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Calcium-sensitivity of the Plasmalemmal Delayed Rectifier Potassium Current Suggests that Calcium Influx in Pulvinar Protoplasts from *Mimosa pudica* L. can be Revealed by Hyperpolarization

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Abstract. Isolated protoplasts from pulvinar motor cells of *Mimosa pudica* were studied using conventional whole-cell patch clamp techniques. With internal solutions weakly buffered for Ca^{2+} (0.2 mM EGTA), a rundown of the outward delayed rectifier K^+ current was induced by hyperpolarizing the holding potential, and this effect was strongly promoted by high external Ca^{2+} concentrations. This rundown could be reversed by coming back to less hyperpolarized holding potentials or by lowering the external $[Ca^{2+}]$. Such rundown was absent when pipette internal solutions strongly buffered (10 mM EGTA) for Ca^{2+} were used. Ionomycin induced rundown of the K^+ current with internal solutions containing 0.2 mM but not 10 mM EGTA. The hyperpolarization-associated rundown was reversibly blocked by Gd^{3+} and La^{3+}.

Key words: *Mimosa pudica* — Pulvinus — Protoplast — Delayed rectifier potassium current — Calcium influx — Gadolinium

Introduction

The delayed, outwardly rectifying K^+ current (I_{Kout}) is the most prominent voltage-activated current characterized in the plasmalemma of protoplasts from motor cells of the primary pulvinus of *Mimosa pudica* L (Stoeckel & Takeda, 1989). A similar K^+ current has been detected in almost all plant protoplasts studied to date (for reviews, Hedrich & Schroeder, 1989; Tester, 1990; Blatt & Thiel, 1993). In *Mimosa* motor cells, I_{Kout} is especially prominent, and clearly plays an essential role in the repolarization phase of the action potential in these excitable cells (Stoeckel & Takeda, 1993). The inward depolarization-activated currents responsible for triggering action potentials are likely to involve Ca^{2+} and Ca^{2+}-dependent Cl^- channels.

We describe here a sensitivity of I_{Kout} towards cytoplasmic Ca^{2+} which was observed during experiments where internal and external Ca^{2+} concentrations were manipulated, and especially at hyperpolarized holding potentials. This is seen as a rundown in I_{Kout} amplitude. In our previous studies where internal Ca^{2+} was strongly buffered (Stoeckel & Takeda, 1989, 1993), such holding potential-dependent changes in I_{Kout} amplitude were not observed. The experimental conditions used here were initially designed to mimic those employed in studies of guard cell protoplasts, where activation of inward Ca^{2+}-dependent Cl^- currents was favored by elevated external Ca^{2+} concentrations and especially by pipette internal solutions weakly buffered for Ca^{2+} (Hedrich et al., 1990). We hoped that cell excitability would be thus favored, but this was not the case. Rather, the data show that I_{Kout} is decreased in conditions favoring a rise of the internal Ca^{2+} concentration and are consistent with a Ca^{2+} influx being revealed at hyperpolarized holding potentials. This Ca^{2+} influx would be clearly different from the putative depolarization-activated Ca^{2+} entry associated with action potential generation.

Materials and Methods

Plants and Isolation of Protoplasts

Plants were cultivated from seeds in greenhouses at 20–30°C where daylight was supplemented with artificial light. The youngest leaves from 3–4-month old plants were isolated. The motor tissue from the lower (abaxial) half of the primary pulvinus was cut using a scalpel into 3 or 4 fine slices, and rinsed twice with the enzymatic digestion medium (*see below*). Usually 5 half pulvini were incubated in 2 ml of the

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digestion solution for 2.5 hr at 30°C. The digestion medium contained 4% cellulase (2% Cellulase Onozuka R10 from Yakult, Honshu, Japan, and 2% Caylase 345 from Cayla, Toulouse, France), 1% pectinase (Pectinase Rohament 5 from Serva) or 1% macerozyme (Macerozyme Onozuka R10 from Yakult, Honshu, Japan), 0.1% pectolyase (Pectolyase Y23 from Seishin Pharmaceutical, Japan) and 0.2% BSA (Fraction V, protease-free, Sigma) dissolved in an external solution containing (in mM): 600 mannitol, 8 $CaCl_2$, 25 MES, pH 5.6 with KOH ($[K^+]$ was 6 and osmolarity was 720 mOsmol/kg). After digestion, protoplasts were centrifuged (30 min at $100 \times g$) on a discontinuous Ficoll gradient (20, 10, 0% Ficoll in external solution). Protoplasts collected at the 10–0% interface were kept on ice until use and were similar to those described previously (Stoeckel & Takeda, 1993).

Electrophysiology

Conventional patch clamp techniques (Hamill et al., 1981) were used, as in previous studies (Stoeckel & Takeda, 1989, 1993), with macroscopic currents being recorded in the whole-cell configuration. Pipettes were made from thick-wall borosilicate glass (Hilgenberg, Germany) using a vertical puller (D. Kopf 720) and were coated with beeswax to reduce associated capacitance. Pipette resistances were in the range of 10 MΩ with the solutions used. The standard pipette internal solution contained (in mM): 150 KCl, 100 HEPES, 10 or 2 or 0.2 EGTA, pH 7.8 with KOH; 2 or 5 ATP and/or 0.2 or 0.4 GTP were usually added. In some cases, pH was 6.8. An internal solution with a pCa_i of 6 was made using 9.9 mM $CaCl_2$ and 10 mM EGTA. The osmolarity of all internal solutions was adjusted to 760–780 mOsm/kg with mannitol. External bath solutions contained (in mM): 550 mannitol, 30 $CaCl_2$, 25 MES/6 KOH pH 5.6 or 20 HEPES/6 KOH pH 7.0 (high Ca^{2+}), or 550 mannitol, 8 $CaCl_2$, 22 $MgCl_2$, 25 MES/6 KOH pH 5.6 or 20 HEPES/6 KOH pH 7 (high Mg^{2+}); the osmolarity of these solutions was 715 mOsm/kg. When indicated, the osmolarity of the high Ca^{2+} solution was increased to 770 mOsm/kg with mannitol. E_{Cl} was 23 mV, except for internal pCa 6 solution (25 mV). E_K was −94.5 mV for internal 10 mM EGTA, pH 7.8 solution, −92.5 mV for internal 0.2 mM EGTA, pH 7.8 solution and −92 mV for internal 0.2 mM EGTA, pH 6.8 solution.

Protoplasts were placed in a polylysine-coated Petri dish (volume 2–3 ml), continuously perfused with external medium (≈2–3 ml/min) and viewed with a microscope equipped with phase contrast optics (Nikon Diaphot) and a green filter. Cell capacitance and series resistance were compensated for using the inbuilt circuitry of the patch clamp amplifier (List EPC-7). Data were acquired on DAT (Biologic DTR1200) for later offline analysis using a Goupil 386DX lab computer with a CED 1401 interface and CED software. Leak subtraction was in general not employed. Typically, I_{Kout} was activated by 1-sec depolarizing steps to a constant test pulse amplitude of 80 mV, given at 10-sec intervals. Increasingly hyperpolarized holding potentials were used, usually −60, −100, −140 and −180 mV. At each holding potential, at least 10 test pulses were applied. Experiments were made at room temperature. Data from 107 pulvinar protoplasts are presented here. In the figures, outward currents are shown upwards, and 0 current levels are indicated by dashed lines.

Results

Hyperpolarization-associated and Calcium-dependent Rundown of I_{Kout}

When the holding potential was hyperpolarized to −100, −140 or to −180 mV from an initial value of −60 mV, the peak amplitude of I_{Kout} elicited for successive step depolarizations to 80 mV gradually decreased or ran down. Rundown occurred in 56 of 63 protoplasts using a pipette internal solution weakly buffered for Ca^{2+} (with 0.2 mM EGTA) and an external solution containing a high Ca^{2+} concentration (30 mM). This is illustrated in Figure 1*A*, where the largest I_{Kout} was obtained from a −60 mV holding potential (control) and the progressively smaller amplitude currents (numbered 1–7) were successively obtained after switching the holding potential to −100 mV (all currents were for test pulses to 80 mV). After stabilization of the amplitude of I_{Kout} for a given holding potential, a further rundown could be induced by using a more hyperpolarized holding potential (e.g., Fig. 3*B* and *C;* Fig. 6*B*). Note that I_{Kout} amplitude was stable with a −60 mV holding potential (Fig. 1*B*). Recovery from rundown of I_{Kout} was observed when the holding potential was returned to a less hyperpolarized value (Fig. 1*B*). This reversal was not always complete but was always slower in time course compared to the onset of rundown (Fig. 1*B*).

Rundown was not dependent on the repetitive application of step depolarizations: I_{Kout} elicited after hyperpolarization of the holding potential for varying periods in the absence of test pulses was not different from I_{Kout} observed after the same delay when test pulses were continuously given (*not shown*).

Rundown of I_{Kout} was favored by the presence of a high external $[Ca^{2+}]$. For example, as illustrated in Fig. 2*A*, peak amplitudes of I_{Kout} were always greater in high Mg^{2+} (22 mM Mg^{2+}, 8 mM Ca^{2+}; *see* Materials and Methods) compared to high Ca^{2+} (30 mM Ca^{2+}, 0 Mg^{2+}) external bath solutions at hyperpolarized holding potentials (n = 13), and this was observable even with a −60 mV holding potential in some cells (9 of 20 protoplasts). In some cases, rundown in high Mg^{2+} solution was not observed unless the holding potential was made more hyperpolarized compared to that in high Ca^{2+} solution (*not shown;* n = 14). Finally, rundown of I_{Kout} induced by hyperpolarization of the holding potential in high Ca^{2+} external solution could be totally or partially reversed by changing to high Mg^{2+} solution (e.g., Fig. 2*A* where the protoplast was first exposed to high Ca^{2+} bath solution, followed by high Mg^{2+} solution). None of these effects are likely to be related to differences in surface charge screening as the total divalent cation concentrations were identical in these solutions.

As illustrated in Fig. 2*B*, when pipette internal solutions strongly buffered for Ca^{2+} (with 10 mM EGTA) were used, almost no rundown of I_{Kout} was induced upon hyperpolarization of the holding potential in the presence of high Ca^{2+} external solutions (23 of 28 protoplasts). However, even in the presence of internal 10 mM EGTA, when Ca^{2+} was buffered to pCa 6, hyperpolarization induced rundown could be detected at holding potentials of −100 mV in 2 of 3 protoplasts.

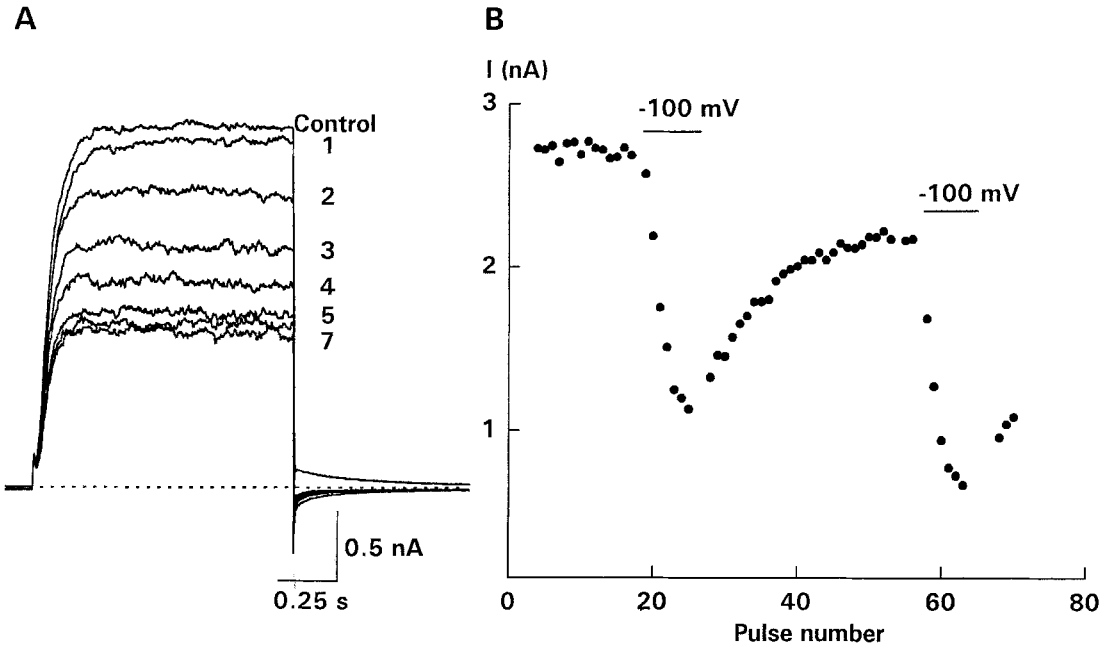

Fig. 1. Rundown of I_{Kout} in *Mimosa pudica* pulvinar protoplasts following hyperpolarization of the holding potential. (*A*) Outward K^+ currents observed for successive voltage pulses to 80 mV given every 10 sec. The largest current was evoked from a holding potential of −60 mV (Control), and the currents numbered 1–7 were consecutively obtained after switching the holding potential to −100 mV. (*B*) Time course of I_{Kout} peak amplitude for step depolarizations to 80 mV from a −60 mV holding potential and when the holding potential was switched to −100 mV (bars). Total duration represented is 12 min. Same protoplast as in *A*. High Ca^{2+} (30 mM) external solution; pipette internal solution contained *inter alia* 0.2 mM EGTA, 2 mM ATP and 0.4 mM GTP.

Quasi steady-state current-voltage (*I–V*) relationship for I_{Kout} obtained using ramp commands from holding potentials of −60, −100 and −140 mV in the presence of high Ca^{2+} and high Mg^{2+} external solutions are shown in Fig. 3. For this protoplast, no significant differences were seen between the two different external solutions with a control holding potential of −60 mV (Fig. 3*A*). At more hyperpolarized holding potentials, I_{Kout} was decreased compared to control in both solutions, with this decrease being greater in high Ca^{2+} external solution (Fig. 3*B* and *C*). *I–V* relationships for this protoplast at three different holding potentials in high Ca^{2+} external solution are shown superimposed (Fig. 4*A*) and after normalization to the peak control I_{Kout} amplitude (Fig. 4*B*). The lack of change both in the threshold for activation of I_{Kout} (Fig. 3) and in the overall form of the *I–V* relation (Fig. 4) are again consistent with the idea that surface charge screening effects are not involved in the decrease in I_{Kout} amplitude.

As both the time- and voltage-dependence of I_{Kout} activation remained unchanged after rundown was induced (Fig. 4), it appears reasonable to conclude that the current after rundown is still essentially K^+-selective. Direct confirmation is provided in Fig. 5, where it can be seen that the tail current reversal potential is unaffected after hyperpolarization-associated rundown of I_{Kout} (n = 5). The reversal potential remains close to E_K, consistent with the outward current being carried mainly by K^+. A similar result was obtained for I_{Kout} after rundown induced by ionomycin (*not shown;* n = 2).

Rundown was not related to pH, as it was also observed when the external pH was raised to 6.8 or 7 (n = 10), when the internal pH was lowered to 6.8 (n = 4) and in the absence of a plasmalemmal pH difference (n = 2). A change of intracellular pH during the time course of our experiments is also very unlikely, given the large concentration of HEPES used in the pipette internal solutions. The rundown was observed in the presence (n = 16) and in the absence (n = 35) of ATP and/or GTP in the pipette internal solution. It was also observed in protoplasts not attached to the polylysine-coated bath bottom (n = 1), in the absence of polylysine (n = 4) and also in the absence of bath perfusion (n = 4). In all of the above conditions, rundown was most prominent upon hyperpolarization of the holding potential with a high Ca^{2+} external solution and a pipette internal solution with 0.2 mM EGTA.

Effects of Ionomycin on I_{Kout}

The calcium ionophore ionomycin was used to test the hypothesis that an increase in cytoplasmic free $[Ca^{2+}]$ underlies the decrease in I_{Kout} amplitude. Local microperfusion of 20 μM ionomycin (for 3–5 sec) induced a rapid and pronounced decrease in the peak amplitude of

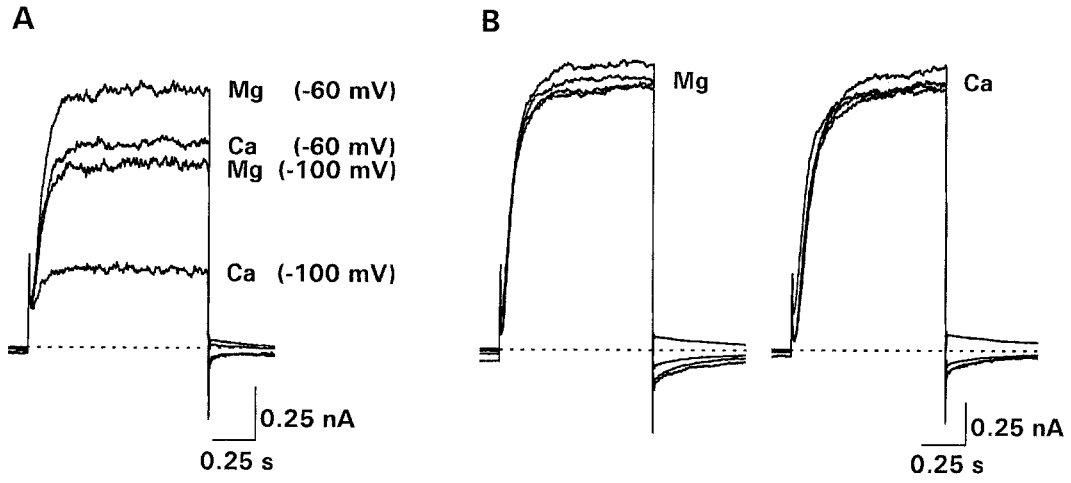

Fig. 2. The hyperpolarization-associated rundown of I_{Kout} depends on external $[Ca^{2+}]$ and on internal Ca^{2+} buffering capacity. (*A*) Outward K^+ currents in a single protoplast elicited by steps to 80 mV from holding potentials of −60 mV and about 1 min after switching to −100 mV, as indicated; the bath contained initially a high Ca^{2+} external solution, and was then changed to a high Mg^{2+} external solution during a period of 10 min without stimulation. The pipette internal solution contained *inter alia* 0.2 mM EGTA, 2 mM ATP and 0.2 mM GTP. Note that even with a −60 mV holding potential, I_{Kout} amplitude is larger in high Mg^{2+} compared to high Ca^{2+} solution. (*B*) Rundown is not seen upon hyperpolarization of the holding potential when the pipette internal solution is strongly buffered for Ca^{2+} (with 10 mM EGTA). Currents elicited from a single protoplast by steps to 80 mV from holding potentials of −60 mV and about 1 min after switching to −100, −140 and −180 mV in the presence of initially external high Mg^{2+} (left) and then high Ca^{2+} (right) solutions. In this protoplast, the largest I_{Kout} was obtained from a −180 mV holding potential in both external solutions.

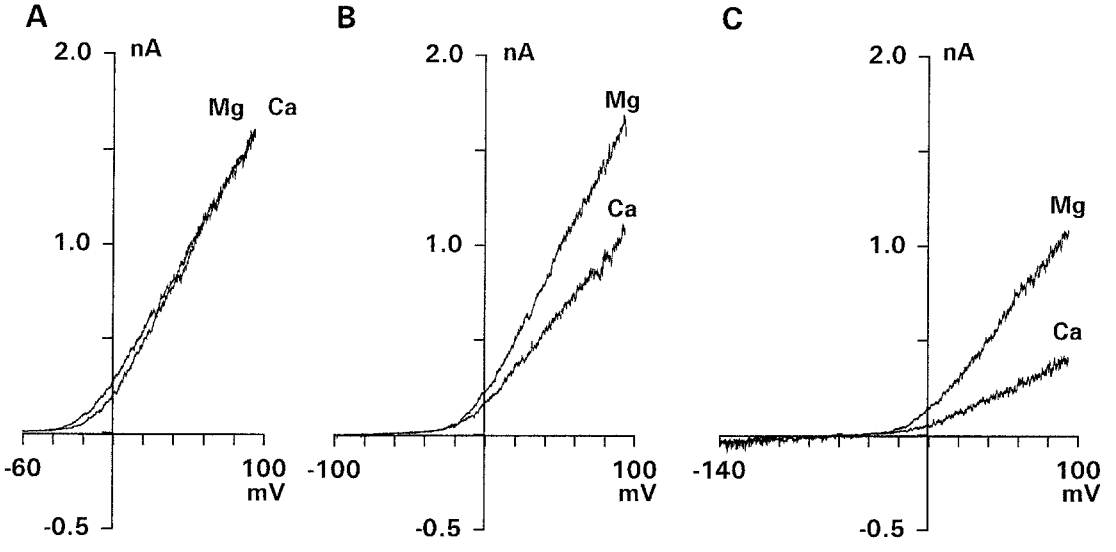

Fig. 3. Quasi steady-state ramp *I–V* relations for I_{Kout} for a single protoplast at holding potentials of −60 (*A*), −100 (*B*) and −140 mV (*C*), in external high Ca^{2+} and high Mg^{2+} solutions. Internal solution contained *inter alia* 0.2 mM EGTA, 5 mM ATP and 0.2 mM GTP. Ramp commands (having a constant *dV/dt* of 35 mV/sec) were applied at least 1 min after switching to the different holding potentials.

I_{Kout} (Fig. 6). The applications of ionomycin (n = 11) were done in high Ca^{2+} external solutions (pH 7), with (n = 4) or without (n = 7) nucleotides being present in the pipette internal solution (which contained 0.2 mM EGTA). With the same experimental conditions, rundown associated with hyperpolarization was also observable (Fig. 6*B*), although both the onset and recovery were much slower compared to that for ionomycin. Note that the effect of ionomycin was reversible (Fig. 6*B;* n = 7). As before, no effect of ionomycin was detected when the pipette internal solution was strongly buffered for Ca^{2+} with 10 mM EGTA (*not shown;* n = 5). As in Figs. 3 and 4, ramp *I–V* relationships in the presence of ionomycin revealed that I_{Kout} was decreased in a similar manner, with no change in the activation threshold nor in the overall form of the *I–V* relation (not shown; n = 2).

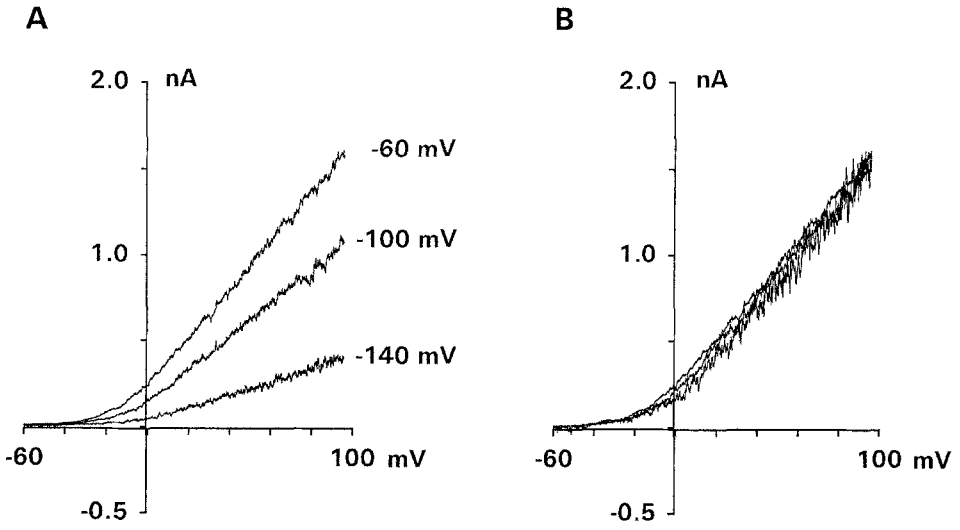

Fig. 4. *I–V* relations for I_{Kout} in external high Ca^{2+} solution superimposed for the indicated holding potentials of −60, −100 and −140 mV (*A*) and after normalization of peak amplitudes (*B*); I_{max} corresponds to the I_{Kout} peak amplitude obtained with a −60 mV holding potential. Same ramp conditions and same protoplast as Fig. 3.

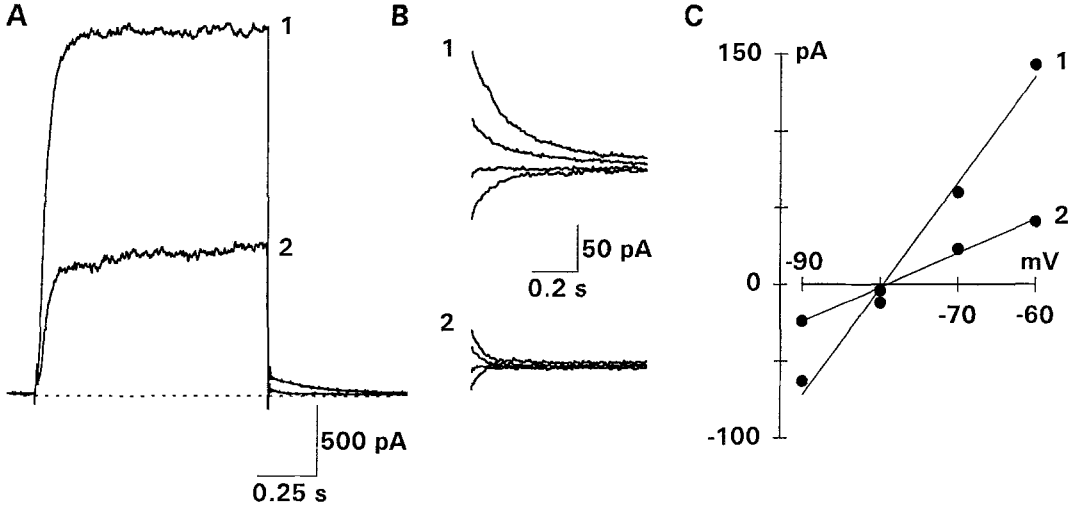

Fig. 5. The tail current reversal potential is unchanged after rundown of I_{Kout}. (*A*) Currents triggered by steps to 80 mV from a holding potential of −60 mV before (1) and just after (2) hyperpolarization for 30 sec to a −140 mV holding potential. High Ca^{2+} external solution, and internal solution with *inter alia* 0.2 mM EGTA and no nucleotides. (*B*) Tail currents for return potentials of −60 to −90 mV before (1) and after (2) hyperpolarization-associated rundown. (*C*) Corresponding *I/V* relationship of the tail currents before (1) and after (2) rundown.

Effects of Calcium Channel Antagonists on I_{Kout} Rundown

The rundown of I_{Kout} associated with changing the bath from high Mg^{2+} to high Ca^{2+} external solution at a holding potential of −100 mV was blocked when the high Ca^{2+} solution also contained either 2 mM La^{3+} (n = 6) or 0.5 to 2.0 mM Gd^{3+} (n = 4), as illustrated in Fig. 7. Interestingly, Gd^{3+} was also effective in preventing rundown at 200 μM (n = 3), while La^{3+} was not at 1 mM (n = 2). In protoplasts where rundown had been induced by hyperpolarization, 200 μM Gd^{3+} caused a partial reversal of I_{Kout} rundown (n = 2; *not shown*).

A variety of organic Ca^{2+} channel antagonists were also tested (25 and 50 μM bepridil, 5 μM nitrendipine and 50 and 100 μM verapamil). However, these antagonists produced complex effects on I_{Kout}. A decrease of I_{Kout} was seen with 5 μM nitrendipine (*not shown;* n = 5), while a reduction in peak amplitude and the appearance of a rapid "inactivation" was observed with 25 μM bepridil and 50 μM verapamil as illustrated in Fig. 8. Note that even at high concentrations, neither La^{3+} (5 mM; *not*

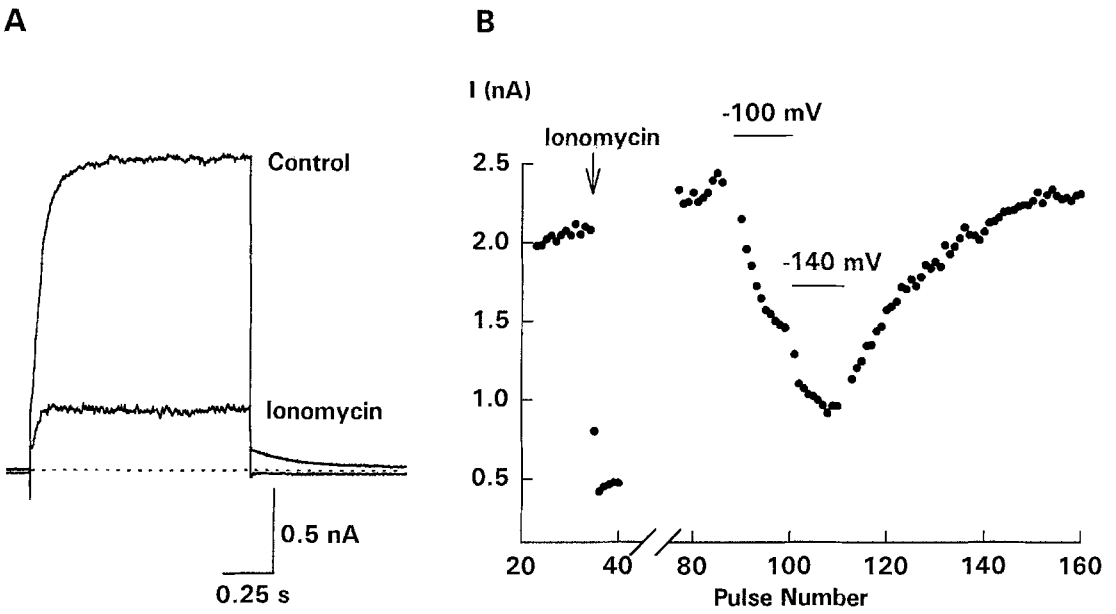

Fig. 6. Ionomycin induces a rapid decrease in I_{Kout} amplitude. (*A*) Currents triggered by steps to 80 mV from a holding potential of −60 mV, before (Control), and 20 sec after puffer application of ionomycin for 3–5 sec. The 0-current level is indicated by the dotted line. High Ca^{2+} external solution, and internal solution with *inter alia* 0.2 mM EGTA and no nucleotides. (*B*) Time course of ionomycin and hyperpolarization induced rundown of I_{Kout}. Currents were elicited by steps to 80 mV every 10 sec from a −60 mV holding potential, ionomycin was applied for 3–5 sec (arrow), and the holding potential was changed to −100 and −140 mV as indicated. Total duration represented is 26 min. External solution high Ca^{2+}, internal solution contained *inter alia* 0.2 mM EGTA and no nucleotides. Different protoplast from *A*.

shown) nor Gd^{3+} (2 mM; e.g., Fig. 7) produced such "inactivation."

Discussion

We report here a Ca^{2+} sensitivity of I_{Kout} in protoplasts of *Mimosa* pulvinar motor cells that is most notable at hyperpolarized holding potentials using a high external [Ca^{2+}] and a pipette internal solution containing 0.2 mM EGTA. The morphological organization of protoplasts from higher plant cells often results in the cytoplasmic compartment being reduced to a thin layer between the plasmalemma and the tonoplast. It is well known that the vacuole, which can occupy up to 90% of the cellular volume, is characterized by an acidic pH and by a high [Ca^{2+}]. To avoid modifications of the cytoplasmic compartment due to any leak from the vacuole, internal solutions were strongly buffered for H^+ and for Ca^{2+} in previous studies (Stoeckel & Takeda, 1989, 1993). In those conditions, no holding potential dependence of I_{Kout} was observed. The rundown of I_{Kout} characterized here was found only with pipette internal solutions having weak Ca^{2+} buffering.

Calcium Effect on I_{Kout}

Clearly, internal free Ca^{2+} is involved in the decrease of I_{Kout} seen when hyperpolarized holding potentials were used, being favored by high external [Ca^{2+}] and not being observed with pipette internal solutions containing 10 mM EGTA. The ionomycin-induced decrease of I_{Kout} (Fig. 6) appears to unambiguously confirm the role played by Ca^{2+}.

In many types of animal cells, elevation of cytoplasmic free Ca^{2+} increases K^+ conductance by opening Ca^{2+}-sensitive K^+ channels (for review, Latorre et al., 1989). In only a few cases are outward K^+ currents inhibited by internal Ca^{2+}. For example, elevated cytoplasmic Ca^{2+} reduces outward A-type K^+ current in T-lymphocytes (Bregestovski et al., 1986), as is the case for a maintained outward K^+ current in *Limulus* ventral photoreceptors (Chinn & Lisman, 1984) and in human adenomatous parathyroid cells (Komwatana et al., 1994). However, in general, the delayed outward K^+ rectifier current is unaffected by cytoplasmic Ca^{2+} (Hille, 1992).

In plant cells, Ca^{2+}-sensitivity of plasmalemmal K^+ currents was first described for hyperpolarization-activated inward K^+ currents in stomatal guard cell protoplasts from *Vicia faba,* where raising free internal Ca^{2+} from 0.1 to 1.5 μM blocked I_{Kin} without affecting I_{Kout} (Schroeder & Hagiwara, 1989). However in these cells, it was later reported (Fairley-Grenot & Assmann, 1992) that an increase in $[Ca^{2+}]_i$ from 2 to 180 nM inhibited I_{Kout} (by 59% at 80 mV), with a similar effect in maize guard cells (80% decrease in I_{Kout} at 80 mV; Fairley-Grenot & Assmann, 1992). It should also be noted that

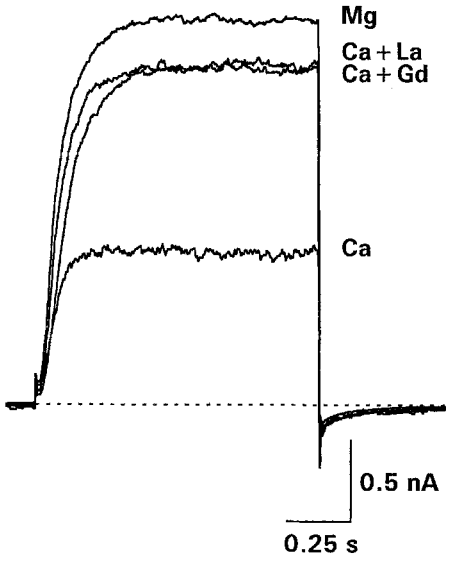

Fig. 7. Gd^{3+} and La^{3+} block the hyperpolarization-associated rundown of I_{Kout}. Currents elicited by steps to 80 mV from a holding potential of −100 mV, initially in external high Mg^{2+} solution and then in high Ca^{2+} solutions, in the absence and presence of 2 mM $LaCl_3$ or 2 mM $GdCl_3$. Internal solution contained *inter alia* 0.2 mM EGTA, 5 mM ATP and 0.4 mM GTP. Data obtained 60–90 sec after switching the holding potential.

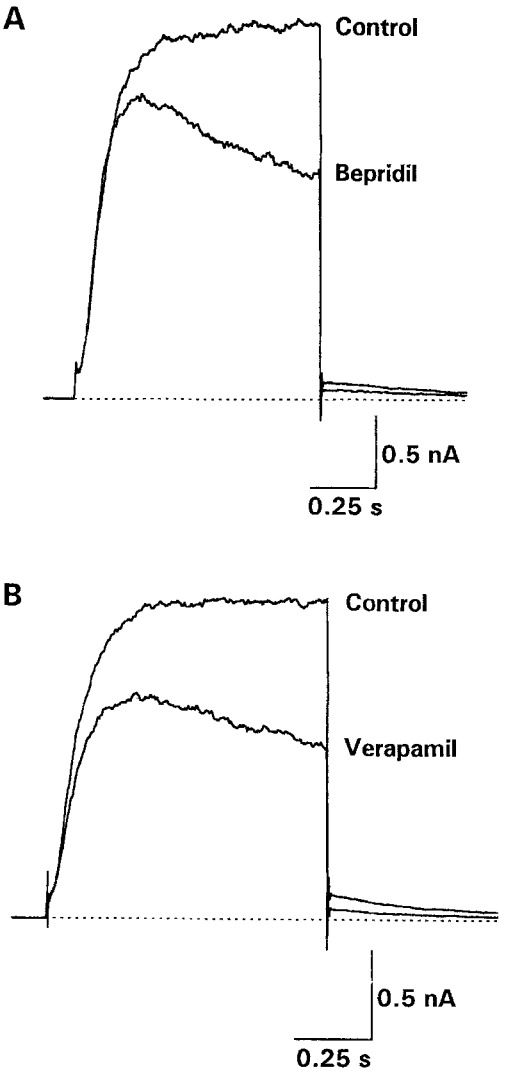

Fig. 8. Effects of bepridil and verapamil on I_{Kout}. Currents elicited by steps to 80 mV from a holding potential of −60 mV in the absence and presence of 25 μM bepridil (*A*) or 50 μM verapamil (*B*). External high Ca^{2+} solution. Internal solution contained *inter alia* 0.2 mM EGTA and no nucleotides. Data from 2 different protoplasts.

in mesophyll cell protoplasts from *Vicia faba*, I_{Kout} was greater with 5 mM BAPTA in the pipette internal solution compared to when Ca^{2+} was buffered to 20 μM (Li & Assmann, 1993), and in suspension-cultured corn cell protoplasts, I_{Kout} was enhanced when internal Ca^{2+} was buffered to 400 nM *vs.* 40 nM, but with 1 mM free Ca^{2+}, I_{Kout} rapidly decreased (Ketchum & Poole, 1991).

The Ca^{2+} dose-response on single I_{Kout} open channel probability was not determined here, as inside-out patches are exceedingly difficult to make from our protoplasts. Because both hyperpolarization-associated and ionomycin-induced rundown of I_{Kout} were reversible and could be triggered repeatedly without nucleotides in the pipette solution, the effect of Ca^{2+} on I_{Kout} here does not seem to involve kinase- or phosphatase-mediated steps, unlike in *Vicia faba* guard cell (Thiel & Blatt, 1994).

Recently, I_{Kout} sensitivity to cytoplasmic pH (Blatt, 1992; Blatt & Armstrong, 1993) and to external pH (Ilan et al., 1994) has been reported. The pH of both our internal and external solutions was strongly buffered (with respectively 100 mM HEPES and 25 mM MES or 20 mM HEPES). Nevertheless, as ionomycin promotes Ca^{2+}/H^+ exchange, local acidification may occur externally, perhaps thereby resulting in a pH-mediated decrease of I_{Kout}. At least, when internal Ca^{2+} was strongly buffered with 10 mM EGTA, the effects of ionomycin were prevented, consistent with a rise in free internal Ca^{2+} being responsible for the rundown of I_{Kout}.

Calcium Influx and Effects of Calcium Channel Antagonists

While Gd^{3+} selectively blocks mechanically-activated channels in animal (Yang & Sachs, 1989) and plant cells (Ding & Pickard, 1993), it also blocks voltage-sensitive Ca^{2+} channels in a variety of animal cells (Docherty, 1988; Canzoniero et al., 1993; Mlinar & Enyeart, 1993; Lacampagne et al., 1994). In plant cells, Gd^{3+} blocks Ca^{2+} influx (Johannes et al., 1992; Allen & Sanders, 1994; Marshall et al., 1994; Rengel, 1994), as does La^{3+} (Huang et al., 1994; Marshall et al., 1994). Here, the rundown of I_{Kout} observed at hyperpolarized holding potentials depends not only on a low [EGTA] in the pipette

internal solution, but also was strongly favored by a high external [Ca^{2+}]. As the driving force for passive Ca^{2+} entry increases with hyperpolarization, together with the blocking actions of La^{3+} and Gd^{3+}, it appears reasonable to suggest that Ca^{2+} influx is associated with I_{Kout} rundown. The Gd^{3+} and La^{3+} induced decrease of I_{Kout} amplitude described in several types of protoplasts (Moran et al., 1990; Ketchum & Poole, 1991; Terry et al., 1992) was not observed here.

Depolarization-activated Ca^{2+} channels appear not to be involved in the rundown of I_{Kout} observed here, as there was an increasing effect with hyperpolarization of the holding potential up to −180 mV, in accord with a passive type of Ca^{2+} influx. In higher plant cells, voltage-activated Ca^{2+} entry has been reported to be maximal at about −80 mV (Marshall et al., 1994), −85 mV (Thuleau et al., 1994) and −100 mV (Huang et al., 1994). In carrot cell protoplasts, Ca^{2+} uptake was inhibited by verapamil and bepridil but not by dihydropyridines (Graziana et al., 1988).

Verapamil, bepridil and nitrendipine produced complex blocking effects on I_{Kout} in our *Mimosa* protoplasts, presumably unrelated to antagonism of Ca^{2+} channels. Similar actions of Ca^{2+} channel antagonists on outward rectifying K^+ currents have already been described in plant cells (Ketchum & Poole, 1991; Terry et al., 1992; Thomine et al., 1994). Such effects make questionable the interpretation of experiments where the involvement of Ca^{2+} fluxes in physiological processes in higher plants has been tested only using these antagonists.

As Gd^{3+} was most efficient in preventing the hyperpolarization-associated rundown, stretch-activated channels may be involved. Gd^{3+}-sensitive, Ca^{2+}-permeable stretch-activated channels have been described in plant plasmalemma (Ding & Pickard, 1993), and in *Mimosa* motor pulvini such channels likely underly the mechanosensitivity of these excitable cells. Usually, the difference in osmolarity between our internal and external solutions was about 50 mOsmol/kg, in the range sufficient for inducing the activation of hydrostatic- and osmotic-dependent channels in the tonoplast of red beet vacuoles (Alexandre & Lassalles, 1991). However, hyperpolarization-associated rundown was also seen when this difference in osmolarity was abolished (by raising the osmolarity of the external solution), suggesting that Ca^{2+} influx is not likely mediated by stretch-activated channels. Thus, the exact mechanisms underlying the hyperpolarization-associated Ca^{2+} influx remain an open question for the moment, and require further study.

In situ, the resting potential of these motor cells is very hyperpolarized (around −180 mV) and action potentials can be triggered by depolarization from a hyperpolarized resting potential, although this was found in only a very few protoplasts under the experimental conditions used in our previous studies (Stoeckel & Takeda, 1993). A likely first step underlying the action potential is a depolarization-activated, transient Ca^{2+} influx, associated with the development of regenerative inward current through Ca^{2+}-sensitive Cl^- channels. Such Ca^{2+} influx would appear to be clearly different from the putative Ca^{2+} influx at hyperpolarized holding potentials accounting for the decrease in I_{Kout} revealed here. Disappointingly, the different experimental protocols employed here, which mimic those favoring Ca^{2+}-dependent Cl^- current activation in guard cell protoplasts (Hedrich et al., 1990), clearly did not increase the number of excitable *Mimosa* protoplasts.

The physiological relevance of this putative hyperpolarization-associated Ca^{2+} influx is unclear. A possible function for such influx may be linked to the known elevated intravacuolar Ca^{2+} concentrations in higher plant cells. One also wonders whether such Ca^{2+} entry might be related to the passive Ca^{2+} influx through I_{CRAC} channels described in animal cells (Fasolato et al., 1994). Our observations raise questions concerning both the cytoplasmic Ca^{2+} buffering capacity and the apoplastic free Ca^{2+} activity *in vivo.* Furthermore whether the procedures used to enzymatically isolate protoplasts from motor tissue modulate cell function is an important point, as there is no assurance that for example, cellular responses to osmotic stresses or elicitation-like responses do not occur during protoplast isolation. In any case, if the levels of cytoplasmic [Ca^{2+}] induced in our experiments occur under physiological conditions, they would clearly influence the maximum amplitude of I_{Kout}, and thus would modulate the duration and the repolarizing phase of the action potential.

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