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Calcineurin, a Type 2B Protein Phosphatase, Modulates the Ca^{2+}-Permeable Slow Vacuolar Ion Channel of Stomatal Guard Cells

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The slowly activating vacuolar (SV) channel of plant vacuoles is gated open by cytosolic free Ca^{2+} and by cytosol-positive potentials. Using vacuoles isolated from broad bean guard cell protoplasts, SV-mediated currents could be measured in the whole-vacuole configuration of a patch clamp as the time-dependent increase in current at cytosol-positive voltages. Time-dependent deactivation of the SV currents when changing from activating to nonactivating voltages (tail currents) was used to calculate the selectivity of the channel to Ca^{2+} and Cl^- with respect to K^+. Changing the equilibrium potential for each permeant ion (Ca^{2+}, Cl^-, and K^+) at least once for individual vacuoles allowed the relative permeabilities (P) of each of these ions to be calculated in a single experiment. The resulting $P_{Ca}:P_{Cl}:P_K$ ratio was close to 3:0.1:1. In accord with its characterization as a weakly selective Ca^{2+} channel, the SV-mediated current density decreased with increasing Ca^{2+} activity in the vacuole lumen. SV currents were potently modulated by the Ca^{2+}-dependent, calmodulin-stimulated protein phosphatase 2B (calcineurin). At low concentrations (≤0.4 units per mL), calcineurin stimulated SV currents by ~60%, whereas at higher concentrations the phosphatase was inhibitory, reaching ~90% inhibition at 3 units per mL. Bovine calmodulin had no direct effect on SV-mediated currents, although calcineurin stimulated by exogenous calmodulin inhibited SV currents at all concentrations tested with half-maximal inhibition for calcineurin at 0.16 units per mL. The inhibitory effect of calcineurin could be blocked by the pyrethroid deltamethrin, indicating inhibition of SV channels by calcineurin via dephosphorylation. A model is discussed in which vacuolar Ca^{2+} release through SV channels is subject to both positive feedforward and negative feedback control through cytosolic Ca^{2+} and dephosphorylation, respectively.

INTRODUCTION

Changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) are responsible for linking many stimuli to their specific responses in plant cells (Gilroy et al., 1993). The change in $[Ca^{2+}]_{cyt}$ can vary in terms of magnitude, duration, and spatial arrangement within the cell, depending on the nature of the stimulus (Gilroy et al., 1993; Price et al., 1994). This variability probably contributes, at least in part, to encoding the specificity of the response.

Vacuoles comprise an excellent source of Ca^{2+} for sustained elevation of $[Ca^{2+}]_{cyt}$ because they account for >80% of the intracellular volume of a mature plant cell, and they accumulate Ca^{2+} to millimolar levels. The resultant driving force on Ca^{2+} into the cytosol is in excess of 100 mV, given the steady state values of $[Ca^{2+}]_{cyt}$ on the order of 200 nM and a membrane potential of −20 mV (cytosol negative).

Large and prolonged elevations of $[Ca^{2+}]_{cyt}$ are thought to be generated via a mechanism for propagating and amplifying an initial Ca^{2+} signal. It has been suggested that for guard cells, this amplification occurs through a mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) via slowly activating vacuolar (SV) ion channels in the vacuolar membrane (Ward and Schroeder, 1994). The SV channel, which is widely distributed in plant vacuoles (Hedrich et al., 1988), might provide a pathway for CICR, because it is activated by increases in $[Ca^{2+}]_{cyt}$ (Hedrich and Neher, 1987; Ward and Schroeder, 1994) and by positive shifts in the vacuolar membrane potential, which occur following activation of Ca^{2+}-dependent K^+-selective channels (Ward and Schroeder, 1994).

The ionic selectivity of the SV channel is a matter of some controversy. The SV channel in guard cells and beet taproot cells has been demonstrated to be weakly Ca^{2+} selective ($P_{Ca}:P_K$ = 3:1, where P indicates ion permeability) and to have a negligible Cl^- permeability (Ward and Schroeder, 1994). On the other hand, in previous studies of the SV channel from a wide range of plant cells, including guard cells, researchers concluded that the channel possesses a poor selectivity between monovalent cation and anions, the selectivity for divalent cations being ignored (*Coyaud et al., 1987; see Hedrich et al.*, 1988, for overview; Schulz-Lessdorf et al., 1994). All selectivity determinations to date have measured only the relative permeability of two ions in any given experiment; as stated by Bertl et al. (1992a), this measurement does not allow an

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accurate assessment of the relative Ca^{2+} and anion permeabilities. In this study, we employed a method utilizing tail currents of the SV channel in guard cell vacuoles in which the selectivity ratio for all three permeant ions (Ca^{2+}:Cl^-:K^+) could be determined simultaneously for individual vacuoles. A similar approach has been described by Bertl and Slayman (1992) for selectivity determination of the yeast Ca^{2+} release channel YVC1.

The large electrochemical gradient for Ca^{2+} release from the vacuole and the high potential for activity of SV channels in guard cells necessitate acute regulation of CICR to prevent excess flooding of Ca^{2+} into the cytosol, which would result in cellular damage. The feedback mechanism involved is most likely to involve a Ca^{2+}-responsive element in the cytosol. Serine/threonine protein phosphatases have been shown to act as such elements in animal cells in a wide variety of signal transduction cascades (Cohen and Cohen, 1989). There are three classes of serine/threonine protein phosphatases in eukaryotic cells that can be characterized by the following criteria: type 1 (PP 1) can utilize the β subunit of phosphorylase kinase as their substrate, are potently inhibited by okadaic acid, and are not dependent on divalent cations for activity; type 2 (PP 2) can utilize the α subunit of phosphorylase kinase as their substrate and can be subdivided depending on their requirement for divalent ions and their regulation. Thus, although PP 2A is not dependent on divalent ions and is okadaic acid sensitive, type 2B (calcineurin) is dependent on Ca^{2+}, is not okadaic acid sensitive, and is stimulated by calmodulin; PP 2C is dependent on Mg^{2+} and is not okadaic acid sensitive (Cohen, 1989).

Based on okadaic acid responses, evidence indicates that protein phosphatases akin to types 1 and 2A regulate plasma membrane K^+ channels in guard cells and other cells (Li et al., 1994; Thiel and Blatt, 1994). Furthermore, the product of an abscisic acid–induced gene in guard cells (ABI1) has been identified as structurally similar to PP 2C but also containing a Ca^{2+} binding domain (Leung et al., 1994; Meyer et al., 1994). Clearly, these plant protein phosphatases cannot be classified simply according to conventional schemes for animal protein phosphatase types. Nevertheless, there are clear grounds for the experimental application of animal protein phosphatases to explore functional analogies in plant systems (Poole, 1993; Bowler and Chua, 1994). In the case of the Ca^{2+}-dependent protein phosphatase calcineurin, this notion is exemplified by the finding that cyclophilins, which interact with calcineurin in vivo, are present in the cytosol of plant cells (Lippuner et al., 1994; Luan et al., 1994). In addition, calcineurin inhibition of plasma membrane inwardly rectifying potassium (K^+_{in}) channels and blocking of this effect by specific inhibitors of calcineurin have demonstrated that plant Ca^{2+}-dependent protein phosphatases could link increases in cytosolic Ca^{2+} to inhibition of physiological responses in guard cells by altering the phosphorylation state of plasma membrane K^+_{in} channels (Luan et al., 1993).

In this study, we used the time-dependent increase in whole-vacuole current at cytosol positive potentials as an assay of SV channel activity. Modulation of these currents via phosphorylation was tested using calcineurin, calmodulin, and calcineurin/calmodulin mixtures.

RESULTS

Reference and Sign of Voltages and Currents

In accord with Bertl et al. (1992b), transvacuolar membrane potentials in this study are referenced to the extracytosolic medium (that is, the vacuolar lumen). Inward currents are therefore those that pass into the cytosol from the lumen. As the two primary pumps at the vacuolar membrane pass current (positive charge) out of the cytosol (Rea and Sanders, 1987), cytosol-negative potentials correspond to the steady state physiological situation, although instances in which the potential might swing transiently into the positive range are discussed later.

Calculation of P_{Ca}:P_{Cl}:P_K Selectivity of SV Channels Using Tail Currents

For voltage-gated channels such as the SV channel, ionic selectivity can be determined in the whole-vacuole (equivalent to whole-cell) mode of patch clamp by measuring the reversal potential (E_{rev}) of the time-dependent deactivation of current (tail current) on switching from an activating to a less activating voltage. For determination of SV selectivity, most studies have only considered two permeant ions at one time, either K^+ to Cl^- (Coyaud et al., 1987; Colombo et al., 1988; Hedrich and Kurkdjian, 1988; Hedrich et al., 1988; Schulz-Lessdorf et al., 1994) or Ca^{2+} to K^+ and Ca^{2+} to Cl^- (Ward and Schroeder, 1994). In addition, to determine Ca^{2+} selectivity, high Ca^{2+} concentrations were used in the vacuolar lumen (Ward and Schroeder, 1994). This reduces the magnitude of time-dependent current at positive potentials, making it necessary to use very large potentials (+160 mV and higher) to measure large tail currents. We found that pipette-to-membrane seals became unstable at these high potentials.

Therefore, we devised a protocol that allows measurement of SV channel selectivity for more than two ions in one experiment using less extreme potentials. This protocol has three essential criteria. (1) The currents in whole-vacuole mode are measured with high concentrations of K^+ and Cl^- on both sides of the membrane. This ensures that large SV channel–mediated currents are measured at non-extreme potentials (+80 to +100 mV); therefore, large tail currents are measured and seals are not destabilized by high voltages. (2) Changes in the cytosolic (bath) concentrations of ions are used to determine the selectivity of such channels as the SV channel, despite the fact that when open in vivo, they are believed to pass current from lumen to cytosol (Bertl and Slayman, 1992). (3) For a single vacuole, determination of the E_{rev} of tail

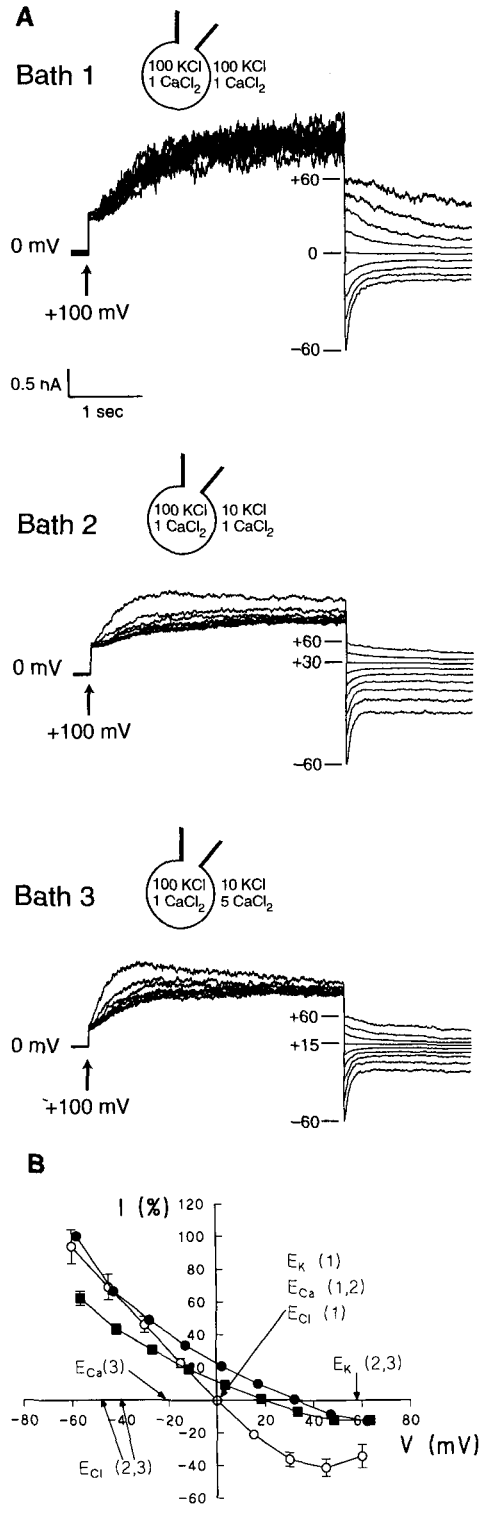

Figure 1. Tail Currents of Whole-Vacuole SV Channel Activity.

(A) SV channel tail currents recorded in three separate bath solutions from a single guard cell vacuole. The ionic composition of the solutions is provided here and in Methods. The respective ionic activities (α) are as follows: pipette and bath 1, α_{Ca} = 0.4 mM, α_{Cl} = 76.2 mM, and α_K = 74.7 mM; bath 2, α_{Ca} = 0.6 mM, α_{Cl} = 10.5 mM, and α_K = 8.7 mM; bath 3, α_{Ca} = 2.8 mM, α_{Cl} = 16.9 mM, and α_K = 8.4 mM. Numbers denote holding potentials. The potentials at which tail currents were measured were between −60 mV and +60 mV in 15-mV steps. The potential closest to E_{rev} is also indicated. Similar results were obtained from 14 other vacuoles.

(B) Plot of tail current versus potential in bath 1 (○), bath 2 (●), and bath 3 (■). The holding potentials in baths 2 and 3 are corrected for E_L values of +2.1 and +3.3 mV, respectively. The approximate equilibrium potential for each ion is also indicated for each bath solution (bath solution number given in parentheses). Data are the mean of 15 vacuoles. Currents are standardized to the current at −60 mV in bath 2. The E_{rev} values in baths 1, 2, and 3 are −0.4 ± 0.7, +31.7 ± 0.8, and +19.0 ± 1.0 mV, respectively.

currents in at least two different bath solutions allows calculation of the selectivity sequence for more than two ions if the equilibrium potential (E_{ion}) of all potentially permeant ions is varied at least once (see later discussion).

In Figure 1, tail currents are shown that were measured under conditions in which these criteria were applied. Figure 1A shows tail currents of SV activity for a single vacuole in three separate bath solutions. In bath 1, the transvacuolar gradients of Ca^{2+}, Cl^-, and K^+ were zero, and hence, tail currents reversed at 0 mV. In bath 2, the cytosolic KCl concentration was lowered by a factor of 10; this shifted the equilibrium potential (E_{ion}) for K^+ to +58 mV, E_{Cl} to −50 mV, while E_{Ca} remained close to 0 mV (at −5 mV). The observed E_{rev} of the tail currents shifted to approximately +30 mV, which lies close to E_K, therefore indicating a permeability to K^+. The channel is not absolutely selective for K^+, however, because E_{rev} did not coincide with E_K. To determine whether E_{rev} was not reaching E_K due to a permeability to Cl^-, Ca^{2+}, or both Cl^- and Ca^{2+}, tail currents were measured in bath 3, where the same external K^+ gradient was maintained but the external $CaCl_2$ concentration was increased from 1 to 5 mM. This shifted E_{Ca} to −25 mV, whereas E_K remained essentially unchanged and E_{Cl} shifted slightly to −38 mV. The observed E_{rev} now shifted to a less positive potential (close to +15 mV), indicating a finite Ca^{2+} permeability. Figure 1A also shows that in baths 2 and 3, the magnitude of the SV-mediated current at +100 mV was less than in bath 1, because the activation potential was closer to the reversal potential of the most abundant charge carrier (K^+). Figure 1B shows a plot of the mean tail currents measured in baths 1, 2, and 3 for 15 separate vacuoles. Mean reversal potentials were −0.4 ± 0.7, +31.7 ± 0.8, and +19.0 ± 1.0 mV in baths 1, 2, and 3, respectively. These values were used to calculate the selectivity ratio $P_{Ca}:P_{Cl}:P_K$ as described in Methods, taking bath 2 as solution A and bath 3 as solution B.

Solving equations 3, 4, and 5 (Methods) using the mean values of E_{rev} from Figure 1, values of $P_{Ca}:P_K$ = 2.73 and $P_{Cl}:P_K$ = 0.105 are derived. Allowing for any small error in measuring E_{rev}, this may be simplified to $P_{Ca}:P_{Cl}:P_K$ = 3:0.1:1.

These findings unequivocally demonstrate, in agreement with Ward and Schroeder (1994), that the SV channel in broad

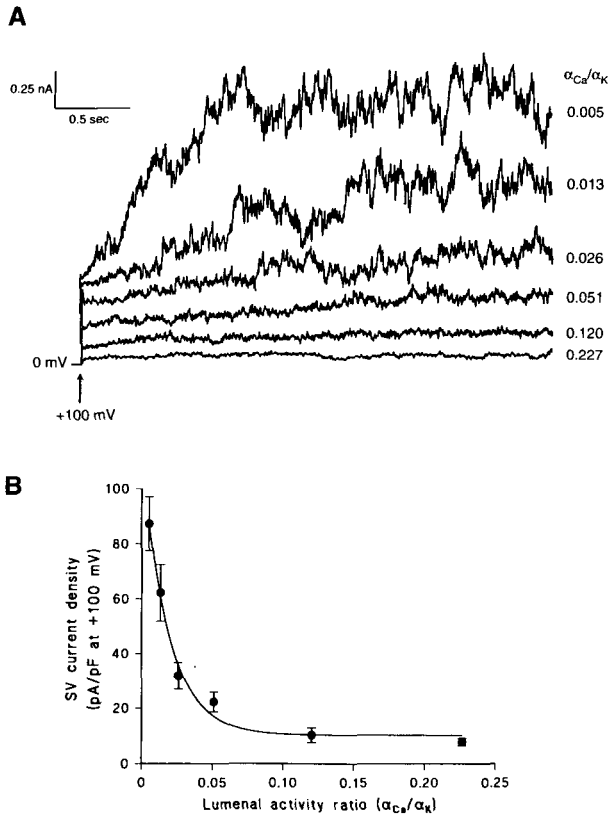

Figure 2. Effect of Lumenal α_{Ca}:α_K Ratio on SV-Mediated Currents.

(A) Whole-vacuole SV channel currents measured in response to a 3.5-sec voltage pulse of +100 mV. Each trace is from a separate vacuole of similar size (13 to 15 pF) superimposed on each other. For each pipette α_{Ca}:α_K ratio, the bath solution was identical (bath 1, see Figure 1). Numbers to the right of traces indicate the lumenal α_{Ca}:α_K ratio. **(B)** Whole-vacuole SV-mediated current densities at +100 mV (pA/pF) measured at various lumenal (pipette) α_{Ca}:α_K ratios. Each point is the mean ± SE of 10 to 15 vacuoles. For each vacuole, the current at +100 mV was recorded 10 times and the mean taken. The differences in E_L values between the solutions are small (<5% of the pulse potential) and so were ignored.

bean guard cell vacuoles is a cation channel with a moderate selectivity for Ca^{2+} over K^+.

SV-Mediated Currents Are Reduced by Increases in Lumenal Ca^{2+}

With K^+ as the principal charge carrier, calcium channels commonly show reduced conductance with increasing Ca^{2+} activity as Ca^{2+} binds to the selectivity filter and impedes current flow (Rosenberg and Chen, 1991; Johannes and Sanders, 1995). Because the SV channel in guard cells is a moderately selective Ca^{2+} channel, the effect of increases in lumenal Ca^{2+} on SV-mediated current density was measured. This can

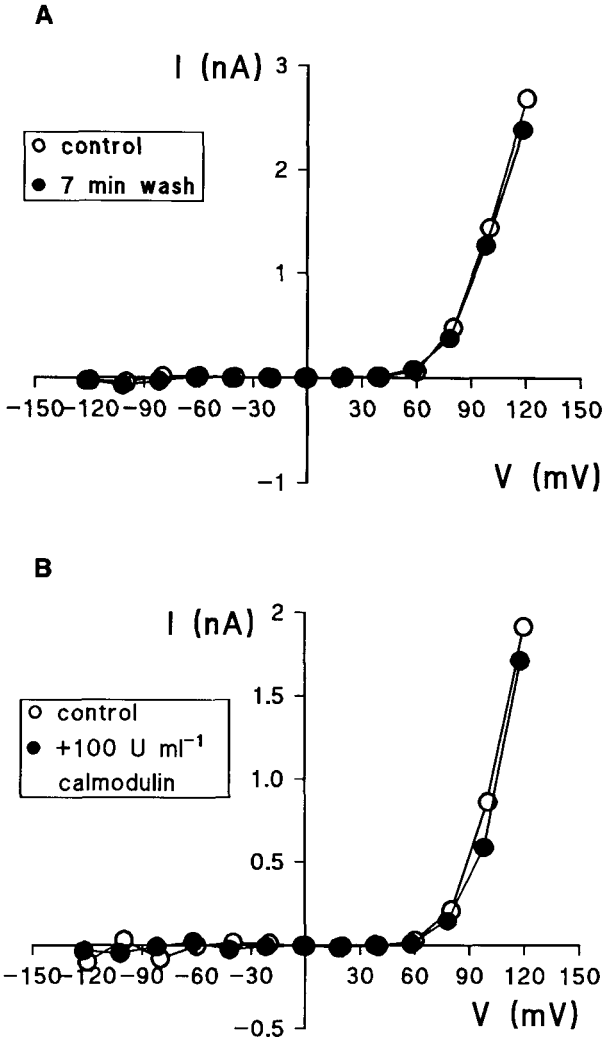

Figure 3. Effect of Bovine Calmodulin on SV-Mediated Currents.

(A) Whole-vacuole I/V relationships for SV currents from a typical guard cell vacuole before (○) and after (●) a 7-min bath perfusion (wash) using control bath media (composition given below), indicating that there was a slight rundown of current over this period. SV currents were measured in identical conditions for 21 vacuoles; the mean percentage rundown, averaged over +80 to +120 mV, was 18 ± 3%. **(B)** Whole-vacuole I/V relationships for SV currents from a typical guard cell vacuole before (○) and after (●) a 7-min bath perfusion using a solution containing 100 units per mL (U mL^{-1}) of bovine calmodulin, indicating that there was no significant change in current compared with controls (see **[A]**). Similar results were obtained from 11 vacuoles; the mean rundown, averaged over +80 to +120 mV, was 19 ± 5%.

In **(A)** and **(B)**, the pipette solution contained 5 mM $CaCl_2$, 200 mM KCl, 5 mM Mes-Tris, pH 5.5, and sorbitol to 485 mosmol/kg. The bath solution contained 1 mM $CaCl_2$, 200 mM KCl, 5 mM Tris-Mes, pH 7.5, and sorbitol to 485 mosmol/kg. In these conditions, E_L was <1 mV and so was ignored. Points before perfusion (○) are offset by −2 mV for clarity.

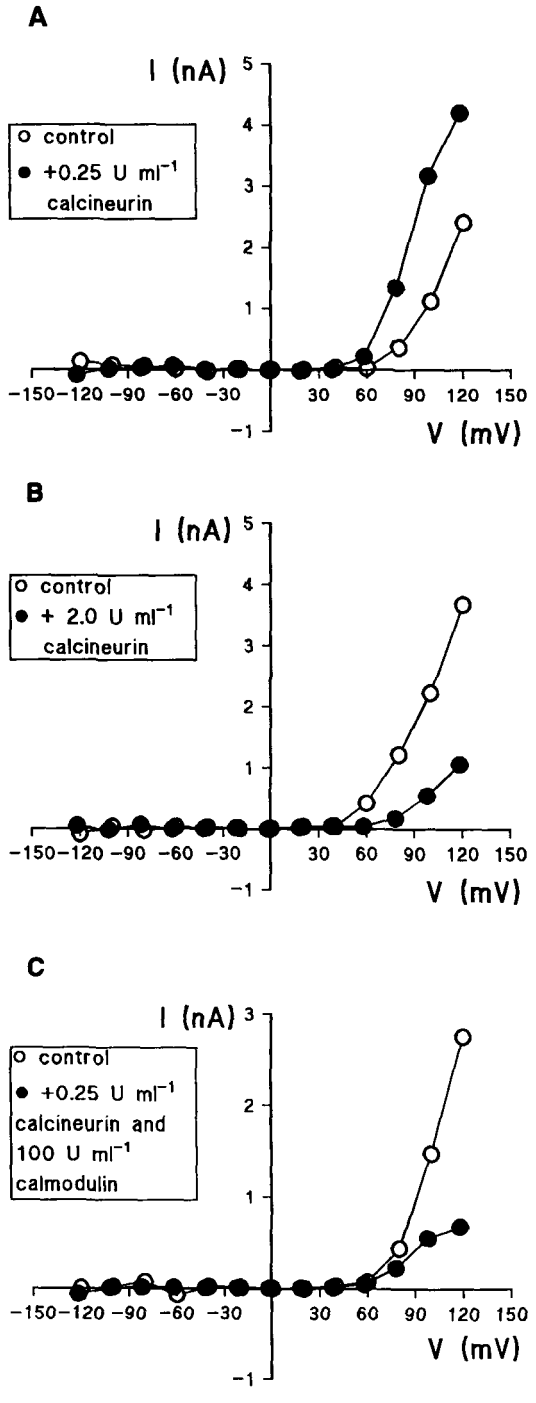

Figure 4. Effect of Cytosolic Calcineurin on SV-Mediated Currents.

(A) Whole-vacuole I/V relationships for SV currents from a typical guard cell vacuole before (○) and after (●) a 7-min perfusion with bath solution containing 0.25 units per mL (U mL^{-1}) of calcineurin, indicating that there was significant stimulation of current averaged over +80 to +120 mV. Similar results were obtained from 10 separate vacuoles, with the mean stimulation being 58 ± 11%.

be achieved in the whole-vacuole configuration with different pipette solutions by expressing currents from different vacuoles on a pA/pF basis, which effectively normalizes with respect to surface area. The current traces in Figure 2A indicate a marked effect of the lumenal ionic activity ratio (α_{Ca}:α_K) on SV-mediated currents (the α_{Ca}:α_K ratio is used rather than lumenal α_{Ca} alone, because the α_K varies slightly with increasing ionic strength of the pipette solution). The higher the proportion of Ca^{2+}, the lower the SV current. This effect is quantified in Figure 2B, where half-maximal inactivation is calculated for an activity ratio of 0.02. This rather low value is indicative of tight Ca^{2+} binding to the channel selectivity filter.

SV Channels Are Not Modulated by Bovine Calmodulin

Large SV channel–mediated currents can be measured in the whole-vacuole configuration at positive potentials with 200 mM KCl and 5 mM $CaCl_2$ on the lumenal side and 200 mM KCl and 1 mM $CaCl_2$ on the cytosolic side (165 ± 13 pA/pF at +120 mV; n = 54 vacuoles). Figure 3A demonstrates that these currents showed little change with time over standard wash periods of 7 min (equivalent to 22 bath volume exchanges, with the flow of the bath buffer initiated 5 min after whole-vacuole configuration was achieved). Thus, a small change, amounting to an 18 ± 3% (n = 21) rundown of the currents averaged over +80 to +120 mV, was recorded.

The addition of bovine calmodulin (100 units per mL) to vacuoles in the whole-vacuole configuration did not significantly alter the currents compared with the controls, with the rundown amounting to 19 ± 5% (n = 11) of the current averaged over +80 to +120 mV.

Modulation of SV Channel–Mediated Currents by Calcineurin and Calcineurin/Calmodulin Mixtures

Figures 4A and 4B demonstrate that the Ca^{2+}-dependent protein phosphatase 2B (calcineurin) has a dual effect on

(B) Whole-vacuole I/V relationships for SV currents from a typical guard cell vacuole before (○) and after (●) a 7-min perfusion with bath solution containing 2.0 units per mL (U mL^{-1}) of calcineurin, indicating that there was significant inhibition of current averaged over +80 to +120 mV. Similar results were obtained from six separate vacuoles, with the mean inhibition being 82 ± 4%.

(C) Whole-vacuole I/V relationships for SV currents from a typical guard cell vacuole before (○) and after (●) a 7-min perfusion with bath solution containing 0.25 units per mL (U mL^{-1}) of calcineurin activated with 100 units per mL calmodulin, indicating that there was significant inhibition of current averaged over +80 to +120 mV. Similar results were obtained from seven separate vacuoles, with the mean percentage inhibition being 60 ± 5%.

In **(A)** to **(C)**, the pipette and bath solutions are identical to those given in the legend to Figure 3. In these conditions, E_L was <1 mV and so was ignored. Points after perfusion (●) are offset by −2 mV for clarity.

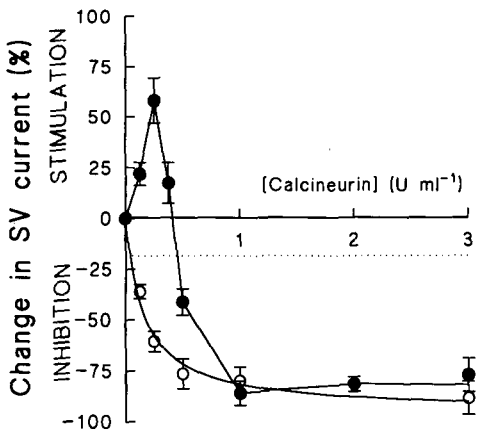

Figure 5. Effect of Calcineurin and Calcineurin/Calmodulin Mixtures on SV-Mediated Currents.

The dependence of SV currents (averaged over +80 to +120 mV) on the concentration of calcineurin alone (●) and calcineurin stimulated with exogenous calmodulin (○) at 100 units per mL (U mL^{-1}) is shown. The data for calmodulin-stimulated calcineurin were fitted by a monophasic function with half-maximal inhibition at 0.16 units per mL. Each data point is the mean ± SE of between five and 11 separate vacuoles. Each vacuole was perfused for 7 min with the respective bath solution. The dotted line indicates the control rundown (n = 21) when the bath was perfused with media that contained no additions of calcineurin or calmodulin. The pipette and bath solutions are identical to those given in the legend to Figure 3.

SV-mediated currents. At low concentrations, the addition of calcineurin to the bath stimulated the SV currents. For a concentration of 0.25 units per mL, the stimulation was 58 ± 11% (n = 10) of the currents averaged over +80 to +120 mV (Figure 4A). However, higher calcineurin concentrations inhibited SV currents, amounting to 82 ± 4% (n = 6) inhibition at 2 units per mL calcineurin (Figure 4B).

Whereas bovine calmodulin itself does not modulate the activity of guard cell SV channels (Figure 3B), the stimulation by calcineurin at low concentrations could be dramatically reversed by the concomitant addition of 100 units per mL of calmodulin. At 0.25 units per mL calcineurin and 100 units per mL of calmodulin, SV currents were inhibited by 61 ± 5% (n = 7; Figure 4C).

These responses were studied in more detail by measuring SV-mediated currents after treatment with a range of calcineurin concentrations in the presence and absence of 100 units per mL of calmodulin. As shown in Figure 5, in the absence of calmodulin, concentrations of calcineurin up to ~0.4 units per mL stimulated SV currents. Concentrations higher than this were inhibitory. When calcineurin was stimulated with exogenous calmodulin before addition to the bath, it inhibited SV currents at all concentrations tested (Figure 5). This inhibition follows simple monophasic kinetics with half-maximal inhibition at 0.16 units per mL.

Inhibitory Effect of Calcineurin on SV-Mediated Currents Is Blocked by Deltamethrin

It could be postulated that the inhibitory effect of calcineurin is mediated via high-affinity binding of calcineurin to endogenous calmodulin associated with the SV channel that normally mediates activation of the channel by Ca^{2+} (Weiser et al., 1991; Bethke and Jones, 1994). Thus, association of calcineurin could "strip" the channel of calmodulin and prevent activation by Ca^{2+}. Experiments were therefore performed with deltamethrin, a pyrethroid insecticide, that specifically inhibits calcineurin by binding to the calcineurin/calmodulin complex. Thus, complex formation is not prevented (Enan and Matsumura, 1992). Figure 6 demonstrates that the inhibitory effect of calcineurin on SV-mediated currents was completely blocked by pretreating the enzyme with 1 nM deltamethrin before perfusion into the bath. If inhibition by calcineurin were due to stripping of calmodulin from the channel, then inhibition would not be blocked by deltamethrin. Because deltamethrin was a potent blocker of inhibition of SV currents by calcineurin, it can be concluded that calcineurin acts via a specific dephosphorylation event.

DISCUSSION

Calcium Selectivity of SV Channels and Ca^{2+} Release

The ability of the SV channel of guard cell vacuoles to mediate Ca^{2+} release into the cytosol requires that the SV channel

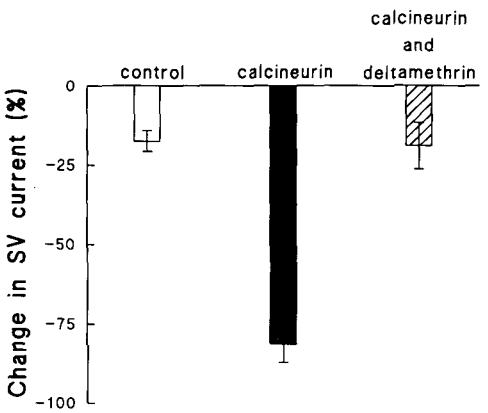

Figure 6. The Inhibitory Effect of Calcineurin on SV-Mediated Currents Is Blocked by Deltamethrin.

Data are shown from SV-mediated currents (averaged over +80 to +120 mV) after a 7-min perfusion with bath solutions that contain no enzyme (control) or are in the presence of 2 units per mL calcineurin, or 2 units per mL of calcineurin and 1 nM deltamethrin. Data are from 21, six, and seven vacuoles, respectively. The pipette and bath solutions are identical to those given in the legend to Figure 3.

exhibit a significant degree of Ca^{2+} selectivity. Our study demonstrated that SV channels are moderately Ca^{2+} selective in the simultaneous presence of the potentially permeant ions K^+ and Cl^- on both sides of the vacuolar membrane. Thus, the SV channel emerges as a cation-selective channel, with a 10- to 30-fold selectivity for K^+ and Ca^{2+} over Cl^- and a significant (3:1) selectivity for Ca^{2+} over K^+. These findings are similar to those of Ward and Schroeder (1994), who have also demonstrated that the guard cell SV channel has a $P_{Ca}:P_K$ of 3:1. Discrimination of the channel against Cl^- has also been shown by Amodeo et al. (1994) and Ward and Schroeder (1994) in guard cells, although the degree of Cl^- discrimination could not be quantified from any of their experiments. Our study also highlighted the similarity between the plant vacuolar SV channel and the Ca^{2+}-dependent YVC1 channel at the vacuolar membrane of yeast, which, using protocols similar to those described here, has been demonstrated to select for Ca^{2+} over K^+ and to discriminate against Cl^- by a factor of 30 (Bertl and Slayman, 1992).

Nevertheless, given the $P_{Ca}:P_K$ of 3:1, the Ca^{2+} selectivity of the SV channel was lower than that of a distinct type of voltage-gated Ca^{2+} channel at the same membrane for which $P_{Ca}:P_K$ is 6:1 (Allen and Sanders, 1994a) and much lower than that of vacuolar ligand-gated channels for which $P_{Ca}:P_K \geq$ 100:1 and 20:1 for inositol 1,4,5 trisphosphate–gated channels and for cyclic ADP-ribose–gated channels, respectively (Allen and Sanders, 1994b; Allen et al., 1995). However, all these more selective Ca^{2+} channels are opened by negative-going voltages at which there is a significant lumen-to-cytosol driving force on K^+ (because E_K resides in the range 0 to +60 mV, depending on the lumenal K^+ activity; MacRobbie and Lettau, 1980). The high values of $P_{Ca}:P_K$ might therefore be seen as a requirement to prevent excessive K^+ flux through these channels. In contrast, the more weakly Ca^{2+}-selective SV channel operates at voltages closer to E_K, and this lack of significant driving force on K^+ removed the requirement for discrimination against K^+. Furthermore, as proposed by Ward and Schroeder (1994), the relatively high K^+ permeability through the SV channel helps retain the membrane potential sufficiently displaced from E_{Ca} (normally approximately +100 mV) so that the driving force for Ca^{2+} flux through the channel is large.

Even though in our experiments current was flowing from cytosol to vacuole, the energetic arguments outlined earlier indicate that Ca^{2+} acts as the biological substrate for the SV channel (Slayman and Bertl, 1994), and it mediates Ca^{2+} release from the vacuole in vivo, despite the molar excess of K^+ over Ca^{2+} in the lumen.

The SV channel–mediated current density decreases as the proportion of Ca^{2+} on the lumenal side increases. This effect has been shown to be due to a reduction in single channel current (Ward and Schroeder, 1994) and indicates a Ca^{2+} binding site in the channel selectivity filter (Rosenberg and Chen, 1991; Johannes and Sanders, 1995).

The unequivocal finding that the SV channel is Ca^{2+} selective warrants a note of caution concerning the utilization of channel blockers to determine the identity of a channel. The SV channel in plant cells has been shown previously to be inhibited by the "K^+ channel blockers" tetraethylammonium, (+)-tubocurarine, charybdotoxin, and quinine (Weiser and Bentrup, 1993) and also by the "anion channel blockers" 4,4′–diisothiocyanotostilbene-2,2′-disulfonate and by 4-acetamido-4′-isothiocyanotostilbene-2,2′-disulfonate (Hedrich and Kurkdjian, 1988). As suggested previously (Allen and Sanders, 1994a), it is now clear that these compounds have relatively nonspecific effects on plant ion channels; therefore, the physiological role of a channel cannot be inferred from its pharmacology.

Voltage- and Ca^{2+}-Dependent Activation of SV Channel-Mediated Currents

The question remains as to how the SV channel opens in vivo when the normal steady state vacuolar membrane potential is negative (−20 to −50 mV; Rea and Sanders, 1987) and the opening range of the SV channel appears to be restricted to positive potentials. Two possibilities are apparent. First, as proposed by Ward and Schroeder (1994), the Ca^{2+}-dependent opening of highly K^+-selective channels might enforce a positive swing in membrane potential toward E_K. This would comprise an indirect pathway for SV channel activation by Ca^{2+}. Alternatively, the activation potential of the SV channel might depend on the prevailing transvacuolar ionic gradients, as is the case in the storage root of sugar beet, where under certain conditions the SV channel can open at negative potentials (Coyaud et al., 1987). If this capacity were retained in vivo, then Ca^{2+} release by the SV channel could depend solely on direct activation by an increase in $[Ca^{2+}]_{cyt}$ (Hedrich and Neher, 1987), irrespective of any change in membrane potential.

Evidence that activation of the SV channel by Ca^{2+} is mediated by endogenous calmodulin arises from observations that SV currents are inhibited by the calmodulin agonists W-5, W-7, and trifluoperazine (Weiser et al., 1991; Bethke and Jones, 1994). Furthermore, the inhibitory effect of W-7 on the SV channel can be partially reversed by exogenous calmodulin isolated from plant sources, whereas bovine calmodulin is without effect (Weiser et al., 1991). In this study, we found no direct effect of exogenous calmodulin on SV currents in guard cells at high cytosolic Ca^{2+} concentrations, which has also been reported previously for plant calmodulin (Bethke and Jones, 1994). We were able to exploit this lack of calmodulin action on the SV channel to investigate calmodulin's role in selective stimulation of calcineurin. This requires the retention of high $[Ca^{2+}]_{cyt}$ in the experiments to isolate calcineurin effects on SV channels independently of direct Ca^{2+}/calmodulin activation.

Downregulation of the SV Channel-Mediated Currents

Our findings demonstrated that the Ca^{2+}-dependent PP 2B (calcineurin) at low concentrations stimulates SV currents,

whereas at higher concentrations calcineurin is inhibitory. When calcineurin is stimulated with exogenous calmodulin, inhibition was observed at all calcineurin concentrations tested. These results can be explained if the SV channel exists in multiple phosphorylation states that regulate activity. When partly dephosphorylated, the activity would be stimulated as observed at low calcineurin concentrations. If the channel is more fully dephosphorylated, SV currents would be inhibited as observed with high concentrations of calcineurin or when the potency of calcineurin was increased with calmodulin.

Calcium-dependent protein phophatases analogous to calcineurin have also been demonstrated to inactivate inward-rectifying K^+ channels at the plasma membrane of guard cells (Luan et al., 1993) and to modulate Ca^{2+} distribution in internal pools in yeast (Tanida et al., 1995). Our study furthers the notion that Ca^{2+}-dependent protein phosphatases play a pivotal role in signal transduction in plant cells, although the identity of the phosphatases involved and their relationships to other eukaryotic phosphatase types remain to be determined.

Positive Feedforward and Negative Feedback Regulation of Vacuolar Ca^{2+} Release

The SV channel could mediate CICR during stimulus–response coupling, because the channel is activated indirectly and/or directly by increases in $[Ca^{2+}]_{cyt}$ (Hedrich and Neher, 1987; Ward and Schroeder, 1994). Vacuoles contain Ca^{2+} at high concentrations and in effect are an inexhaustible supply; it is therefore essential that CICR be tightly regulated. Modulation of SV currents by a Ca^{2+}-dependent protein phosphatase reported here suggests a major role for these enzymes in feedback regulation of CICR. Thus, the rise in $[Ca^{2+}]_{cyt}$, which ensures Ca^{2+} release through SV channels, activates Ca^{2+}/calmodulin–dependent protein phosphatases (and other Ca^{2+} responsive elements) in the cytosol. These phosphatases will dephosphorylate their target proteins, which can link the Ca^{2+} signal to the physiological response (Luan et al., 1993) but will also effectively regulate via feedback those channels (in this case, the SV channel) that are generating the Ca^{2+} signal. It therefore seems likely that the complex Ca^{2+} dependence of SV channel activity might be responsible not only for regenerative Ca^{2+} release but also, given suitable time delays in the dephosphorylation response, for transient downregulation of the Ca^{2+} signal, as has been observed during oscillatory behavior (Berridge, 1993).

METHODS

Isolation of Guard Cell Protoplasts and Release of Vacuoles

Broad bean (cv Dreadnought) plants were grown for 2 to 4 weeks in a growth cabinet at 16/8 hr light/dark cycle at 25/20°C and 60 to 70% humidity. Guard cell protoplasts were isolated by the method of Raschke and Hedrich (1989), with modifications as described by Allen and Sanders (1994a).

Protoplasts were commonly 98% of guard cell origin, as determined by the presence and morphology of chloroplasts. A 200-μL sample was placed in a patch clamp chamber, and the protoplasts were allowed to adhere to the glass bottom. They were then washed with a solution containing 100 mM KCl, 10 mM Hepes-KOH, pH 8.0, and 2 mM EGTA (200 mosmol/kg). This caused the protoplasts to rupture and release vacuoles. The appropriate bath solution for patch clamping was then perfused into the chamber. Only those vacuoles that could be positively identified as coming from guard cells (by having the remnants of the small, gray-green guard cell chloroplasts attached) were selected for seal formation.

Patch Clamp Media and Protocols

In the experiments to determine the selectivity of slow vacuolar (SV) channels using tail currents, the pipette (vacuolar) medium contained 1 mM $CaCl_2$, 100 mM KCl, 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)-Tris, pH 5.5, and sorbitol to an osmotic pressure of 485 mosmol/kg. The ionic activities (α_{ion}) were calculated using the Debye-Hückel relationship and have the values for the pipette medium as follows: α_{Ca} = 0.4 mM, α_{Cl} = 76.2 mM, and α_K = 74.7 mM. The three bath media contained the following: bath 1, identical to the pipette medium but with 5 mM Tris-Mes, pH 7.5, as the buffer; bath 2, 1 mM $CaCl_2$, 10 mM KCl, 5 mM Tris-Mes, pH 7.5, and sorbitol to 485 mosmol/kg (α_{Ca} = 0.6 mM, α_{Cl} = 10.5 mM, and α_K = 8.7 mM); bath 3, 5 mM $CaCl_2$, 10 mM KCl, 5 mM Tris-Mes, pH 7.5, and sorbitol to 485 mosmol/kg (α_{Ca} = 2.8 mM, α_{Cl} = 16.9 mM, and α_K = 8.4 mM).

In experiments to measure the effect of lumenal Ca^{2+} activity on SV currents, the bath solution was bath 1 (see previous description). The pipette solution was also the same, except that the $CaCl_2$ concentration was varied to 1, 2.5, 5, 10, 25, and 50 mM (α_{Ca} = 0.40, 0.98, 1.92, 3.71, 8.50, and 15.28 mM).

For experiments with calcineurin and calmodulin, the pipette solution contained 5 mM $CaCl_2$, 200 mM KCl, 5 mM Mes-Tris, pH 5.5, and sorbitol to 485 mosmol/kg, and the bath solution contained 1 mM $CaCl_2$, 200 mM KCl, 5 mM Tris-Mes, pH 7.5, and sorbitol to 485 mosmol/kg. Calmodulin (bovine brain; Sigma) and calcineurin (bovine brain; Sigma or Fluka Chemie AG, Bushs, Switzerland) were made as stock solutions (stored at −20°C in aliquots) and diluted into the bath media as required. Deltamethrin (Alomone Labs, Jerusalem, Israel) was dissolved in absolute ethanol and diluted to a final concentration of 1 nM in bath solution containing 1% (v/v) ethanol.

Patch pipettes were pulled from thin-walled borosilicate glass capillaries (Kimax, Vineland, NJ), coated with Sylgard (Dow Corning, Seneffe, Belgium), and fire polished. Pipette resistance in the experimental solutions was commonly 30 to 50 MΩ. After pipette-vacuolar membrane seals of 5 GΩ or greater were formed following gentle suction to the pipette, the whole-vacuole configuration was achieved by rupturing the membrane with voltage pulses up to 1 V delivered by an A310 Accupulser (World Precision Instruments, New Haven, CT). The pipette solution was allowed to exchange with the lumenal solution for exactly 5 min before recording commenced, to ensure complete exchange.

Whole vacuole current/voltage (I/V) relationships were determined by clamping the membrane potential using a bipolar pulse protocol of 20-mV steps for 6 sec (from a holding potential of −20 mV) between +120 and −120 mV. The SV channel–mediated currents were defined as the time-dependent current at positive potentials sampled between 50 msec and 6 sec after initiation of a voltage pulse. A change in SV

current for any vacuole was defined as the mean percentage change in time-dependent current at +80, +100, and +120 mV. Results are cited in the form mean ±SE (number of observations from which mean was derived). For the determination of selectivity with tail currents, the voltage was pulsed from a holding potential of 0 mV to +100 mV for 3.5 sec, and the potential was then changed to −60 mV for 3 sec. This protocol was repeated eight times, the second pulse increasing by 15 mV on each occasion. The reversal current (tail current) was determined as the difference in current from samples 50 msec and 3 sec after the second pulse. The reversal potentials of the tail currents were determined from plots of tail current versus potential.

Solution changes were achieved via two low-noise peristaltic pumps (Gilson Minipuls 3; Gilson Medical Electronics, Middleton, WI), with the exchange (22 times bath volume) being complete after 7 min. Controls were washed for 7 min with identical bath solution to measure any channel rundown.

Calculation of P_{Ca}:P_{Cl}:P_K for Experiments Utilizing SV Channel Tail Currents

From the Goldman-Hodgkin-Katz current equation (Fatt and Ginsborg, 1958), the current (I) carried by any permeant ion (S) through a channel is given by

$$I_s = P_s z^2 \frac{EF^2}{RT} \frac{[S]_c - [S]_v e^{(-zEF/RT)}}{1 - e^{(-zEF/RT)}}, \quad (1)$$

where E is the membrane potential (in volts), F is the Faraday constant, R is the gas constant, T is the absolute temperature, z is the valency of the ion S, $[S]$ is the activity (in molar concentration) of the ion on the vacuolar (v) and cytosolic (c) side of the membrane, and P is the permeability of the ion.

Because the current, I, passing through a channel is the sum of the currents carried by the individual ions, at equilibrium ($E = E_{rev}$),

$$I = I_K + I_{Cl} + I_{Ca} = 0. \quad (2)$$

These current terms can be expanded to yield the following identities for a given solution (A) on the cytosolic side of the membrane (that is, bath solution):

$$I_K = P_K Q_K^A,\ I_{Cl} = P_{Cl} Q_{Cl}^A,\ I_{Ca} = P_{Ca} Q_{Ca}^A;$$

and for a separate bath solution (B),

$$I_K = P_K Q_K^B,\ I_{Cl} = P_{Cl} Q_{Cl}^B,\ I_{Ca} = P_{Ca} Q_{Ca}^B,$$

where the Q terms are defined from Equation 1 as

$$z^2 \frac{EF^2}{RT} \frac{[S]_c - [S]_v e^{(-zEF/RT)}}{1 - e^{(-zEF/RT)}}.$$

Therefore, at equilibrium in solution A, Equation 2 becomes

$$Q_K^A + \left[\frac{P_{Cl}}{P_K}\right] Q_{Cl}^A + \left[\frac{P_{Ca}}{P_K}\right] Q_{Ca}^A = 0. \quad (3)$$

Similarly for solution B, Equation 2 becomes

$$Q_K^B + \left[\frac{P_{Cl}}{P_K}\right] Q_{Cl}^B + \left[\frac{P_{Ca}}{P_K}\right] Q_{Ca}^B = 0. \quad (4)$$

Combining Equations 3 and 4,

$$-\frac{P_{Cl}}{P_K} = \frac{Q_K^A}{Q_{Cl}^A} + \frac{P_{Ca} Q_{Ca}^A}{P_K Q_{Cl}^A} = \frac{Q_K^B}{Q_{Cl}^B} + \frac{P_{Ca} Q_{Ca}^B}{P_K Q_{Cl}^B},$$

which rearranges to give

$$\frac{P_{Ca}}{P_K} = \frac{(Q_{Cl}^A Q_K^B - Q_{Cl}^B Q_K^A)}{(Q_{Cl}^B Q_{Ca}^A - Q_{Cl}^A Q_{Ca}^B)}. \quad (5)$$

Having solved Equation (5) for P_{Ca}:P_K, the value of P_{Cl}:P_K can be determined from Equation (3) or (4).

Patch Clamp Apparatus and Analysis

Patch clamp recordings were made as described by Hamill et al. (1981), with the reference Ag/AgCl half cell connected to the bath by a 3% agar bridge in pipette medium. Pipette potentials (V_p) were controlled with an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Data were digitized at 1 kHz (1401 A/D converter; Cambridge Electronic Design, Cambridge, UK) and recorded directly onto computer disk after low-pass filtering at 200 to 400 Hz. Data analysis used software packages from Cambridge Electronic Design. The actual potential across the membrane (V_m) was calculated by correcting V_p for the liquid junction potential (E_L), such that $V_m = -(V_p - E_L)$. The value of E_L was calculated using a relationship that accounts for the presence of polyvalent ions (Equation 12 in Barry and Lynch, 1991). The values of E_L obtained for each solution are given in the figure legends. Because pipette solutions were used in the reference electrode, V_p was corrected for E_L for each solution change. This is because E_L appears across the bath/reference junction, which is not the case when a 3 M KCl reference is used (Neher, 1992).

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