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.
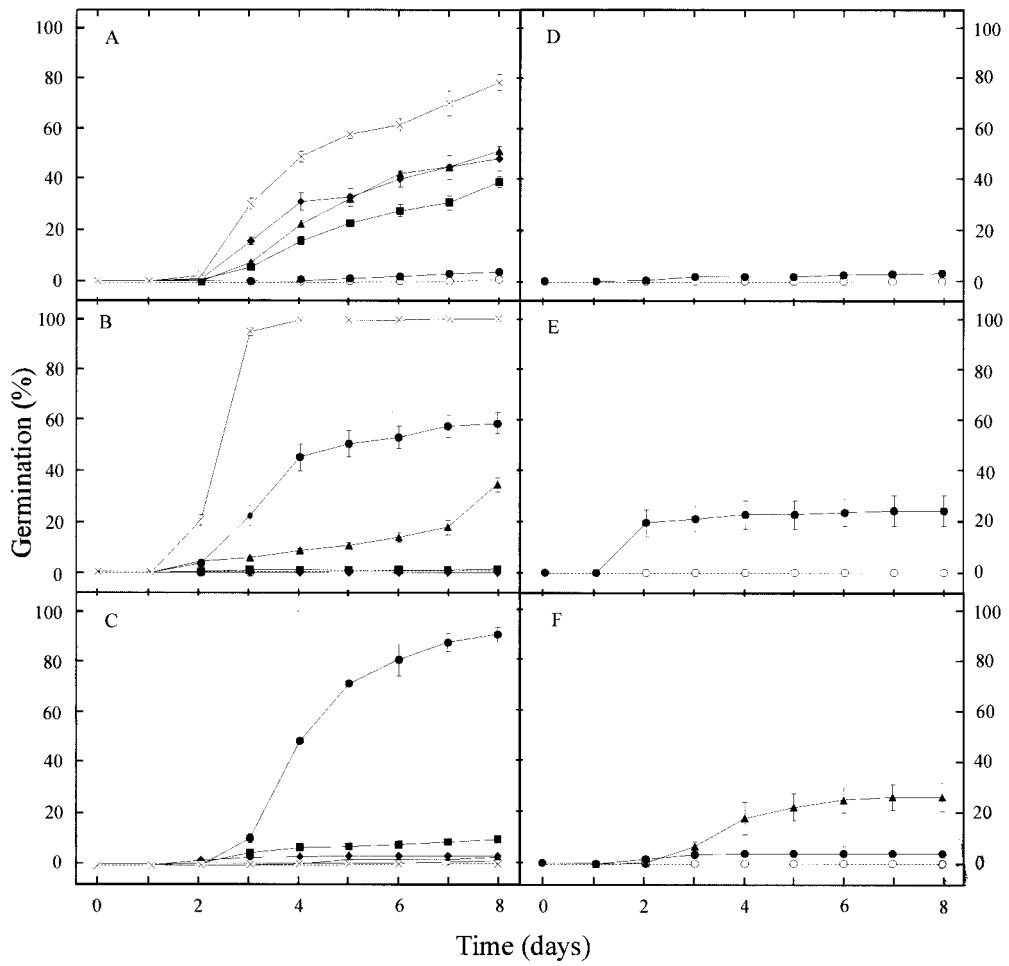
Figure 4. Time course of germination for freshly harvested seed lots (d 9 after harvest; compare with Fig. 3B). In A, ○, L*er*, ●, *tt1*; ▲, *tt2*; ■, *tt3*; ◆, *tt4*; and ×, *tt5*. In B, ○, L*er*, ●, *tt6*; ▲, *tt7*; ■, *tt9*; ◆, *tt10*; and ×, *ttg1*. In C, ○, L*er*, ●, *tt11*; ▲, *tt13*; ■, *ats*; ◆, *ap2*; and ×, *gl2*. In D, ○. Ws and ●, *tt12*. In E, ○, Col and ●, *tt14*. In F, ○, En; ●, *tt8*; and ▲, *ban*.
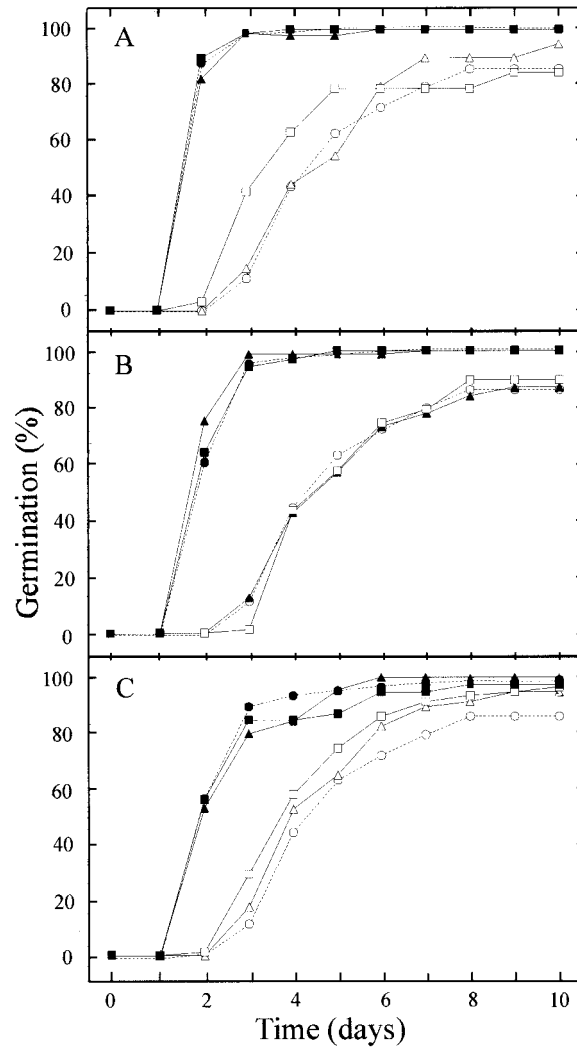
Figure 5. Genetic determinism of the high germination rate encountered in the mutants *tt2*, *tt4*, and *tt7*. The time course of germination after 16 d of dry storage is presented. The parent mentioned first was used as female parent and the second as pollen parent. Seeds from a bulk of nine siliques 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,

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 Mac-Robbie, 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).
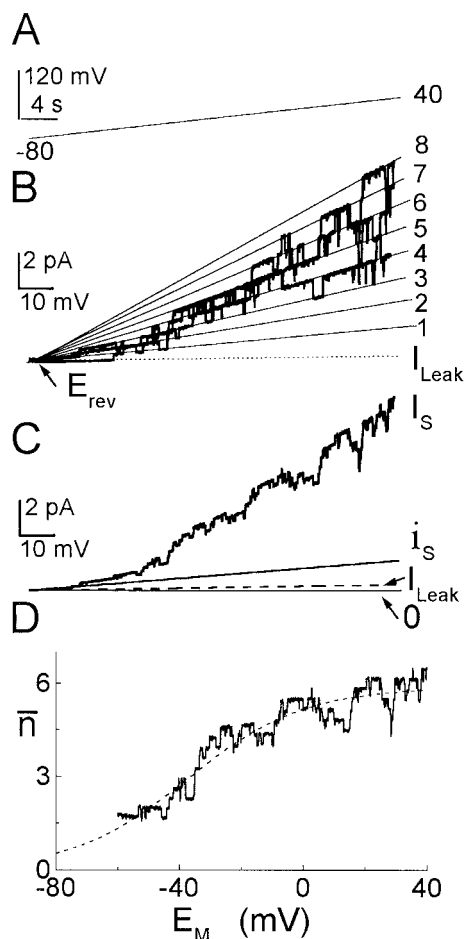
Figure 1. Unitary outward K^+ currents via K_D channels versus E_M, from a representative "inside-out" patch of a *S. saman* extensor cell protoplast, during a slow voltage ramp. A, A linearly increasing voltage ramp applied to the patch membrane. B, Three traces of current (superimposed) during the 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.
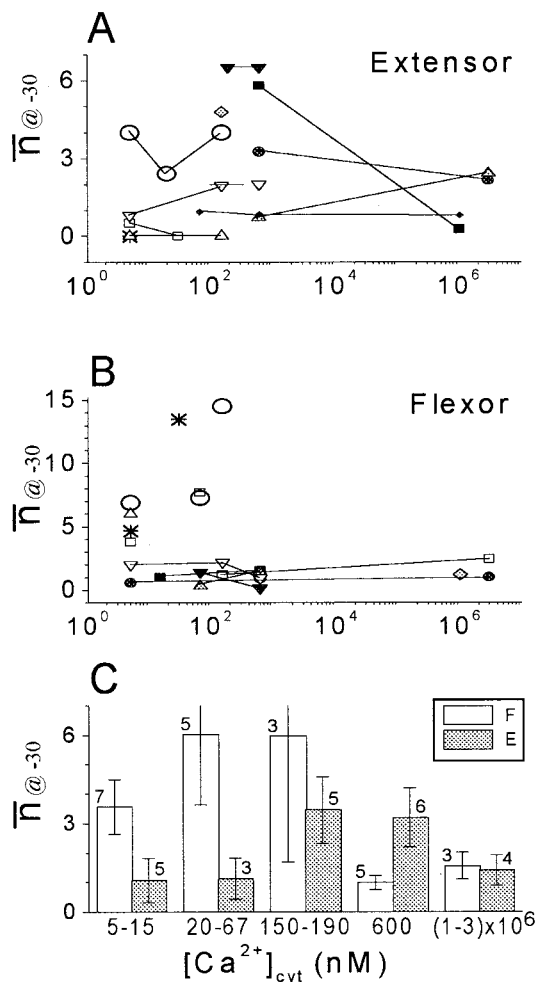
Figure 2. The effect of $[Ca^{2+}]_{cyt}$ on the average activity of K_D channels at −30 mV, $\bar{n}_{@-30}$. Different symbols (connected by lines) denote data from different patches. A, Extensor cells. B, Flexor cells. C, Average values of $\bar{n}_{@-30}$ obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ (±SE). F, Flexor; E, extensor.
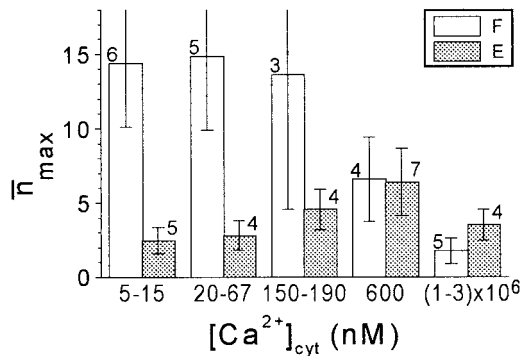
Figure 3. The effect of [Ca^{2+}]$_{cyt}$ on the $\bar{n}_{max}$. Mean values of $\bar{n}_{max}$ obtained from *n* patches at the indicated ranges of [Ca^{2+}]$_{cyt}$ (±SE). F, Flexor; E, extensor.

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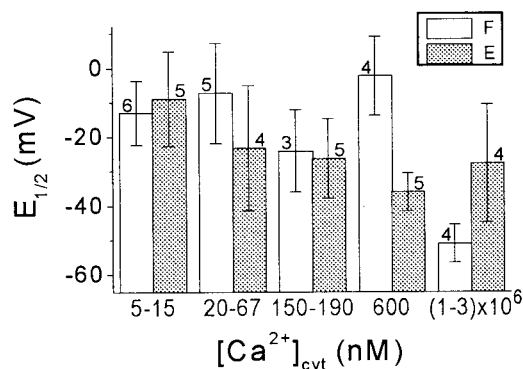
Figure 4. The effect of $[Ca^{2+}]_{cyt}$ on the $E_{1/2}$. Mean values of $E_{1/2}$ obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$). F, Flexor; E, extensor.

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
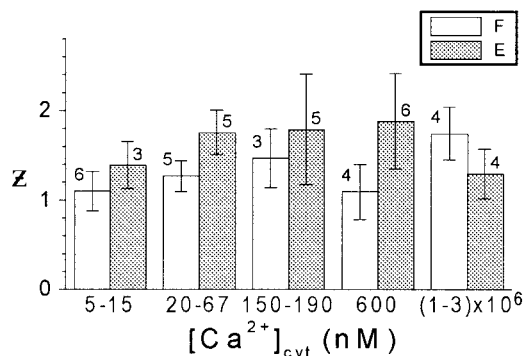
Figure 5. The effect of $[Ca^{2+}]_{cyt}$ on the effective number of charges, z. Mean values of z obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm SE$). F, Flexor; E, extensor.
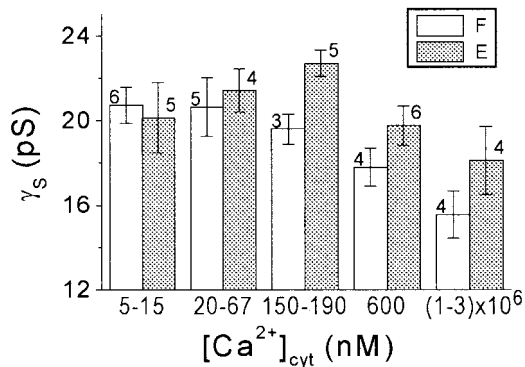
Figure 6. The effect of $[Ca^{2+}]_{cyt}$ on the γ_S. Mean values of γ_S obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm$SE). F, Flexor; E, extensor.

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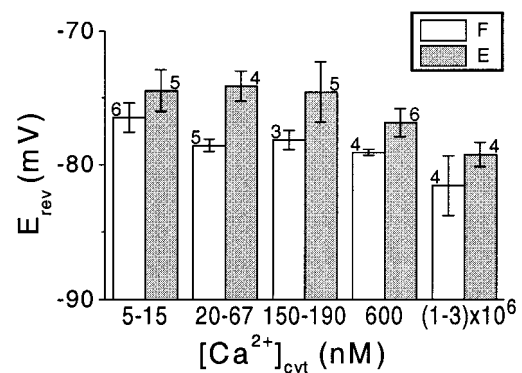
Figure 7. The effect of $[Ca^{2+}]_{cyt}$ on the E_{rev} of the unitary currents. Mean values of E_{rev} obtained from n patches at the indicated ranges of $[Ca^{2+}]_{cyt}$ ($\pm$SE). F, Flexor; E, extensor.

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):

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

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):

$$I_S = \bar{n} \cdot i_S + I_{Leak} \tag{2}$$

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),

$$\bar{n} = \bar{n}_{max} \cdot P_O \tag{3}$$

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

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

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 $[Ca^{2+}]_{cyt}$ con-

centrations (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:

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

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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