca^{2+} response. The data plotted are means (±SEM) from three or four independent trials. C, Treatment of roots with La^{3+} blocked the Glu-induced depolarization without affecting the resting V_m. Roots were treated with 5 mM $LaCl_3$ before and during exposure to 1 mM K-Glu.

gated phenomena should be considered an open question. An alternative test would be to determine if EGTA treatment also inhibits the depolarization triggered by Glu, but stable recordings of V_m are difficult to obtain when extracellular Ca^{2+} is depleted to an extent that significantly affects its availability for inward fluxes. Patch-clamp studies of the ionic currents activated by Glu would be the preferred means of obtaining a biophysical description of the depolarization mechanism.

Glu is the primary natural ligand of iGluRs in the central nervous system although other non-native ligands are effective and have been used to classify receptor subtypes. The effectiveness of different ligands was tested using the aequorin reporter plants. Figure 3 demonstrates that Glu was clearly the most effective agonist tested (note the logarithmic y-scale). α-amino-3-hydroxy-5-methylisoxazole-4-propionate and N-methyl-D-aspartate, potent agonists of animal iGluRs, did not induce a response above the control treatment (approximately 2% of the L-Glu response). These non-native agonists of animal iGluRs also did not activate the *Synechocystis* GLU0 (Chen et al., 1999). It may be that affinity for α-amino-3-hydroxy-5-methylisoxazole-4-propionate and N-methyl-D-aspartate evolved in Glu receptors after the divergence of plants and animals. An alternative possibility is that Glu-gated Ca^{2+} entry in Arabidopsis does not involve iGluR-like molecules, but instead some unrelated Ca^{2+}-permeable pathway lacking affinity for typical iGluR agonists is responsible for the phenomenon.

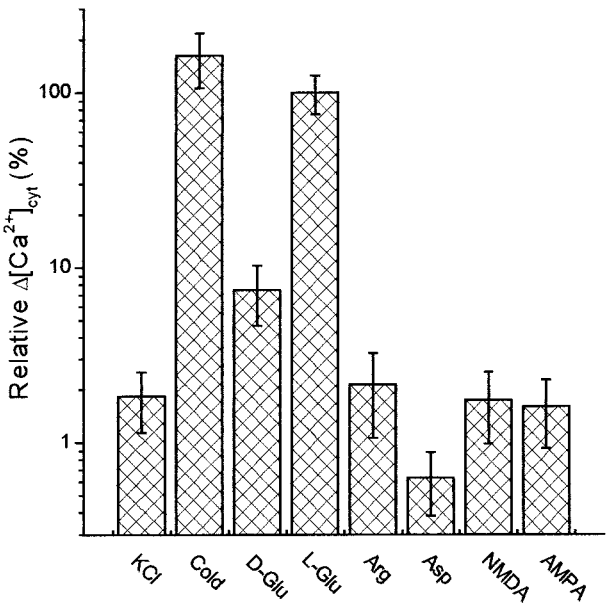

Figure 3. Relative effectiveness of related compounds. L-Glu was much more effective than other potential agonists, including D-Glu and the animal iGluR agonists, NMDA and AMPA. Note the logarithmic scale of the *y* axis, and that the response magnitudes are shown relative to the response induced by L-Glu. All compounds were administered at a final concentration of 1 mM. The plotted values are the means (±SE) of six independent trials.

The fact that the response to D-Glu was less than 10% of the L-Glu response indicates high stereochemical specificity of the binding site(s) on whatever molecules are responsible. Although Glu is clearly an effective ligand in a plausible concentration range, other ligands may be more physiologically important. The Arabidopsis genome contains several iGluR-like genes (Chiu et al., 1999) and that diversity may be matched by a similar diversity of agonists.

Information on the effective concentration range of Glu would help to establish a physiological context for this ligand-gated response in plants. The change in $[Ca^{2+}]_{cyt}$ induced by Glu increased between 0.3 and 3 mM, with the concentration for half-maximal response (EC_{50}) being approximately 1 mM (data not shown). This value is approximately 10-fold greater than the typical value for prokaryotic and animal iGluRs, but very similar to the EC_{50} of Cl^--permeable iGluRs from nematodes (Cully et al., 1994).

If Glu or some other related small organic acid is the primary endogenous ligand, then it is important to consider how and when the external ligand-binding site would experience 0.3 to 3 mM concentrations. Anion channels at the plasma membrane of plant cells are known to function in the transduction of several signals important to plant growth and development (Ward et al., 1995). These channels are relatively non-selective among anions and may conduct significant efflux of dicarboxylic anions such as malate (Hedrich, 1994; Schmidt and Schroeder, 1994), and therefore perhaps Glu, as well. When environmental or endogenous signals activate such anion channels, apoplastic Glu concentration may rise into the effective range, causing a transient change in $[Ca^{2+}]_{cyt}$ that serves to couple a stimulus to downstream responses. This hypothetical scenario may be most plausible in roots, where anion-channel mediated release of dicarboxylic acids has been proposed as a mechanism for combating Al^{3+} stress (Delhaize and Ryan, 1995; Ryan et al., 1997). Perhaps it is no coincidence that dissection experiments revealed most of the Ca^{2+} signal recorded from intact Arabidopsis seedlings was contributed by the root; leaves and cotyledons of young plants displayed smaller Glu responses (data not shown).

The results presented here form the basis of our proposition that a key element of a mechanism for altering $[Ca^{2+}]_{cyt}$ in plant cells during signaling is similar to that responsible for neurotransmitter action in the central nervous system of animals. The evidence would be bolstered considerably if mutational studies revealed a link between specific iGluR-like genes and Glu-gated Ca^{2+} fluxes. Plant biologists interested in Ca^{2+} signaling are presently particularly well equipped to test this connection because there is a wealth of published details about iGluR-mediated Ca^{2+} signaling in neurons, the Arabidopsis genome is essentially sequenced and searchable, so-

phisticated reverse-genetic strategies are very practical, and electrophysiological techniques can measure function with high resolution. The stage for exciting developments in Ca^{2+} signaling is set.

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LITERATURE CITED

Allen GJ, Muir SR, Sanders D (1995) Science **268:** 735–737

Baudry M, Lynch G (1993) *In* Baudry M, Thompson RF, Davis JL, eds, Synaptic Plasticity. MIT Press, Cambridge, MA, pp 87–115

Bliss TVP, Collinridge GL (1993) Nature **361:** 31–39

Boorer KJ, Frommer WB, Bush DR, Kreman M, Loo DDF, Wright EM (1996) J Biol Chem **271:** 2213–2220

Chen G-Q, Cui C, Mayer ML, Gouaux E (1999) Nature **402:** 817–821

Chiu J, DeSalle R, Lam H-M, Meisel L, Coruzzi G (1999) Mol Biol Evol **16:** 826–838

Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LHT, Schaeffer JM, Arena JP (1994) Nature **371:** 707–711

Delhaize E, Ryan PR (1995) Plant Physiol **107:** 315–321

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) Pharmacol Rev **51:** 7–61

Haley A, Russell AJ, Wood N, Allan AC, Knight M, Campbell AK, Trewavas AJ (1995) Proc Natl Acad Sci USA **92:** 4124–4128

Hedrich R (1994) Curr Top Membr **42:** 1–33

Hille B (1992) Ionic Channels of Excitable Membranes. Sinauer, Sunderland, MA, pp 140–169

Hollmann M, Heinemann S (1994) Ann Rev Neurosci **17:** 31–108

Kirischuk S, Kirchoff F, Matyash V, Kettenmann H, Verkhratsky A (1999) Neuroscience **92:** 1051–1059

Knight H, Trewavas AJ, Knight MR (1996) Plant Cell **8:** 489–503

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Nature **352:** 524–526

Lam H-M, Chiu J, Hsieh M-H, Meisel L, Oliveira IC, Shin M, Coruzzi G (1998) Nature **396:** 125–126

Legué V, Blancaflour E, Wymer C, Perbal G, Fantin D, Gilroy S (1997) Plant Physiol **114:** 789–800

Lewis BD, Karlin-Neumann G, Davis RW, Spalding EP (1997) Plant Physiol **114:** 1327–1334

Lewis BD, Spalding EP (1998) J Membr Biol **162:** 81–90

Malhó R, Moutinho A, van der Luit A, Trewavas AJ (1998) Phil Trans R Soc Lond B **353:** 1463–1473

Obrietan K, van den Pol A (1999) J Neurophysiol **82:** 94–102

Ryan PR, Skerrett M, Findlay GP, Delhaize E, Tyerman SD (1997) Proc Natl Acad Sci USA **94:** 6547–6552

Sanders D, Brownlee C, Harper JF (1999) Plant Cell **11:** 691–706

Schmidt C, Schroeder JI (1994) Plant Physiol **106:** 383–391

Spalding EP, Hirsch RE, Lewis DR, Qi Z, Sussman MR, Lewis BD (1999) J Gen Physiol **113:** 909–918

Ward JM, Pei Z-M, Schroeder JI (1995) Plant Cell **7:** 833–844

CORRECTIONS

Vol. 122: 403–413, 2000

Debeaujon, I., Léon-Kloosterziel K.M., and Koornneef, M. Influence of the Testa on Seed Dormancy, Germination, and Longevity in Arabidopsis.

In Figure 1, flavan-4-ols should be replaced with flavan-3-ols. The legends for Figures 4 and 5 were incorrect. Figures 4 and 5 and their correct legends have been reprinted on pp 1140–1141.

Vol. 124: 911–919, 2000

Moshelion, M., and Moran, N. Potassium-Efflux Channels in Extensor and Flexor Cells of the Motor Organ of *Samanea saman* Are Not Identical. Effects of Cytosolic Calcium.

An incorrect figure was erroneously printed for Figure 1. The article has been reprinted with the correct figure on pp 1142–1150.

Vol. 124: 1511–1514, 2000

Dennison, K.L., and Spalding, E.P. Glutamate-Gated Calcium Fluxes in Arabidopsis.

Figure 1 was erroneously printed in black and white. Figure 1 has been reprinted in color on p 1151.

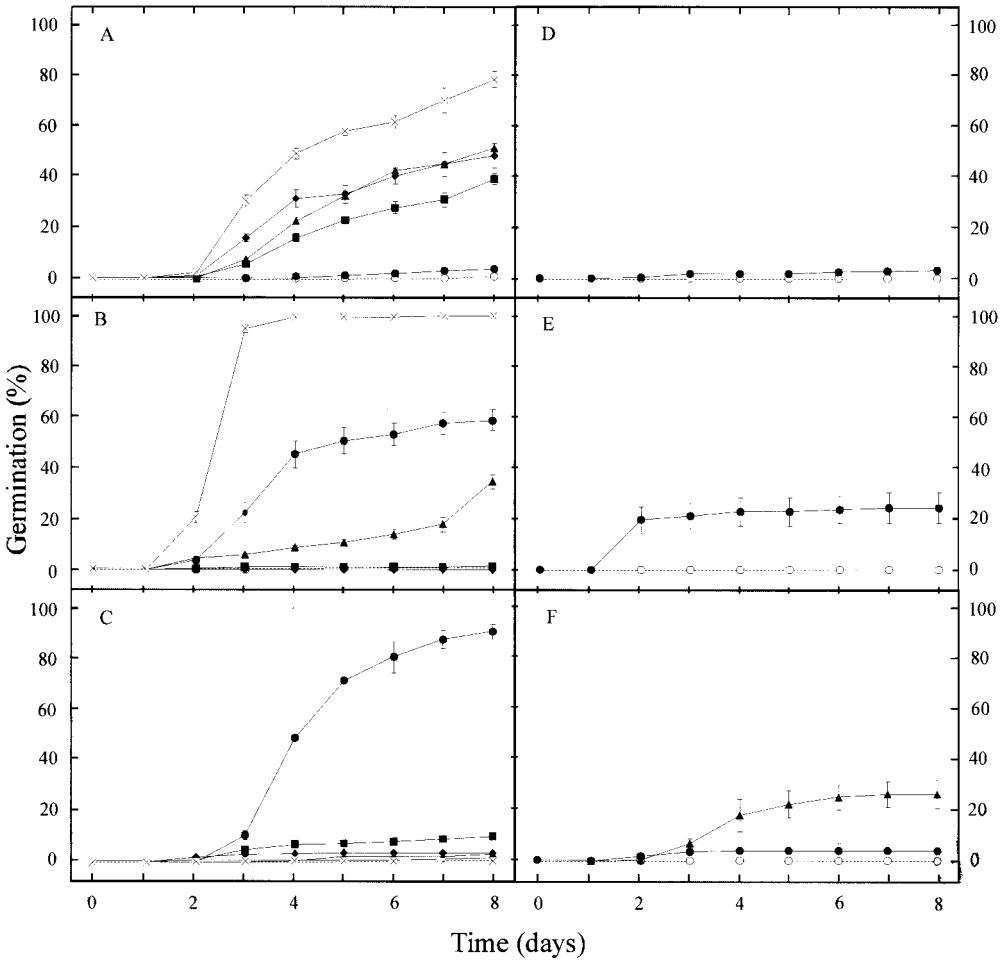

Figure 4. Time course of germination for freshly harvested seed lots (d 9 after harvest; compare with Fig. 3B). In A, ○, L*er*, ●, *tt1*; ▲, *tt2*; ■, *tt3*; ◆, *tt4*; and ×, *tt5*. In B, ○, L*er*, ●, *tt6*; ▲, *tt7*; ■, *tt9*; ◆, *tt10*; and ×, *ttg1*. In C, ○, L*er*, ●, *tt11*; ▲, *tt13*; ■, *ats*; ◆, *ap2*; and ×, *gl2*. In D, ○. Ws and ●, *tt12*. In E, ○, Col and ●, *tt14*. In F, ○, En; ●, *tt8*; and ▲, *ban*.

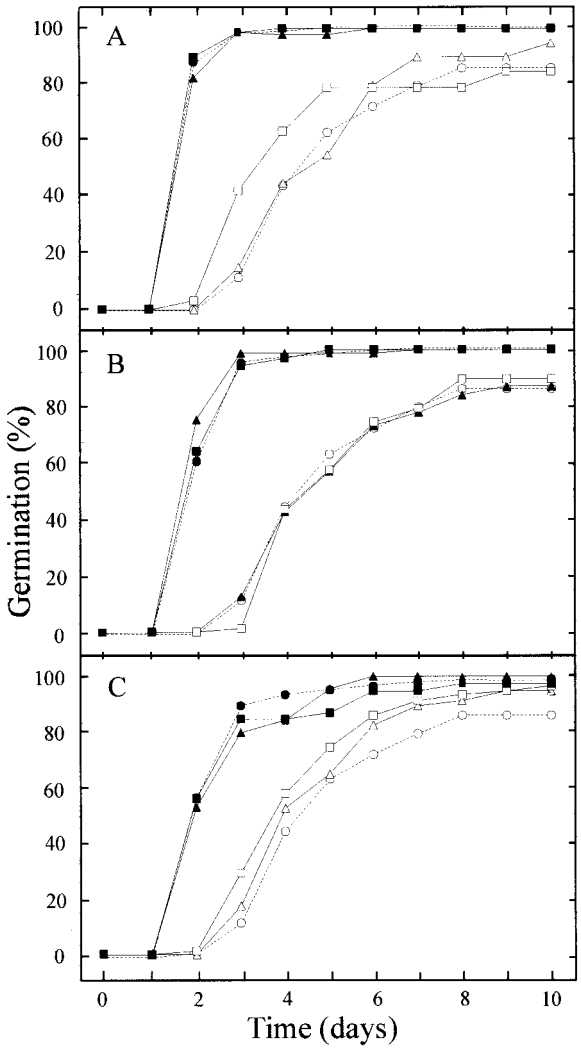

Figure 5. Genetic determinism of the high germination rate encountered in the mutants *tt2*, *tt4*, and *tt7*. The time course of germination after 16 d of dry storage is presented. The parent mentioned first was used as female parent and the second as pollen parent. Seeds from a bulk of nine siliques derived from crosses were used. In A, ○, L*er*; △, L*er* × L*er*; □, L*er* × *tt2*; ●, *tt2*; ▲, *tt2* × *tt2*; ■, *tt2* × L*er*. In B, ○, L*er*, △, L*er* × L*er*, □, L*er* × *tt4*; ●, *tt4*; ▲, *tt4* × *tt4*; ■, *tt4* × L*er*. In C, ○, L*er*, △, L*er* × L*er*; □, L*er* × *tt7*; ●, *tt7*; ▲, *tt7* × *tt7*; ■, *tt7* × L*er*.

Potassium-Efflux Channels in Extensor and Flexor Cells of the Motor Organ of *Samanea saman* Are Not Identical. Effects of Cytosolic Calcium[1]

Menachem Moshelion and Nava Moran*

Department of Agricultural Botany, Institute of Plant Sciences and Genetics in Agriculture, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

Leaflet movements in the mimosa-family tree *Samanea saman* stem from coordinated volume changes of cells in the leaf motor organs in the adaxial and abaxial motor cells ("flexors" and "extensors"). Shrinking, initiated by dissimilar light signals in extensors and in flexors, depends in both cell types on K^+ efflux via depolarization-dependent potassium (K_D) channels. To compare between flexor and extensor K_D channels and to test for a possible interaction of these channels with the Ca^{2+}-mobilizing phosphoinositide cascade evoked in these motor cells by the "shrinking signals," we probed the channels with varying (5 nM–3 mM) cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in patch-clamped inside-out excised membrane patches. Ca^{2+} was not required for K_D channel activation. $[Ca^{2+}]_{cyt}$ of 600 nM decreased the mean number of open K_D channels in flexors, as monitored at −30 mV. Detailed analysis revealed that in flexors millimolar $[Ca^{2+}]_{cyt}$ decreased the maximum number of open channels, but simultaneously increased K_D channel opening probability by negatively shifting the half-maximum-activation voltage by 40 to 50 mV. Thus, the promoting and the inhibitory effects at millimolar $[Ca^{2+}]_{cyt}$ practically cancelled-out. In contrast to flexors, none of the gating parameters of the extensor K_D channels were affected by $[Ca^{2+}]_{cyt}$. Irrespective of $[Ca^{2+}]_{cyt}$, the steady-state gating of extensor K_D channels was slightly but significantly more voltage sensitive than that of flexors. The unitary conductances of flexor and extensor K_D channels were similar and decreased by approximately 20% at millimolar $[Ca^{2+}]_{cyt}$. It is intriguing that the extensor K_D channels were significantly less K^+ selective than those in flexors.

Considerable insight into the regulation of plant K^+-efflux channels (K_{out} or K_D channels) has been achieved in one particularly well-studied model system, the stomatal guard cell (for reviews, see MacRobbie, 1998; Assmann and Shimazaki, 1999, and refs. therein), but even there the underlying mechanisms are not completely understood. To gain insight into the regulation of the K_D channels we study another model system: the motor cells in a leaf-moving organ, the pulvinus, in the mimosa-family tree *Samanea saman*. The pulvinus moves leaves and leaflets by virtue of osmotic volume and turgor changes of its motor cells, resulting from the movement of ions, chiefly K^+ and Cl^-, into and out of the cells (Satter and Galston, 1981; Satter et al., 1988). Signals causing leaf unfolding (e.g. blue light) cause cell shrinking in the top (adaxial, flexor) one-half of the pulvinus and swelling in the bottom (abaxial, extensor) one-half. Signals causing leaf folding (e.g. red light followed by dark) cause the reverse responses. In both cell types, K^+ is released passively from the shrinking cell into the apoplast (Moran et al., 1988; Satter et al., 1988; Lowen and Satter, 1989). In flexors, blue light (a "shrinking signal") has been demonstrated recently to promote the opening of K_D channels (Suh et al., 2000). K_D channels open, presumably, in shrinking extensors as well. The abundance of K_D channels in the *S. saman* motor cell membrane is quantitatively more than sufficient to conduct K^+ fluxes needed to account for the osmotic changes. Moreover, K_D channels are essential to the shrinking of the motor cells and hence, to pulvinar movements, as demonstrated by the arrest of movement by the K_D channel blocker, tetraethylammonium (Moran et al., 1988).

In both cell types, the "shrinking signaling" (blue-light illumination of flexor protoplasts, or imposition of darkness on extensor protoplasts) results in the formation of 1,4,5-inositol trisphosphate (Kim et al., 1993, 1996), a second messenger in the phosphoinositide (PI) cascade (Berridge and Irvine, 1989; Berridge, 1997). According to the present paradigm on the roles of the PI cascade and Ca^{2+} mobilization in the shrinking of stomatal guard cells (Blatt et al., 1990; Gilroy et al., 1990; McAinsh et al., 1990; Irving et al., 1992; Lee et al., 1996), K^+-efflux channels in guard cells are activated by depolarization resulting from Ca^{2+} activation of Cl^- efflux (Schroeder and Hagiwara, 1989).

Although a similar paradigm is generally applicable to the shrinking of *S. saman* motor cells (Moran,

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* Corresponding author; e-mail morana@agri.huji.ac.il; fax 972–8–946–7763.

1990), depolarization is not the sole activator of K^+ efflux in these (flexor) cells (Suh et al., 2000), nor is it in guard cells (Blatt, 1990; Lemtiri-Chlieh and MacRobbie, 1994), although the other effector is not known. Direct interaction with Ca^{2+} could be another plausible mode of regulation of K-efflux channels by the PI cascade. Cytosolic Ca^{2+} did promote the activation of K^+-efflux channels in the plasma membrane in corn suspension cell protoplasts (Ketchum and Poole, 1991), in the alga *Mougeotia* (Lew et al., 1990), and in the alga *Eremosphera viridis* (Bauer et al., 1998). In guard cells, K_{out} channels were reported by some to be Ca^{2+} insensitive (Hosoi et al., 1988; Schroeder and Hagiwara, 1989; Lemtiri-Chlieh and MacRobbie, 1994), whereas others reported their inhibition by cytosolic Ca^{2+} of 200 nM (relative to 2 nM; Fairley-Grenot and Assmann, 1992). In addition, Ca^{2+} entry enhanced the rundown of outward-rectifying K^+ channels in pulvinar cells of *Mimosa pudica* (Stoeckel and Takeda, 1995).

In view of these different possibilities, and since the coupling of the PI cascade to K_D channels in shrinking *S. saman* motor cells has not yet been resolved, the sensitivity of flexor and extensor K_D channels to Ca^{2+} needs to be examined. Moreover, since the initiation of shrinking of *S. saman* motor cells is linked to a different photoreceptor in the flexors and extensors, the question arises whether, in each cell type, this cascade is linked differently also at the effector end, to the K^+-efflux channels. In fact, a detailed comparison between the K_D channels of the two cell types has not been carried out and in spite of their mutual resemblance noticed so far (Moran et al., 1988, 1990), these K^+-efflux channels might not even be the same molecular entities in flexors and extensors. For example, the two outward-rectifying K^+ channels, KCO1 and SKOR1, cloned recently from Arabidopsis, display superficially similar behavior in heterologous expression systems (e.g. are activated by depolarization exceeding the K^+ reversal potential [E_{rev}]), although they are encoded by genes from different potassium channel families (Czempinski et al., 1997; Gaymard et al., 1998). Such a possibility merits the comparison of the flexor and extensor K^+-efflux channels.

To address the above questions, we examined the cytosolic [Ca^{2+}] dependence of K_D channels by patch clamp, in inside-out patches excised from both extensor and flexor cells. In this configuration, the [Ca^{2+}] in the vicinity of the channel is controlled much more strictly than in the whole-cell configuration, where the channels in the plasma membrane are in a rather close proximity to the Ca^{2+}-storing (and potentially Ca^{2+}-releasing) organelles: vacuole, mitochondria, chloroplasts, and endoplasmic reticulum. We investigated the Ca^{2+} dependence of the outward-rectifying plant K^+ channels in the plasma membrane at a single channel level. To our knowledge, this is the first such detailed analysis of higher plant K^+-efflux channels in situ. This analysis revealed differences in three properties between the flexor and the extensor K_D channels: in Ca^{2+} sensitivity, in K^+ selectivity, and in voltage sensitivity.

RESULTS

Cytosolic Ca^{2+} Affects Flexor K_D Channel Gating

The activity of single K_D channels in a representative patch from an extensor protoplast, with 600 nM free Ca^{2+} at the cytoplasmic side, is shown in Figure 1. As "befits" K_D channels, the channel activity increased with depolarization. At a saturating depolarization, eight channels were open simultaneously in this patch. From the linear unitary current-voltage relationships we were able to deduce the E_{rev} of −79 mV (Fig. 1B), and the mean single-channel conductance (γ_S) of 17.5 pS (the slope of i_S-E_M, the idealized single-channel current-voltage relationship; Fig. 1C). From the proximity of E_{rev} to E_K (−79 and −78 mV, respectively), we concluded that these channels were K^+ selective {for comparison, the respective equilibrium potentials of the other, potentially permeant, ions—Cl^-, H^+, and Ca^{2+}—were: E_{Cl}, +117 mV; E_H, +72 mV; and E_{Ca}, −13 to +244 mV, depending on the value of the cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_{cyt}$)}.

To evaluate their steady-state gating properties, we plotted the mean number of open channels ($\bar{n}$), versus membrane potential (E_M) and fitted these data with the Boltzmann relationship (Eq. 3; Fig. 1D). At a non-saturating potential of −30 mV, a fraction of K_D channels was usually active. We quantified this activity in patches from 11 extensor and 10 flexor protoplasts, in different $[Ca^{2+}]_{cyt}$, in terms of $\bar{n}_{@-30}$, the $\bar{n}$ at −30 mV, which was read off the Boltzmann curve (Fig. 1D). $\bar{n}_{@-30}$ values were then pooled into five groups of three to seven patches each, according to subranges of $[Ca^{2+}]_{cyt}$ (Fig. 2; "Materials and Methods"). Data from individual patches were connected by lines, to reveal potential trends. We detected no consistent effect of $[Ca^{2+}]_{cyt}$ in the individual patches (Fig. 2, A and B). However, among the averaged values of $\bar{n}_{@-30}$ at the different $[Ca^{2+}]_{cyt}$, flexor $\bar{n}_{@-30}$ values at 15 nM were significantly larger than those at 600 nM (Fig. 2C). Since, potentially, inhibitory effects of $[Ca^{2+}]_{cyt}$ may have masked promoting effects of $[Ca^{2+}]_{cyt}$ on K_D channel opening, we took advantage of the resolution of single channel data to examine such effects separately on the individual gating properties of the K_D channels. We tested the effect of $[Ca^{2+}]_{cyt}$ on the classically defined steady-state properties of channel gating (the half-maximum-activation voltage, the mean number of channels open at saturation potentials, and the effective number of gating charges, z [Eq. 4]; Hille, 1992). In addition, we examined the properties of K^+ permeation through the open channel pore (the γ_S and channel selectivity).

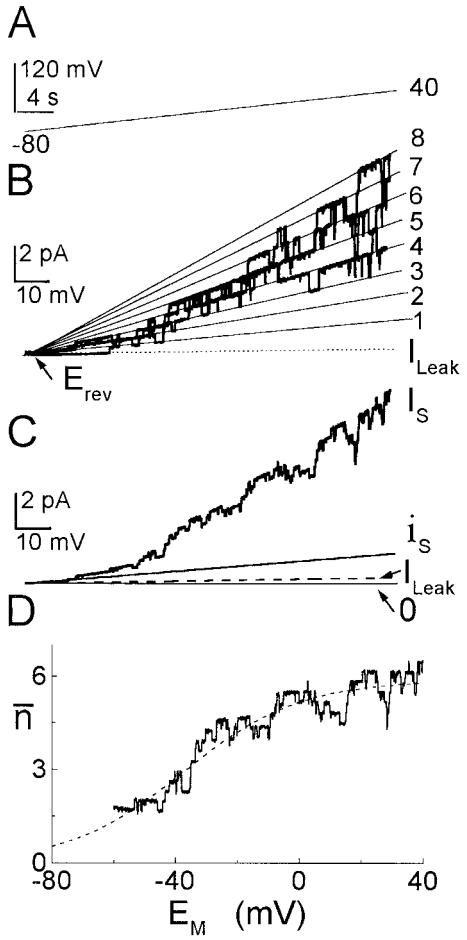

Figure 1. Unitary outward K^+ currents via K_D channels versus E_M, from a representative "inside-out" patch of a *S. saman* extensor cell protoplast, during a slow voltage ramp. A, A linearly increasing voltage ramp applied to the patch membrane. B, Three traces of current (superimposed) during the voltage ramp. Note the "up and down steps" signifying opening and closing of K_D channels. Straight lines—fitted manually to open-channel currents—indicate idealized current levels through "n" (nos. to the right) simultaneously open K_D channels. Note the increase of "n" with the increased depolarization. I_{Leak}, Current recorded when all K_D channels are closed. E_{rev}, −79 mV. C, Initial analysis. I_S, Mean of three current records; i_S, idealized unitary current through a single open channel; I_{Leak}, as in B; 0, the level of zero current. D, Voltage dependence of K_D channel activation. The $\bar{n}$ was calculated as a point-by-point ratio of currents in C (corrected for leak; Eq. 2). Note that $\bar{n}$ increases with membrane depolarization. Dashed line, Boltzmann relationship (Eq. 4), with the following parameters (see "Materials and Methods"): $\bar{n}_{max}$ = 5.9; $E_{1/2}$ = −37 mV; z = 1.4.

$\bar{n}_{max}$ ($\bar{n}$ at saturation potentials) varied considerably from patch to patch, in both cell types, much more than most of the other parameters (Fig. 3), resembling the same phenomenon already noted in broad bean guard cells (Ilan et al., 1994). In extensor cells, $[Ca^{2+}]_{cyt}$ did not have any significant effect on $\bar{n}_{max}$. In flexor cells, $\bar{n}_{max}$ was significantly smaller at $[Ca^{2+}]_{cyt}$ of 1 to 3 mM than at 5 to 67 nM (Fig. 3), and this conclusion holds even if the data from two flexor patches with the most extreme values of $\bar{n}_{max}$ at these low concentrations are ignored. Since $\bar{n}_{max}$ is a product of the total number of channel proteins in the membrane (N), and the voltage-independent probability of their opening (f_O; Ilan et al., 1996), either N or f_O (or both) could be responsible for the $[Ca^{2+}]_{cyt}$-induced $\bar{n}_{max}$ decrease. However, N and f_O can be resolved only in very prolonged recordings in saturation voltages, which was impractical in our experiments. $\bar{n}_{max}$, averaged over the physiological $[Ca^{2+}]_{cyt}$ of 20 to 600 nM, was approximately 11 in flexors, significantly more than approximately 5, in extensors (Table I). Since the flexors and extensors do not differ in size (Moran et al., 1988) or in the values of whole-cell steady-state current levels (not shown), they would be expected to have the same K_D channel density. Further work is required to reconcile this with the observed difference in $\bar{n}_{max}$ between the flexor and extensor membrane patches.

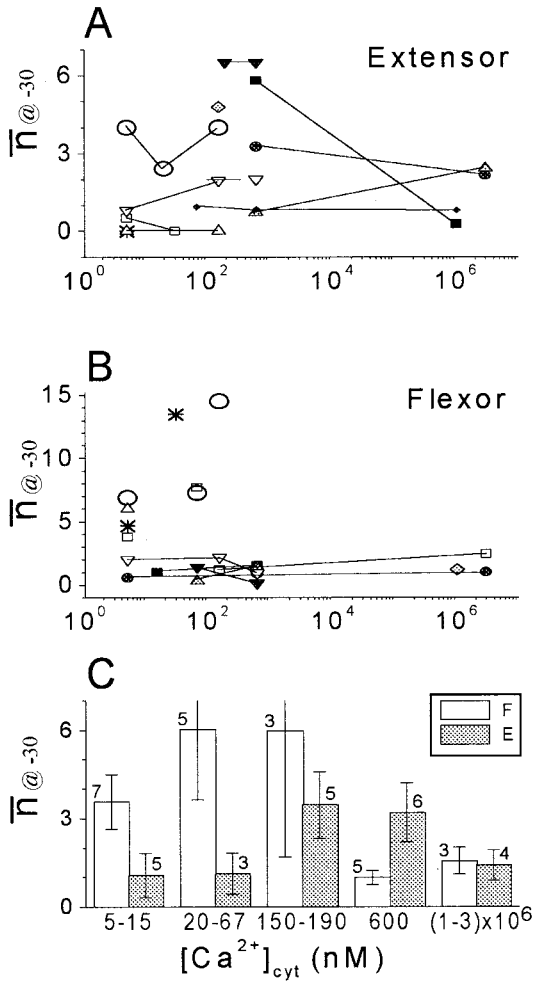

Figure 2. The effect of $[Ca^{2+}]_{cyt}$ on the average activity of K_D channels at −30 mV, $\bar{n}_{@-30}$. Different symbols (connected by lines) denote data from different patches. A, Extensor cells. B, Flexor cells. C, Average values of $\bar{n}_{@-30}$ obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.

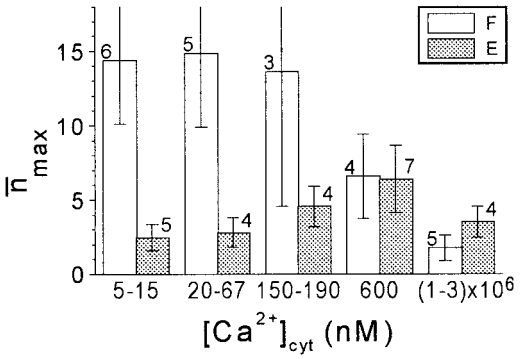

Figure 3. The effect of $[Ca^{2+}]_{cyt}$ on the $\bar{n}_{max}$. Mean values of $\bar{n}_{max}$ obtained from *n* patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.

The values of half-maximum-activation potential ($E_{1/2}$) varied only a little less than $\bar{n}_{max}$ (Fig. 4). Nevertheless, at millimolar $[Ca^{2+}]_{cyt}$, $E_{1/2}$ in flexor cells was significantly smaller than at 5 to 15 nM or at 600 nM (by approximately 40 and approximately 50 mV, respectively). In extensors cells, $E_{1/2}$ did not seem to be affected (Fig. 4). Extensor and flexor cells did not differ significantly in the overall mean values of $E_{1/2}$ at $[Ca^{2+}]_{cyt}$ of 20 to 600 nM, (24 and 14 mV, respectively; Table I).

In contrast, the values of *z* varied very little among patches of one cell type and they did not change with the $[Ca^{2+}]_{cyt}$ (Fig. 5). At the range of 20 to 600 nM, *z* was 1.9 in extensor cells and 1.2 in flexor cells (Table I), indicating that, although in both cell types the gating process involved the cross-membranal movement of at least two electrical charges, the gating of extensor K_D channels was slightly but significantly more voltage sensitive than that of flexor K_D channels.

The Effect of $[Ca^{2+}]_{cyt}$ on K_D Channel Selectivity and Conductance

The two cell types did not differ with respect to the mean values of γ_S (the unitary conductance) at any one of the concentration ranges (Fig. 6). At the range of physiological $[Ca^{2+}]_{cyt}$ of 20 to 600 nM, the mean γ_S was approximately 20 pS (Table I). High $[Ca^{2+}]_{cyt}$ decreased γ_S slightly (by approximately 20%) in both cell types (Fig. 6). Thus in extensor cells at $[Ca^{2+}]_{cyt}$ of 600 nM, γ_S was smaller than at 150 to 190 nM, and in flexor cells at millimolar $[Ca^{2+}]_{cyt}$, γ_S was smaller than at $[Ca^{2+}]_{cyt}$ 190 nM ($P < 0.05$). This decrease of γ_S could be due to open-channel block by Ca^{2+} (e.g. Vergara and Latorre, 1983).

The mean values of E_{rev} (of the i_S), determined at five ranges of $[Ca^{2+}]_{cyt}$ in flexor cells, were largely indistinguishable from the predicted K^+ Nernst potential (E_K) of −78 mM (Fig. 7). In extensor cells, a small though significant deviation of 4 mV from E_K could be noted at the lower $[Ca^{2+}]_{cyt}$ (20–67 nM; Fig. 7). When averaged over the physiological $[Ca^{2+}]_{cyt}$ range of 20 to 600 nM, the mean E_{rev} of extensor cells (−75 mV), was also significantly more positive than that of flexor cells (which was equal to E_K; see also Table I).

Summary

K_D channels in both cell types did not require $[Ca^{2+}]_{cyt}$ for their activity. However, in flexor cells (but not in extensors), the steady-state gating properties were affected by the higher $[Ca^{2+}]_{cyt}$. In addition to the different sensitivity to cytosolic Ca^{2+}, flexors and extensors differed perceptibly in two more details: in the steepness of their voltage dependence (*z*) and in their K^+ selectivity.

DISCUSSION

Differentiation between Flexor and Extensor K_D Channels Based on Ca^{2+} Effects

No information exists, as yet, about the molecular identity of the *S. saman* K^+-efflux channels. Therefore, their functional characterization in planta is needed to provide the database against which such identification will ultimately need to be tested. In particular, sensitivity to Ca^{2+} may be a useful criterion for revealing possible differences between *S. saman* K^+-efflux channels in the two cell types, and between them and other K_{out} channels. Contrary to extensor cells, a gating-promoting effect (a negative shift of $E_{1/2}$) was indeed resolved in flexor cells at millimolar $[Ca^{2+}]_{cyt}$ (Fig. 4). This negative shift of $E_{1/2}$ could be theoretically attributed to non-specific screening of negative surface charges at the internal side of

Table I. *Steady-state properties of the Samanea K_D channels*

Extensor and flexor cells compared at a physiological range of free $[Ca^{2+}]_{cyt}$ (20–600 nM). Permeation properties (resulting from fit of Eq. 1 to the steady-state unitary current-voltage relationship, as in "Materials and Methods" and Fig. 1B). Gating properties (resulting from the fit of Eq. 4 to the steady-state voltage dependence of channel opening, as in "Materials and Methods" and Fig. 1C). Data are means ± SE (no. of cells).

Cell Type	Parameter				
	γ_S	E_{rev}	$\bar{n}_{max}$	$E_{1/2}$	*z*
	pS	*mV*		*mV*	
Extensor	20.9 ± 0.8 (10)	−75 ± 1 (10)	5.3 ± 2 (10)	−24 ± 8 (10)	1.9 ± 0.3 (10)
Flexor	19.6 ± 0.8 (8)	−78 ± 1 (8)	11.2 ± 4 (8)	−14 ± 8 (8)	1.2 ± 0.2 (8)

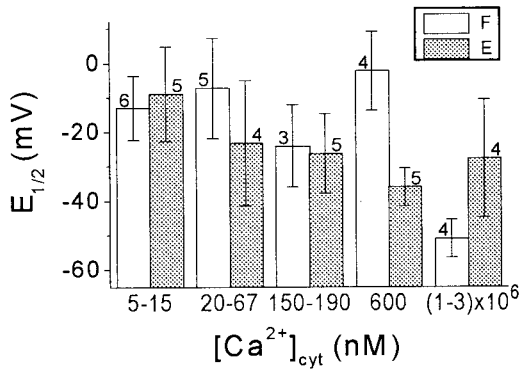

Figure 4. The effect of $[Ca^{2+}]_{cyt}$ on the $E_{1/2}$. Mean values of $E_{1/2}$ obtained from *n* patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.

the membrane by the roughly thousand-fold increase of $[Ca^{2+}]_{cyt}$. We deem it unlikely, however, since, in addition to the high ionic strength of the "internal" solutions, the maximum increase in total divalent ion concentration in our experiments was less than 3-fold (2 mM Mg^{2+} was present in all the "internal" solutions). Both the high ionic strength and the small change in total divalent ion concentration would predict a negligible $E_{1/2}$ shift (Gilbert and Ehrenstein, 1969; Kell and DeFelice, 1988).

A more likely explanation is a specific Ca^{2+} action, by binding. In lieu of any information about the molecular identity of the K_D channel, the target of Ca^{2+} binding remains in the realm of speculation. If we assume direct binding of Ca^{2+} to the flexor K_D channel, we need to assume also that this channel has Ca^{2+} binding domains, such as the "EF hands" in the Arabidopsis KCO1 channel (Czempinski et al., 1997). We may assume alternatively that Ca^{2+} affects the channel indirectly, via other Ca^{2+}-activated proteins. Phosphorylation, for example, has been shown to cause $E_{1/2}$ shifts in voltage-dependent outward-rectifying K^+ channels (Esguerra et al., 1994; Levitan, 1994). Furthermore, phosphorylation can occur even in fragmented membranes (for example, extensor K_D channels were regulated by phosphorylation in excised inside-out patches; Moran, 1996).

Difference in Selectivity between Flexor and Extensor K_D Channels

We were surprised to discover that the extensor K_D channels were less selective toward K^+ than flexor channels (Fig. 7; Table I). This could be due, for example, to a partial permeability of the extensor K_D channels to Ca^{2+} ions. For example, a departure of 4 mV from E_K, with $[Ca^{2+}]_{cyt}$ of 20 nM, may be accounted for by a Ca^{2+} permeability one-third (0.35) as large as the permeability to K^+ (Lewis, 1979). The incomplete selectivity to K^+ of the extensor K_D channel resembles, in fact, that of the K_D (K_{out}) channel in broad bean guard cells (Ilan et al., 1994) and the K_{out} channels of Arabidopsis mesophyll cells (Romano et al., 1998), where E_{rev} values were also several mV above E_K. Thus, although the K_{in} channel in guard cells did not conduct Ca^{2+} influx (Grabov and Blatt, 1998, 1999), this has not been excluded for the K_{out} channels in other plant systems. There are also various weakly Ca^{2+} permeant outward-rectifying K channels in animal systems (e.g. Hille, 1992). A variety of mechanisms could underlie this selectivity difference between flexor and extensor K_D channels, such as mutations, mRNA editing or post-translational modifications, and this remains to be resolved.

For the *S. saman* motor system, the physiological implication of such permeability to Ca^{2+} would be, perhaps, the influx of Ca^{2+} through the K_D channels open during the extensors shrinking phase, and enhancement of extensor shrinking via a positive feedback (activating more chloride channels, increasing depolarization, etc.). This, in turn, would enhance leaflet folding.

Although differing in selectivity, K_D channels of both cell types were very similar in their unitary conductance (Fig. 6). To a first approximation, they were similar also in their kinetics (the latter was determined by fitting the activation and deactivation time courses of currents recorded in a whole-cell configuration with single exponentials, at $[Ca^{2+}]_{cyt}$ of approximately 150 nM; data not shown).

Physiological Relevance of Ca^{2+} Effects on K_D Channels

Since K_D channels are essential to the shrinking of *S. saman* motor cells and therefore, to pulvinar movements (Moran et al., 1988), it could be expected that a significant Ca^{2+} effect on K_D channels would be ultimately reflected in its effects on the movement of leaves. The reported observations in leaf-moving trees related to *S. saman* (*M. pudica, Albizzia lophanta, Cassia fasciculata*, and *Robinia pseudoaccacia*) all support a leaf-movement-enhancing role of Ca^{2+} (Campbell and Thompson, 1977; Roblin and Fleurat-Lessard, 1984; Moysset and Simon, 1989; Gomez and

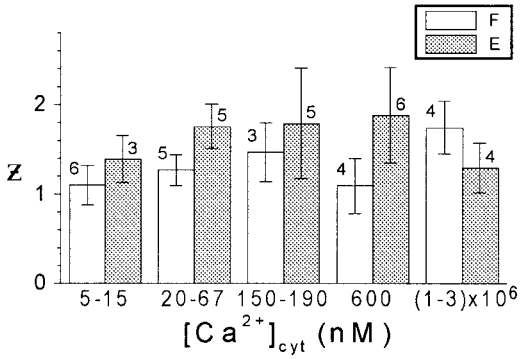

Figure 5. The effect of $[Ca^{2+}]_{cyt}$ on the effective number of charges, *z*. Mean values of *z* obtained from *n* patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.

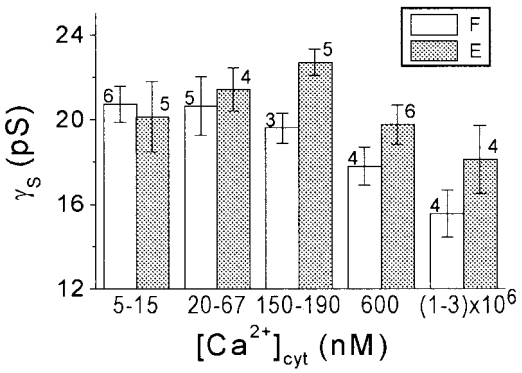

Figure 6. The effect of $[Ca^{2+}]_{cyt}$ on the γ_S. Mean values of γ_S obtained from *n* patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.

Simon, 1995). However, the overall effects of Ca^{2+} on K_D channels in our experiments were either absent or minor. In fact, Ca^{2+} did not appear to be essential at all for the activity of the pulvinar K_D channels, resembling the reported insensitivity of their counterparts in the guard cells. The promoting effect of the negative $E_{1/2}$ shift in flexors at the highest concentrations of $[Ca^{2+}]_{cyt}$ (if such a concentration could be reached in the vicinity of K_D channels) would be probably offset by the decrease of γ_S and of $\bar{n}_{max}$. Thus in whole shrinking pulvinar cells in situ, K_D channels are activated either via a Ca^{2+}-independent mode, or, if $[Ca^{2+}]_{cyt}$ is involved, an additional soluble cytosolic factor (absent in our experiments) may be required to mediate an activating action of Ca^{2+}.

Moreover, at 600 nM in flexors, the apparent effect of $[Ca^{2+}]_{cyt}$ on the gating of K_D channel was a depression of activity: a small but significant decrease of $\bar{n}_{@-30}$ (relative to that at the lower concentration of 5–15 nM). This effect on $\bar{n}_{@-30}$ was consistent with the observation of rundown of pulvinar K_D channels caused by Ca^{2+} influx in a *S. saman* close relative, *M. pudica* (Stoeckel and Takeda, 1995). Based on our findings in *S. saman*, it might be expected that increased cytosolic Ca^{2+} would decrease flexor K_D channel activity and consequently impede flexor cell shrinking and leaf unfolding. This prediction is in conflict with the reported enhancement of leaf movements by Ca^{2+}. Is it possible that $[Ca^{2+}]_{cyt}$ level during flexor shrinking does not even reach the depressing concentration of 600 nM? This remains to be determined directly during the motor cell volume changes.

MATERIALS AND METHODS

Plant Material

Samanea saman (Jacq.) Merr. trees (recently referred to also as *Pithecellobium saman* [Jacq.] Benth.; Little and Wadsworth, 1964), were grown in a greenhouse under a 16-h-light/8-h-dark schedule; leaves were harvested and protoplasts were isolated as described previously (Moran, 1996). The procedure for protoplast isolation has been further modified to include an additional rinse of the freshly chopped tissue pieces on a 20-μm mesh filter with solution containing 0.1% (w/v) polyvinylpyrrolidone to neutralize the possible effects of endogenous phenolics.

Patch-Clamp Experimental Procedure

Patch-clamp experiments were performed in a standard inside-out configuration (Hamill et al., 1981; Moran, 1996). Patch-clamp pipettes were prepared from borosilicate glass (catalog no. BF150–86–10, Sutter Instrument, Novato, CA) by a two-stage pull and fire polishing (both the micropipette puller and microforge were from Narashige [Tokyo]). The pipette was filled with an external solution. The bridge of the reference electrode was filled with an internal solution. After establishing a tight seal with the cell membrane, the bath was flushed with 10 volumes of the internal solution and the patch was excised into an inside-out configuration. The Ca^{2+} concentration of the bath solution was changed by flushing at least 10 volumes of the new solution. The order of Ca^{2+} concentrations applied was varied to eliminate systematic error due to possible time dependence (such as rundown or up-regulation). The i_S current was filtered at 20 Hz (the −3db cutoff frequency of a four-pole Bessel filter), and digitized at a sampling rate of 50 Hz (Axon Instruments, Foster City, CA). To simplify comparisons with published experiments performed in different configurations, channel openings and current directed outward (with respect to the membrane) are shown as positive upward deflections from the closed-level (baseline) current. Likewise, in all of the experiments presented here, depolarization means increasing (more positive) potential at the cytoplasmic side.

The stimulation protocols were as follows. K_D channels were activated by depolarization, applied in the form of ramps (Fig. 1A). The ramps were 40 s long and varied linearly with time from −80 to +40 mV. Channel activity was assumed to have attained a steady state at each point during this slow rate of change of the E_M (3 mV s^{-1}). Between the depolarizations, the membrane was held for 20 s at a "resting" or "holding" potential of −80 or −100

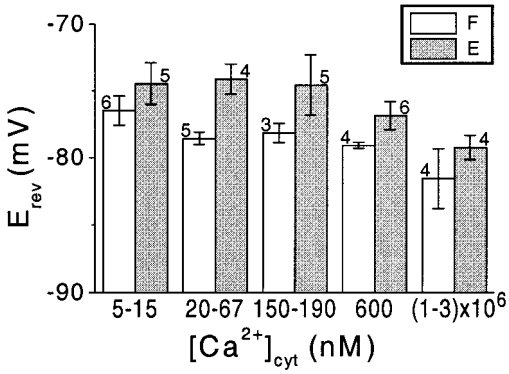

Figure 7. The effect of $[Ca^{2+}]_{cyt}$ on the E_{rev} of the unitary currents. Mean values of E_{rev} obtained from *n* patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.

mV (cytoplasmic side negative), at which K_D channels were closed (Moran et al., 1988; Moran, 1996).

Analysis of Patch-Clamp Data

Determination of the Unitary Conductance and E_{rev} in Inside-Out Patches

The slow voltage ramps and the resulting linear current-voltage (i_S-E_M) relationships between the i_S and the E_M served for the simultaneous determination of the E_{rev}, the unitary conductance and the steady-state level of channel activity (Moran, 1996; Suh et al., 2000):

$$i_S = \gamma_S(E_M - E_{rev}) \quad (1)$$

The E_{rev} was obtained as the common zero-current intercept of several linear regressions (fitted by eye) to the different levels of open-channel current-voltage data points (Fig. 1B). γ_S, the slope of the idealized i_s-E_M relationship of the single channel, was obtained by averaging the differences between the slopes of the linear regressions.

Characterization of Voltage-Dependent Gating in Single-Channel Patches

The total average current through the channels in the patch, I_S, is the function of the average number of open K_D channels, $\bar{n}$, in the patch (Eq. 2):

$$I_S = \bar{n} \cdot i_S + I_{Leak} \quad (2)$$

where I_{Leak} is the linearly fitted baseline (leak current). $\bar{n}$ at each E_M value was calculated by dividing I_S (after the subtraction of I_{Leak}), point by point, by the idealized unitary open-channel current, i_S (Fig. 1C). Provided that the channels are identical and statistically independent (Ehrenstein et al., 1970),

$$\bar{n} = \bar{n}_{max} \cdot P_O \quad (3)$$

where P_O is the voltage-dependent open probability of open channels.

The resulting steady-state $\bar{n}$-E_M relationship (reflecting the P_O-E_M relationship) was then fitted with the Boltzmann relationship

$$\bar{n} = \bar{n}_{max}/(1 + e^{-zF(E_M - E_{1/2})/RT}) \quad (4)$$

These Boltzmann parameter values were obtained for each treatment, 10 to 15 min after a change of solutions. The calculations and fit were performed on the data in the voltage range of −60 to +40 mV, using the commercially available program Origin (Microcal Software, Northampton, MA).

Statistics

Each characteristic parameter of the K_D channels derived from single-channel data (γ_S, E_{rev}, $E_{1/2}$, etc.) initially was examined separately in each cell, at various $[Ca^{2+}]_{cyt}$ concentrations (Fig. 2, A and B). We then grouped the data from all of the experiments in five concentration ranges, 5 to 15 nM, 20 to 67 nM, 150 to 190 nM, 600 nM, and 1 to 3 mM, to compare mean values (Fig. 2C). However, when comparing between flexor and extensor cells, we averaged the data from one "physiological" range of 20 to 600 nM (Table I). Whenever data were pooled together and averaged, a cell contributed no more than once to each average (a single value, or a mean, if there were more determinations than just one from a single patch in a given concentration range). Means are presented with their SES, with n, the number of cells averaged. Differences between means were deemed significant if, using a two-sided Student's t test, $P < 0.05$.

Solutions

The regular extracellular solution contained 5 mM K^+, 9.5 mM MES, at pH 6.0, and 1 mM $CaCl_2$, and was adjusted with sorbitol to osmolarity of 700 mOsm. The cytoplasmic surface was exposed to "internal solution": 125 mM KCl, 20 mM HEPES, 1 mM MgATP and 1 mM $MgCl_2$ (or 1 K_2ATP and 2 mM $MgCl_2$), 2 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-K_4, and variable total $CaCl_2$. The desired concentrations of 600 nM free Ca^{2+}, were calculated using the following equation:

$$[Ca^{2+}]_{cyt} = \frac{k_d}{B_T/Ca_T - 1} \quad (5)$$

where Ca_T is the total concentration of Ca^{2+}, B_T is the total concentration of BAPTA, and k_d is the dissociation constant of BAPTA of 200 nM (in the presence of 2 mM Mg and 0.1 M KCl; Pehtig et al., 1989). One or 3 mM Ca^{2+} was prepared by addition of 1 or 3 mM Ca^{2+} in excess of 2 mM BAPTA. The osmolarity of the "internal solution" was adjusted with sorbitol to 750 mOsm. After addition of ATP and BABTA, the "internal solution" was adjusted with *N*-methylglucamine to pH 7.0 to 7.3 and used within a week of preparation. BAPTA was from Molecular Probes (Eugene, OR) or from Sigma (St. Louis). Other chemicals were from Sigma, Merck (Rahway, NJ), or BDH (AnalaR, Poole, UK).

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LITERATURE CITED

Assmann SM, Shimazaki K (1999) The multisensory guard cell: stomatal responses to blue light and abscisic acid. Plant Physiol **119:** 809–815

Bauer CS, Plieth C, Hansen UP, Simonis W, Schoenknecht G (1998) A steep Ca^{2+}-dependence of a K^+ channel in a unicellular green alga. J Exp Bot **49:** 1761–1765

Berridge MJ (1997) Elementary and global aspects of calcium signalling. J Physiol **499:** 291–306

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. Nature **341:** 197–205

Blatt MR (1990) Potassium channel currents in intact stomatal guard cells: rapid enhancement by abscisic acid. Planta **180:** 445–455

Blatt MR, Thiel G, Trentham DR (1990) Reversible inactivation of K^+ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. Nature **346:** 766–769

Campbell NA, Thompson WW (1977) Effects of lantanum and ethylenediaminetetraacetate on leaf movements of *Mimosa pudica*. Plant Physiol **60:** 635–639

Czempinski K, Zimmermann S, Ehrhardt T, Mueller-Roeber B (1997) New structure and function in plant K^+ channels: KCO1, an outward rectifier with a steep Ca^{2+} dependency. EMBO J **16:** 2565–2575

Ehrenstein G, Lecar H, Nossal R (1970) The nature of the negative resistance in bimolecular lipid membranes containing excitability-inducing material. J Gen Physiol **55:** 119–133

Esguerra M, Wang J, Foster CD, Adelman JP, North RA, Levitan IB (1994) Cloned Ca^{2+}-dependent K^+ channel modulated by a functionally associated protein kinase. Nature **369:** 563–565

Fairley-Grenot KA, Assmann SM (1992) Whole-cell K^+ current across the plasma membrane of guard cells from a grass: *Zea mays*. Planta: 282–293

Gaymard F, Lacombe B, Bouchez D, Bruneau D, Boucherez J, Michaux-Ferriere N, Thibaud JB, Sentenac H (1998) Identification and disruption of a plant *Shaker*-like outward channel involved in K^+ release into the xylem sap. Cell **94:** 647–655

Gilbert DL, Ehrenstein G (1969) Effect of divalent cations on potassium conductance of squid axons: determination of surface charge. Biophys J **9:** 447–463

Gilroy S, Read ND, Trewavas AJ (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature **346:** 769–771

Gomez LA, Simon E (1995) Circadian rhythm of *Robinia pseudoacacia* leaflet movements: role of calcium and phytochrome. Photochem Photobiol **61:** 210–215

Grabov A, Blatt MR (1998) Membrane voltage initiates Ca^{2+} waves and potentiates Ca^{2+} increases with abscisic acid in stomatal guard cells. Proc Natl Acad Sci USA **95:** 4778–4783

Grabov A, Blatt MR (1999) A steep dependence of inward-rectifying potassium channels on cytosolic free calcium concentration increase evoked by hyperpolarization in guard cells. Plant Physiol **119:** 277–287

Hamill OP, Marty A, Neher E, Sakman B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch **391:** 85–100

Hille B (1992) Ionic Channels of Excitable Membranes. Sinauer Associates, Sunderland, MA

Hodgkin AL, Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol **117:** 500–544

Hosoi S, Lino M, Shimazaki KI (1988) Outward-rectifying K^+ channels in stomatal guard cell protoplasts. Plant Cell Physiol **29:** 907–911

Ilan N, Schwartz A, Moran N (1994) External pH effects on the depolarization-activated K channels in guard cell protoplasts of *Vicia faba*. J Gen Physiol **103:** 807–831

Ilan N, Schwartz A, Moran N (1996) External protons enhance the activity of the hyperpolarization-activated K channels in guard cell protoplast of *Vicia faba*. J Membr Biol **154:** 169–181

Irving HR, Gehring CA, Parish RW (1992) Changes in cytosolic pH and calcium of guard cells precede stomatal movements. Proc Natl Acad Sci USA **89:** 1970–1994

Kell MJ, DeFelice LJ (1988) Surface charge near the cardiac inward-rectifier channel measured from single-channel conductance. J Membr Biol **102:** 1–10

Ketchum KA, Poole RJ (1991) Cytosolic calcium regulates a potassium current in corn (*Zea mays*) protoplasts. J Membr Biol **119:** 277–288

Kim HY, Cote GG, Crain RC (1993) Potassium channels in *Samanea saman* protoplasts controlled by phytochrome and the biological clock. Science **260:** 960–962

Kim HY, Cote GG, Crain RC (1996) Inositol 1,4,5-trisphosphate may mediate regulation of K^+ channels by light and darkness in *Samanea saman* motor cells. Planta 279–289

Lee YS, Choi YB, Suh S, Lee J, Assmann SM, Joe CO, Kelleher JF, Crain RC (1996) Abscisic acid-induced phosphoinositide turnover in guard cell protoplasts of *Vicia faba*. Plant Physiol **110:** 987–996

Lemtiri-Chlieh F, MacRobbie EA (1994) Role of calcium in the modulation of *Vicia* guard cell potassium channels by abscisic acid: a patch-clamp study. J Membr Biol **137:** 99–107

Levitan IB (1994) Modulation of ion channels by protein phosphorylation and dephosphorylation. Annu Rev Physiol **56:** 193–212

Lew RR, Serlin BS, Schauf CL, Stockton ME (1990) Calcium activation of *Mougeotia* potassium channels. Plant Physiol **92:** 831–836

Lewis CA (1979) Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. J Physiol **286:** 417–445

Little ERJ, Wadsworth FH (1964) Common Trees of Puerto Rico and the Virgin Islands. U.S. Department of Agriculture, Washington, DC

Lowen CZ, Satter RL (1989) Light-promoted changes in apoplastic potassium activity in the *Samanea saman* pulvinus, monitored with liquid membrane microelectrodes. Planta **179:** 421–427

MacRobbie EAC (1998) Signal transduction and ion channels in guard cells. Philos Trans R Soc Lond **353:** 1475–1488

McAinsh MR, Brownlee C, Hetherington AM (1990) Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. Nature **343:** 186–188

Moran N (1990) The role of ion channels in osmotic volume changes in *Samanea* motor cells analyzed by patch-clamp methods. *In* RL Satter, HL Gorton, TC Vogelmann, eds, The Pulvinus: Motor Organ for Leaf Movement, Ed 1. American Society of Plant Physiologists, Rockville, MD, pp 142–158

Moran N (1996) Membrane-delimited phosphorylation enables the activation of the outward-rectifying K channels in a plant cell. Plant Physiol **111:** 1281–1292

Moran N, Ehrenstein G, Iwasa K, Mischke C, Bare C, Satter RL (1988) Potassium channels in motor cells of *Samanea saman*: a patch-clamp study. Plant Physiol **88:** 643–648

Moran N, Fox D, Satter RL (1990) Interaction of the depolarization-activated K channel of *Samanea saman* with inorganic ions: a patch-clamp study. Plant Physiol **94:** 424–431

Moysset L, Simon E (1989) Role of calcium in phytochrome-controlled nyctinastic movements of *Albizzia lophantha* leaflets. Plant Physiol **90:** 1108–1114

Pehtig R, Kuhn M, Payne R, Adler E, Chen T-H, Jaffe LF (1989) On the dissociation constants of BAPTA-type calcium buffers. Cell Calcium **10:** 491–498

Roblin G, Fleurat-Lessard P (1984) A possible mode of calcium involvement in dark- and light-induced leaflet movements in *Cassia fasciculata* Michx. Plant Cell Physiol **25:** 1495–1499

Romano LA, Miedema H, Assmann SM (1998) Ca^{2+}-permeable, outwardly-rectifying K^+ channels in mesophyll cells of *Arabidopsis thaliana*. Plant Cell Physiol **39:** 1133–1144

Satter RL, Galston AW (1981) Mechanisms of control of leaf movements. Annu Rev Plant Physiol **32:** 83–110

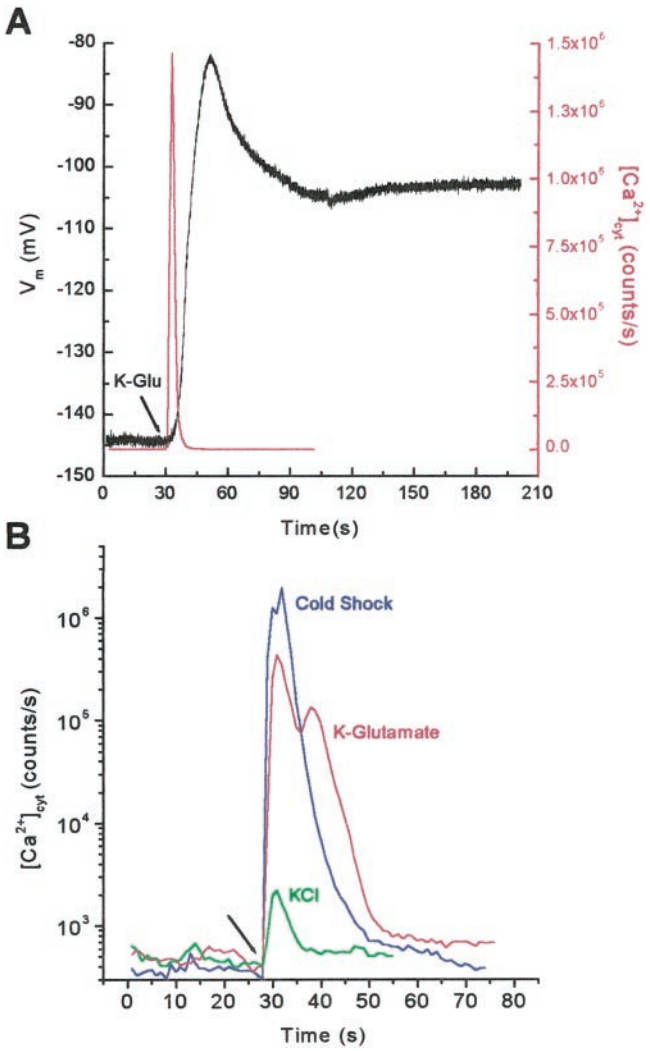
Satter RL, Morse MJ, Lee Y, Crain RC, Cote G, Moran N (1988) Light and clock-controlled leaflet movements in *Samanea saman*: a physiological, biophysical and biochemical analysis. Bot Acta **101:** 205–213

Schroeder JI, Hagiwara S (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. Nature **338:** 427–430

Stoeckel H, Takeda K (1995) Calcium-sensitivity of the plasmalemmal delayed rectifier potassium current suggests that calcium influx in pulvinar protoplasts from *Mimosa pudica* L. can be revealed by hyperpolarization. J Membr Biol **146:** 201–209

Suh S, Moran N, Lee Y (2000) Blue light activates depolarization-dependent K^+ channels in flexor cells from *Samanea saman* motor organs via two mechanisms. Plant Physiol **123:** 833–843

Vergara C, Latorre R (1983) Kinetics of Ca^{2+}-activated K^+ channels from rabbit muscle incorporated into planar bilayers: evidence for a Ca^{2+} and Ba^{2+} blockade. J Gen Physiol **82:** 543–568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2.5x10^5
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[Ca^{2+}]$_{cyt}$ (counts/s)
K-Glu
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Time(s)
B
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10^4
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[Ca^{2+}]$_{cyt}$ (counts/s)
Cold Shock
K-Glutamate
KCl
0
10
20
30
40
50
60
70
80
Time (s)

CORRECTIONS

Vol. 124: 935–939, 2000

Schopfer, C.R., and Nasrallah, J.B. Self-Incompatibility. Prospects for a Novel Putative Peptide-Signaling Molecule.

Figure 2 was erroneously printed in black and white. Figure 2 has been reprinted in color on p 2204.

Vol. 124: 1007–1017, 2000

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., and Mitchell-Olds, T. Induced Plant Defense Responses against Chewing Insects. Ethylene Signaling Reduces Resistance of Arabidopsis against Egyptian Cotton Worm But Not Diamondback Moth.

The GenBank accession number of the β-glucosidase gene was not included when this article was first published. The GenBank accession number is AJ251301.

Vol. 124: 1511–1514, 2000

Dennison, K.L., and Spalding, E.P. Glutamate-Gated Calcium Fluxes in Arabidopsis.

Figure 1 was erroneously printed in black and white in the original publication and again in Vol. 125 on p 1151. Figure 1 has been reprinted in color on p 2205.

Vol. 124: 1532–1539, 2000

Gibson, S.I. Plant Sugar-Response Pathways. Part of a Complex Regulatory Web.

In Table I, the line "*sis5* Is allelic to *aba4*" should have appeared as "*sis5* Is allelic to *abi4*." Table I has been reprinted on p 2206.

Vol. 125: 15–19, 2001

Meyerowitz, E.M. Prehistory and History of Arabidopsis Research.

Professor Georges Bernier of the Universite de Liege (Belgium) kindly sent the following corrections for the photographs that appeared as Figures 1 and 2. In Figure 1, the last person on the right of the first row is Silvano Bonotto, not J. Bouharmont; in the third row, between A.R. Kranz and M. Jacobs, the unidentified person is J. Bouharmont. In Figure 2, in the back row, the person identified as Matigne is in fact R. Matagne. We welcome any additional information on the names of those who appear in the photographs.

Vol. 125: 329–338, 2001

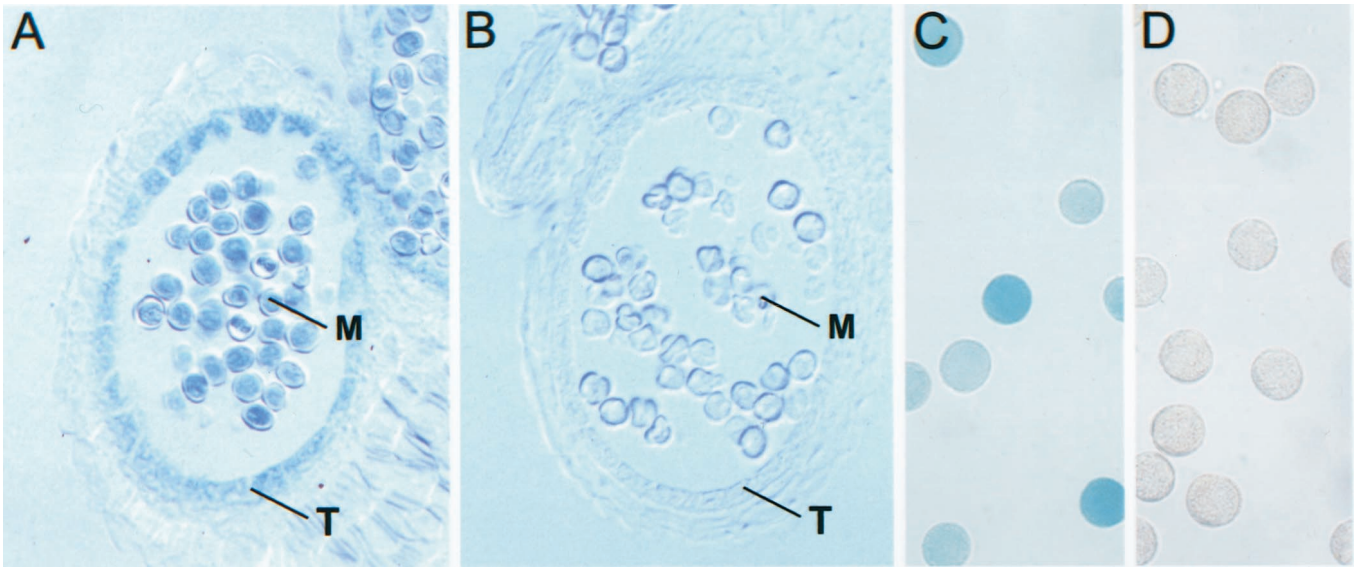
Taylor, A.R., and Assmann, S.M. Apparent Absence of a Redox Requirement for Blue Light Activation of Pump Current in Broad Bean Guard Cells.

Figures 2, 3, and 4 were not printed in the correct order. The correctly numbered figures with legends are reprinted on pp 2207–2209.

Acknowledgment

Vol. 125: No. 1, ii, 2001

We would like to acknowledge Jan Zeevaart, who supplied the photograph of the morning glory flower that appears on the cover of the January 2001 75th Anniversary Special Issue.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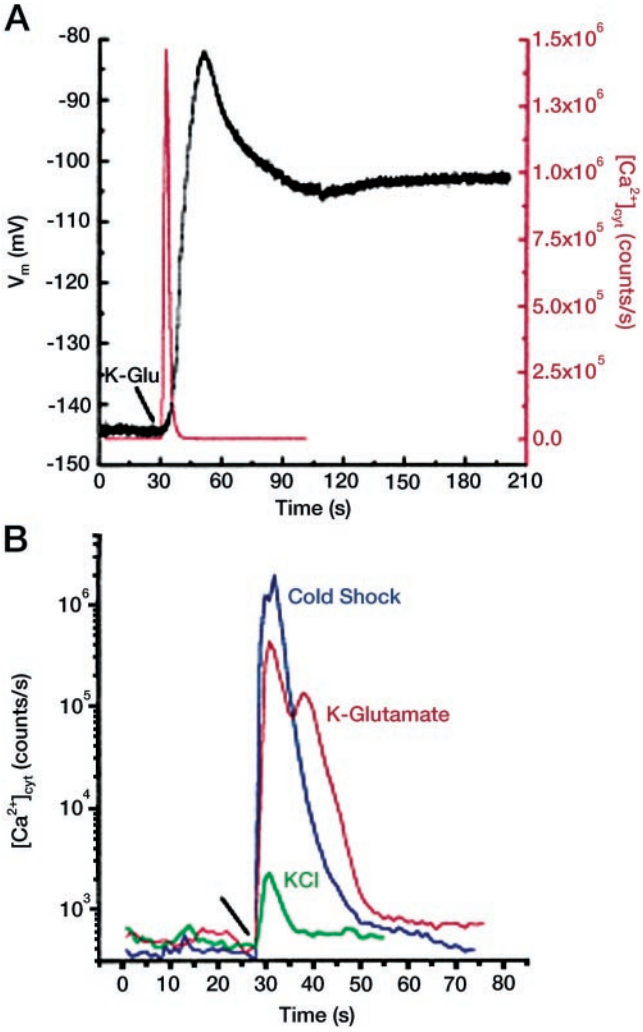
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Time (s)
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1.3x10^6
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7.5x10^5
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2.5x10^5
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10^4
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$[Ca^{2+}]_{cyt}$ (counts/s)
Cold Shock
K-Glutamate
KCl
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Time (s)

Table I. *Sugar-response mutants and corresponding loci*

Mutants	Originals Selection	Loci	References
rsr	Reduced sensitivity to Suc induction of patatin expression		Martin et al., 1997
lba	Reduced sensitivity to Suc induction of β-amylase expression		Mita et al., 1997b
hba	Increased sensitivity to Suc induction of β-amylase expression		Mita et al., 1997a
sun	Reduced sensitivity to Suc repression of plastocyanin expression	*sun6* ls allelic to *abi4*	Dijkwel et al., 1997; Huijser ete al., 2000
sis	Reduced sensitivity to Glc or Suc-mediated inhibition of early seedling development	*sis1* ls allelic to *ctr1* *sis4* ls allelic to *aba2* *sis5* ls allelic to *abi4*	Laby et al., 2000; S. Gibson, R. Laby, and D. Kim, unpublished data
gin	Reduced sensitivity to Glc-mediated inhibition of early seedling development	*gin1* ls allelic to *aba2* *gin6* ls allelic to *abi4*	Zhou et al., 1998; Arenas-Huertero et al., 2000; J. Sheen, personal communication
prl	Increased sensitivity to sugar-mediated inhibition of early seedling development	*PRL1* Encodes a WD-40 protein	Németh et al., 1998; Bhalerao et al., 1999

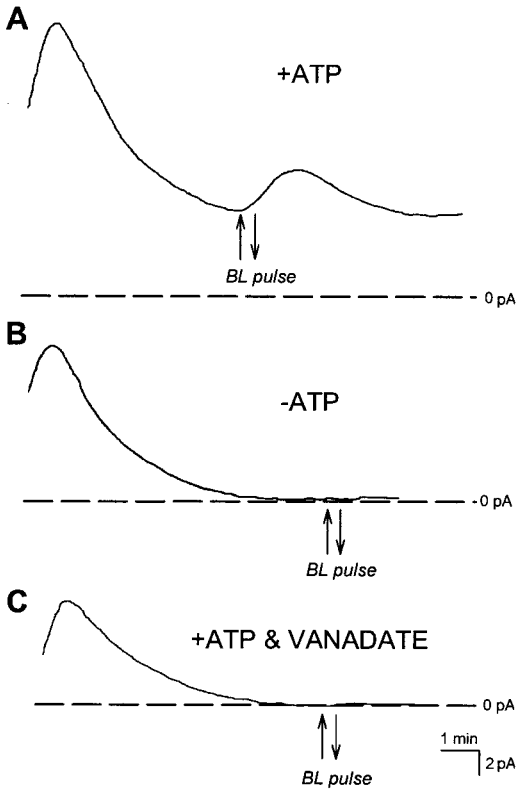

Figure 2. Steady-state- and BL-stimulated pump currents require ATP and are inhibited by vanadate. A, A typical recording with 5 mM ATP in the pipette under saturating RL. The cell responded to a 30-s pulse of BL with a typical transient increase in pump current. B, When ATP is absent from the pipette, cell currents quickly decay to 0 pA under saturating RL and are unresponsive to a pulse of BL. C, Inclusion of ATP and 20 μM vanadate in the pipette causes inhibition of pump current. All cells where pump current was inhibited by vanadate were unresponsive to BL pulses.

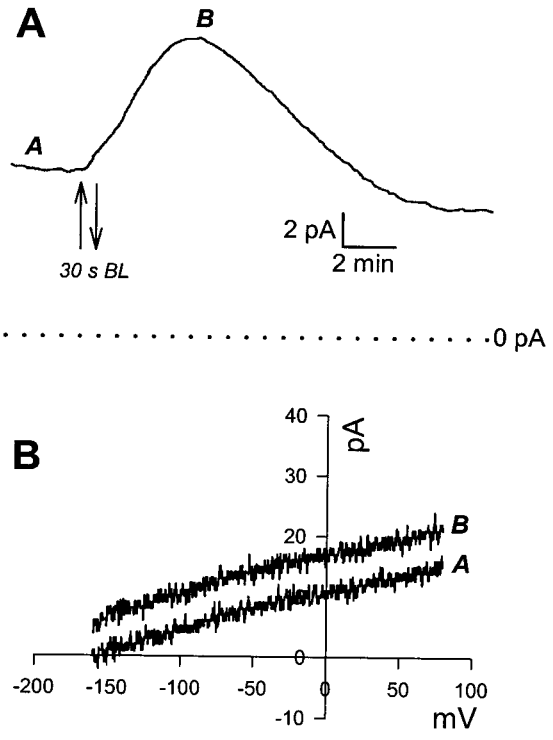

Figure 3. H^+-ATPase activation by a pulse of BL. Saturating RL background illumination was switched on before the beginning of the trace. A, Once stable baseline current is achieved a pulse of BL causes a transient increase in pump current. B, I/V ramps conducted before (*A*) and at the peak (*B*) of the response in A show the parallel shunt in pump current.

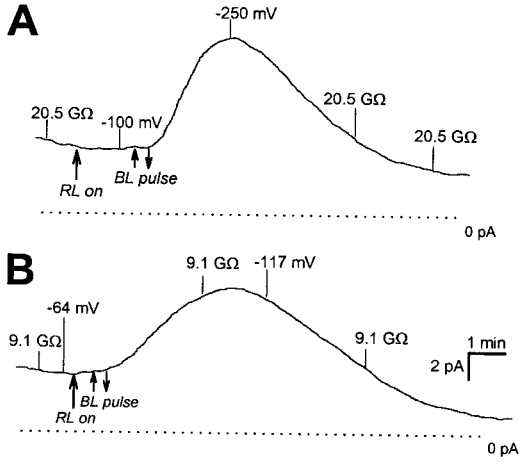

Figure 4. The effect of plasma membrane H^+-ATPase currents on membrane potential. The two traces show the pump current measured with ATP in the pipette. Membrane potential and input resistance are indicated on the traces at steady state and during BL-activated stimulation of pump current. Note the insensitivity to saturating RL illumination.