Extracellular Ba^{2+} and voltage interact to gate Ca^{2+} channels at the plasma membrane of stomatal guard cells

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Abstract **Ca^{2+} channels at the plasma membrane of stomatal guard cells contribute to increases in cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) that regulate K^+ and Cl^- channels for stomatal closure in higher-plant leaves. Under voltage clamp, the initial rate of increase in $[Ca^{2+}]_i$ in guard cells is sensitive to the extracellular divalent concentration, suggesting a close interaction between the permeant ion and channel gating. To test this idea, we recorded single-channel currents across the *Vicia* guard cell plasma membrane using Ba^{2+} as a charge carrying ion. Unlike other Ca^{2+} channels characterised to date, these channels activate at hyperpolarising voltages. We found that the open probability (P_o) increased strongly with external Ba^{2+} concentration, consistent with a 4-fold cooperative action of Ba^{2+} in which its binding promoted channel opening in the steady state. Dwell time analyses indicated the presence of a single open state and at least three closed states of the channel, and showed that both hyperpolarising voltage and external Ba^{2+} concentration prolonged channel residence in the open state. Remarkably, increasing Ba^{2+} concentration also enhanced the sensitivity of the open channel to membrane voltage. We propose that Ba^{2+} binds at external sites distinct from the permeation pathway and that divalent binding directly influences the voltage gate.**

Key words: Ca^{2+} channel, hyperpolarisation-activated; Extracellular Ba^{2+}; Gating, ligand-mediated; Cytosolic free $[Ca^{2+}]$; *Vicia*

1. Introduction

Changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) are ubiquitous as components of second messenger cascades in plants and have been associated, among others, with mechanical and thermal disturbances [1,2], pathogen attack [3], nodulation [4] and hormonal signal transduction [5–7]. In many cases, the mechanisms for these $[Ca^{2+}]_i$ changes are poorly understood, and both Ca^{2+} entry and intracellular release have been proposed to play central roles. It seems likely that the early events include activation of Ca^{2+} channels at the plasma membrane and Ca^{2+} influx that triggers Ca^{2+} release from intracellular stores, much as has been described for Ca^{2+}-induced Ca^{2+} release (CICR) in animal cells [8,9].

Calcium channels that might fill such a role – exhibiting the canonical, depolarisation-mediated activation of their animal counterparts – have been identified at the plasma membrane of carrot suspension culture cells [10] and wheat roots [11], although in neither case has a physiological role been established. Intriguingly, of the few instances in which plasma membrane Ca^{2+} channels are known to be associated with increases in $[Ca^{2+}]_i$ in plants, channel activity is potentiated by membrane hyperpolarisation [12–14]. These characteristics indicate gating properties atypical of Ca^{2+} channels previously identified in plants or animals.

We previously identified a 12 pS Ca^{2+} channel a the plasma membrane of stomatal guard cells [14], following on evidence that membrane hyperpolarisation evokes increases in $[Ca^{2+}]_i$ and contributes to $[Ca^{2+}]_i$ transients associated with the plant water-stress hormone abscisic acid and related signal cascades [7,15]. These changes in $[Ca^{2+}]_i$ depend on Ca^{2+} release from intracellular stores [16–19], as well as on Ca^{2+} entry across the plasma membrane [15,20]. One intriguing feature of the $[Ca^{2+}]_i$ rise in guard cells is its linear dependence on external Ca^{2+} concentration [15], including concentrations above those that saturate the open channel conductance of the Ca^{2+} channel [14]. This behaviour implies an additional action of the divalent cation on gating. We have examined the gating properties of the guard cell Ca^{2+} channel, using Ba^{2+} as the permeant cation to avoid Ca^{2+} entry and the effects of Ca^{2+} on the inner surface of the membrane that lead to channel inactivation [14]. Analysis of single-channel kinetics as a function of Ba^{2+} concentration inside and outside the membrane indicates the presence of binding sites associated with the outer surface of the channel that interact cooperatively to stabilise the open state of the channel and enhance its sensitivity to membrane voltage.

2. Materials and methods

2.1. Plant material

Epidermal strips of *Vicia faba* L., cv. Bunyard Exhibition were obtained and protoplasts prepared as described [21,22]. All operations were carried out on a Zeiss Axiovert microscope with 40×LWD Nomarski D.I.C. optics (Zeiss, Oberkochen, Germany) at 20–22°C. Solution was added (≈20 chamber volumes/min) by gravity feed and removed by aspiration.

2.2. Electrophysiology

Pipettes were pulled using a Narashige PP-81 puller (Narashige, Tokyo) modified for 3-stage pulls (input resistances, 30–50 MΩ) to reduce the number of channels under a patch. Pipettes were coated

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with Sigmacote (Sigma, Poole, UK) to reduce capacitance. Connections to amplifier and bath were via a 0.1 M KCl/Ag–AgCl liquid junctions, and junction potentials were taken into account [23]. Single-channel currents were recorded using an Axopatch 200B patch amplifier (Axon Instruments, Redwood City) after filtering at 5 kHz and sampled at 44 kHz for analysis. Data were filtered at 1 kHz (Kemo, Beckenham, UK) off line and analysed using N-Pro (WyeScience, Kent) and P/V clamp v. 6 (CED, Cambridge) software. Voltages quoted are referenced to the physiological orientation of the membrane, the voltage on the cytosolic side relative to the extracellular side.

2.3. Numerical analysis

Channel amplitudes were calculated from point-amplitude histograms estimated from open events ≥5 ms duration beyond closed levels determined from periods of no channel activity [24]. Channel numbers were estimated from the maximum number of concurrent openings and from binomial distributions of open events [25]. Channel openings were taken as transitions above a threshold of 60% of the single-channel amplitude and P_o estimated from the open-time fraction corrected for the number of channels. Channel lifetime distributions were determined from dwell-time histograms of open and closed events, and were restricted to patches showing evidence of only a single channel. For analyses of closed lifetimes, the data were limited to the intraburst periods of channel activity and were generally defined by preceding and following closed periods at least 10-fold longer than of those within the burst. Open and closed dwell-time histograms were subjected to non-linear, least-squares exponential fittings [24,26] to determine the number of identifiable states and obtain estimates of relaxation constants in each case. Where appropriate results are given as means ± S.E.M. of (n) independent experiments.

2.4. Chemicals and solutions

We use the terms inside and outside with reference to the physiological sidedness of the membrane. Protoplasts were normally bathed in 2, 10 and 30 mM Ba^{2+}–HEPES, pH 7.5 (HEPES buffer titrated to its pK_a with $Ba(OH)_2$) adjusted to 200 mOsm with sorbitol, and pipettes were filled with similar solutions adjusted to 240 mOsm with sorbitol. $(Mg^{2+})_2ATP$ at 1 mM was included in solutions on the inside of the membrane. Buffers and salts were from Sigma Chemicals (Poole, UK).

3. Results and discussion

3.1. Increasing $[Ba^{2+}]_o$ strongly promotes channel opening events

A previous study of the guard cell Ca^{2+} channel showed that its steady state open probability (P_o) was strongly dependent on membrane hyperpolarisation. However, we found that the extent to which hyperpolarisation promoted channel events was also sensitive to the concentration of divalent available to permeate the channel. Fig. 1A shows single-channel records from one membrane patch in the outside-out configuration with Ba^{2+} as the permeant ion, and Fig. 1B summarises the results from this, and five additional, independent experiments. In general, increasing the Ba^{2+} concentration between 2 and 30 mM resulted in a pronounced rise in the number of channel openings which, on average, gave roughly a 50-fold increase in P_o at a common clamp voltage of −100 mV. The effect of Ba^{2+} also showed a definite sidedness. Similar measurements carried out in the inside-out configuration, varying the the Ba^{2+} concentration on the inside of the membrane with 10 mM and 30 mM Ba^{2+} outside, showed no comparable change in P_o [14]. These effects are also clearly different from the effect of Ca^{2+} inside, which led to a precipitous drop in channel openings as Ca^{2+} concentration rose to micromolar values [14].

Technical difficulties precluded increasing the Ba^{2+} on either side of the membrane to obtain estimates of P_o at concentrations significantly beyond 30 mM. Nonetheless, these data confirmed a sensitivity to Ba^{2+} outside, consistent with a high degree of cooperativity in promoting channel activity. The curve in Fig. 1B shows the results of fitting P_o to the Hill equation [27]. We found that the fitted value for the Hill coefficient, n, was well defined and largely insensitive to constraints on the maximum P_o. Allowing the maximum P_o to vary freely during the fitting yielded a Hill coefficient of 3.7 and, even with P_o constrained to a maximum of 1 gave a value only marginally smaller (see Fig. 1B and legend). Constraining n to values above 4, by contrast, failed to give visually acceptable matches to the data (Fig. 1B). Joint fittings incorporating P_o values gathered at −80 mV and at −110 mV yielded similar, and statistically more satisfactory results with a Hill coefficient of 3.6, both when values for maximum P_o and when $K_{1/2}$ were held in common between data at each voltage (Fig. 1B, inset). Thus, while the data are insufficient to define either of these parameters without points at higher

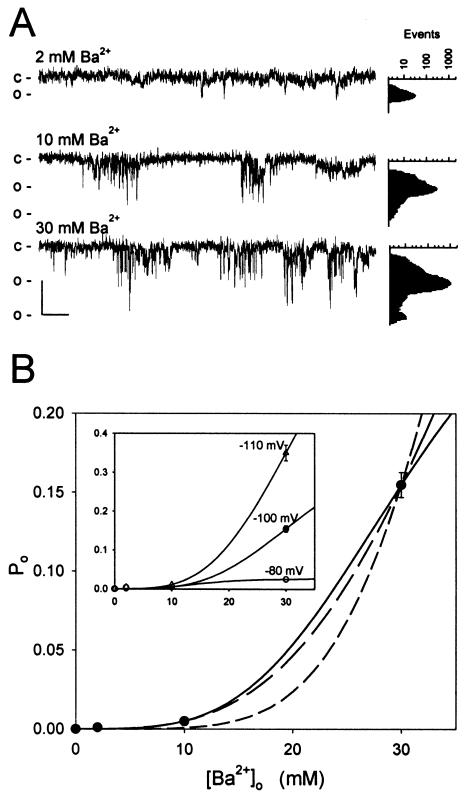

Fig. 1. Increasing Ba^{2+} concentration outside favours Ca^{2+} channel opening in the steady state. A: Single-channel records from one outside-out patch clamped at −100 mV with 30 mM Ba^{2+} inside and 2, 10 and 30 mM Ba^{2+} outside. Note the bursting characteristics of channel openings. Scale: 1 pA and 100 ms. Point-amplitude histograms (right) for 20 s recordings of the corresponding traces (see Section 2). B: Open probability (P_o) as a function of Ba^{2+} concentration at −100 mV from six independent experiments. Data fitted to a Hill function with P_o free (solid line), constrained to a maximum of 1 (long-dashed line), and to a maximum of 1 with n constrained to 5 (short-dashed line) for comparison. Best fittings gave $n = 3.6 \pm 0.2$ irrespective of the maximum P_o. Inset: Best fitting of the same data, joint with measurements at −80 and −110 mV, yielded $n = 3.7 \pm 0.1$, an apparent $K_{1/2}$ of 35 mM at all three voltages and P_o values of 0.07 (2 mM), 0.38 (10 mM) and 0.97 (30 mM Ba^{2+}).

values for $[Ba^{2+}]_o$, they imply the cooperative binding of four Ba^{2+} ions to facilitate opening of the channel.

3.2. Voltage and $[Ba^{2+}]_o$ suppress channel closure

To explore the mechanism of Ba^{2+} action and relationship to the voltage-evoked increase in P_o, we examined the open lifetime distributions of the Ca^{2+} channel using recordings in which only a single open level was clearly evident. Recordings from outside-out patches were selected for analysis that included measurements of 100 s duration at each of three $[Ba^{2+}]_o$, 2, 10 and 30 mM and over a range of membrane voltages. Representative lifetime distributions for one patch at selected voltages are shown in Fig. 2A and the results of

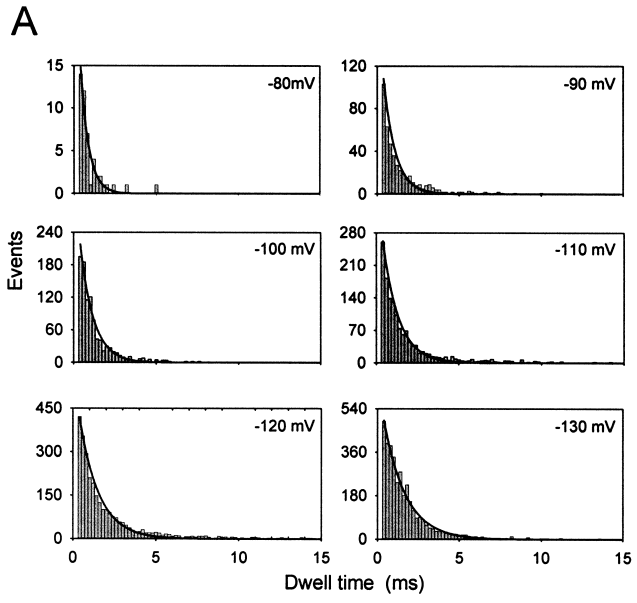

Fig. 2. Mean open lifetime of the Ca^{2+} channel, and its sensitivity to voltage, depends on external Ba^{2+} concentration. A: Open lifetime distributions at voltages between −80 and −130 mV for one outside-out patch with 30 mM Ba^{2+} inside and 10 mM Ba^{2+} outside. Data were well fitted in every case to a single-exponential function. B: Mean open lifetimes as functions of voltage with 2 (●), 10 (○) and 30 mM Ba^{2+} (▼) outside. Data are means ± S.E.M., from six independent experiments and show a roughly linear dependence on voltage. Inset: Slope of voltage dependence as a function of Ba^{2+} concentration.

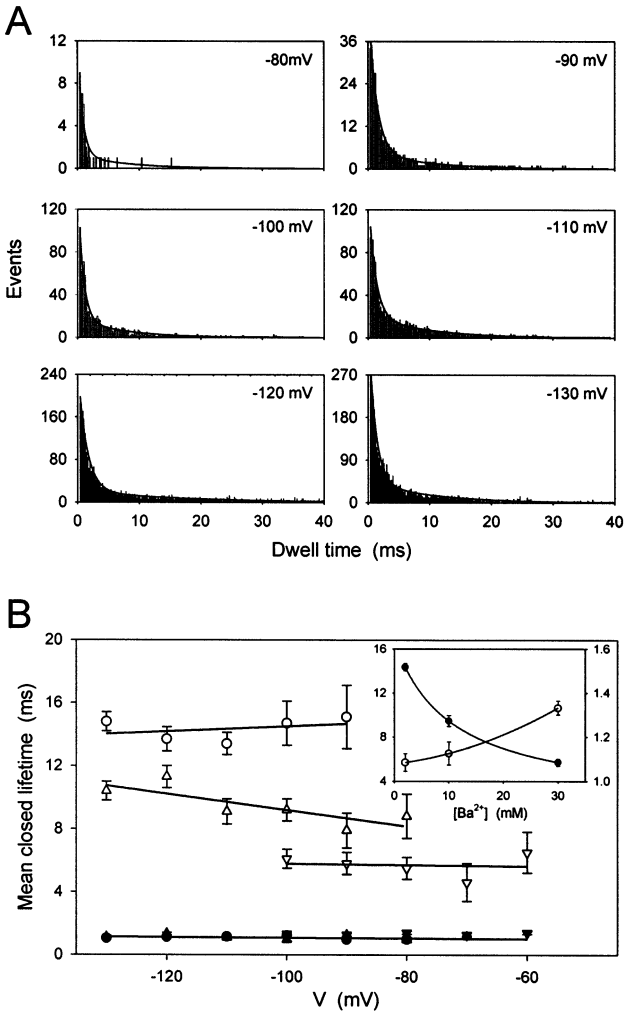

Fig. 3. Mean closed lifetime during bursts of Ca^{2+} channel activity depends on external Ba^{2+} concentration but not on voltage. A: Closed lifetime distributions at voltages between −80 and −130 mV for the same outside-out patch with 30 mM Ba^{2+} inside and 10 mM Ba^{2+} outside as in Fig. 2. Data were well fitted in every case only to a sum of two exponential components. B: Mean closed lifetimes as functions of voltage (components: fast, slow) with 2 (●, ○), 10 (▲, △) and 30 mM Ba^{2+} (▼, ▽) outside. Data are means ± S.E.M. from six independent experiments. Inset: Mean closed lifetime dependence of the fast (○) and slow (●) components as a function of Ba^{2+} concentration. Data were averaged over all voltages at each concentration.

analyses from six independent experiments, including these data, are summarised in Fig. 2B. In general, open lifetime distributions were well described by a single-exponential function for which the mean open lifetime, τ, increased with negative membrane voltage and $[Ba^{2+}]_o$. The analysis also showed that the sensitivity of τ to voltage increased with $[Ba^{2+}]_o$. This voltage sensitivity exhibited roughly a 2-fold rise over the order of magnitude increase in $[Ba^{2+}]_o$ of these experiments (Fig. 2B, inset). Thus, the Ca^{2+} channel could be characterised by the presence of a single open state. Furthermore, increasing negative membrane voltage prolonged the lifetime of the channel once in the open state, and increasing $[Ba^{2+}]_o$ potentiated this effect of voltage, as if Ba^{2+} itself contributed in part to the voltage sensor.

Analysis of the closed lifetimes of the Ca^{2+} channel pointed

to a more complex pattern of non-conducting states. Visual inspection showed a pronounced bursting characteristic to the channel (cf. Fig. 1A) with frequent periods, often of seconds, without opening events. Insufficient data were obtained to carry out a quantitative analysis of this bursting behaviour. Nevertheless, its occurrence indicates at least one long-lived closed state of the Ca^{2+} channel. Within the burst periods, the distribution of closed lifetimes showed little evidence of voltage dependence. Fig. 3A shows the closed lifetime distributions from the same experiment as in Fig. 2A and these are included in the summary in Fig. 3B. Closed lifetime distributions required the inclusion of a second exponential component for fitting. We found little evidence of a sensitivity to clamp voltage for either of the exponential components, but increasing $[Ba^{2+}]_o$ resulted in a significant decrease in the mean closed lifetime of the slower component (Fig. 3B). The lifetime analyses, in this case, must be treated with some caution. Although we took care to select records that appeared to show only a single channel, the very low values for P_o made it possible that more than one channel was present even in these circumstances (see also Hamilton, et al. [14]). With two or more channels in a patch, it becomes impossible to know which opens at the end of a closed period [24]. One consequence is a skewing the analysis towards shorter apparent closed lifetimes which, in this case, may mask more profound effects of experimental manipulations. This caveat aside, the kinetic analysis and characteristics of bursting indicates the presence of at least three closed states.

3.3. *Interpreting the interaction of external Ba^{2+} with voltage in gating*

From Figs. 2 and 3 it is evident that a minimal model for Ca^{2+} channel gating requires a single open state and three separate closed states. At present we have few clues as to whether the open state communicates directly with only one, or with more than one of the closed states. However, the data do point to a strong effect both of voltage and $[Ba^{2+}]_o$ on the open channel lifetime (Fig. 2), the slower of the two closed lifetimes (Fig. 3) and, as a consequence on P_o in the steady state (Fig. 1; see also [14]). Furthermore, because P_o is profoundly dependent on membrane hyperpolarisation [14], we anticipate that the remaining rates of transition between states may also subsume a component of this voltage sensitivity. Overall, the effect of voltage on P_o on the guard cell Ca^{2+} channel is comparable to its influence on Ca^{2+} channels now identified from a variety of cell types, including leaf mesophyll [13] and root hairs [28]. Gating processes for plant Ca^{2+} channels, in general, have not been well defined. For the guard cell Ca^{2+} channel, our results now link voltage to an action on the channel in the open state. In essence, hyperpolarising the membrane stabilises the channel once open, effectively 'locking' the channel in this molecular configuration.

The most striking feature of this Ca^{2+} channel, however, is the effect of external Ba^{2+} concentration and its interaction with the action of membrane voltage. Not only did increasing $[Ba^{2+}]_o$ act to stabilise the open state of the channel, it also enhanced the sensitivity of the open state to membrane hyperpolarisations (Fig. 2B). We are not aware of such a synergy between the chemical and electrical parameters controlling gating in Ca^{2+} channels previously characterised either in plants or animals. For neuronal Ca^{2+} channels, changes of P_o with external Ca^{2+} are known, but these effects have been ascribed to surface-charge masking and generally saturate at concentrations near 2 mM [29,30]. In guard cells and yeast, external alkali cations affect voltage-dependent gating of K^+ channels [31–33], an effect which is mimicked by Ba^{2+} in the yeast YKC1 K^+ channel [32]. However, the actions of external cations in these instances are mediated through voltage-dependent block, albeit with cation binding to allosteric sites distinct from the channel pore. Furthermore, both in these K^+ channels and in the animal Ca^{2+} channels the external cations displace the voltage dependence of channel gating, seemingly altering its voltage 'set-point', but without affecting the underlying sensitivity to voltage. In both respects, therefore, the guard cell Ca^{2+} channel appears unique.

The fact that increasing $[Ba^{2+}]_o$ enhances P_o and the open channel dwell time as a function of voltage does suggest that the external divalent is an integral component of the voltage gate. Plausibly, Ba^{2+} binding to the outer surface of the Ca^{2+} channel may influence local energy landscape traversed by the voltage gate. For example, it could become an integral part of the voltage sensor itself, although other physical mechanisms are also possible. Our data do indicate an external location for Ba^{2+} binding that is separate from the permeation pathway, since changing internal Ba^{2+} concentration did not have an equivalent effect on P_o (compare also [33]). Therefore, we suggest the presence of four binding sites to account for the high apparent cooperativity of $[Ba^{2+}]_o$ action on P_o (Fig. 1), recognising the 4-fold symmetry in the assembly of subunits of many voltage-sensitive cation channels known to date [34–37], and the implicit distribution of binding sites, one per subunit assembly. Clearly, proof of this idea must now await genetic identification of the Ca^{2+} channel.

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