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Voltage-dependent calcium-permeable channels in the plasma membrane of a higher plant cell

Patrice Thuleau, John M.Ward, Raoul Ranjeva[1] and Julian I.Schroeder[2]

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0116, USA and
[1]Université Paul Sabathier, CNRS, 118 Route de Narbonne, Toulouse, France
[2]Corresponding author

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Numerous biological assays and pharmacological studies on various higher plant tissues have led to the suggestion that voltage-dependent plasma membrane Ca^{2+} channels play prominent roles in initiating signal transduction processes during plant growth and development. However, to date no direct evidence has been obtained for the existence of such depolarization-activated Ca^{2+} channels in the plasma membrane of higher plant cells. Carrot suspension cells (*Daucus carota* L.) provide a well-suited system to determine whether voltage-dependent Ca^{2+} channels are present in the plasma membrane of higher plants and to characterize the properties of putative Ca^{2+} channels. It is known that both depolarization, caused by raising extracellular K^+, and exposure to fungal toxins or oligogalacturonides induce Ca^{2+} influx into carrot cells. By direct application of patch–clamp techniques to isolated carrot protoplasts, we show here that depolarization of the plasma membrane positive to –135 mV activates Ca^{2+}-permeable channels. These voltage-dependent ion channels were more permeable to Ca^{2+} than K^+, while displaying large permeabilities to Ba^{2+} and Mg^{2+} ions. Ca^{2+}-permeable channels showed slow and reversible inactivation. The single-channel conductance was 13 pS in 40 mM $CaCl_2$. These data provide direct evidence for the existence of voltage-dependent Ca^{2+} channels in the plasma membrane of a higher plant cell and point to physiological mechanisms for plant Ca^{2+} channel regulation. The depolarization-activated Ca^{2+}-permeable channels identified here could constitute a regulated pathway for Ca^{2+} influx in response to physiologically occurring stimulus-induced depolarizations in higher plant cells.

Key words: calcium channels/cytosolic Ca^{2+}/elicitor/signal transduction

Introduction

Rises in the cytoplasmic Ca^{2+} concentration of higher plant cells have been implicated in the response of plants to many environmental, hormonal and pathogenic signals (Hepler and Wayne, 1985; Leonard and Hepler, 1990). Voltage-dependent Ca^{2+} channels in the plasma membrane of higher plant cells have been suggested to provide a regulated pathway for Ca^{2+} influx. In animal cells and algae, voltage-dependent Ca^{2+} channels are activated by depolarization (Nowycky *et al.*, 1985; Taylor and Brownlee, 1993; for reviews see Tazawa *et al.*, 1987; Tsien and Tsien, 1988; Hess, 1990). Higher plant membrane potentials are normally found in the range of –120 to –180 mV (Bates and Goldsmith, 1983; Felle, 1988). Membrane potential depolarizations positive to –120 mV have been identified as an early response of plant cells to a variety of physiological stimuli. For example, nodulation factors (Ehrhardt *et al.*, 1992), auxin (Bates and Goldsmith, 1983; Marten *et al.*, 1991), abscisic acid (Kasamo, 1981; Ishikawa *et al.*, 1983), light (Racusen and Satter, 1975; Spalding and Cosgrove, 1989) and phytotoxins (Ullrich and Novacky, 1991) produce membrane depolarizations in specific plant cells. Such depolarizations can be produced by H^+ pump inhibition, anion channel activation, non-selective ion channel activation or K^+ channel modulation (for a review see Schroeder and Hedrich, 1989; Tester, 1990) and are associated with Ca^{2+}-dependent signaling (Williamson and Ashley, 1982; Hepler and Wayne, 1985; Felle, 1988; Leonard and Hepler, 1990; Ranjeva *et al.*, 1992; Messiaen *et al.*, 1993).

Carrot cells provide an opportune system to determine whether voltage-dependent Ca^{2+} channels can couple membrane depolarizations with Ca^{2+} influx in a higher plant cell. Previous work has shown that elevation of the extracellular K^+ concentration increases the cytosolic Ca^{2+} concentration in carrot cells (Ranjeva *et al.*, 1992), suggesting that depolarization may induce Ca^{2+} influx across the plasma membrane of these cells. In addition, Ca^{2+} influx into carrot cells is stimulated by the fungal toxin zinniol and by oligogalacturonides (Thuleau *et al.*, 1988; Messiaen *et al.*, 1993). While indirect observations suggest that voltage-dependent Ca^{2+} channels may function in plants, voltage-clamp data unequivocally showing the existence and mechanisms of operation of such Ca^{2+} channels are lacking in higher plants and have been reported in algae (Taylor and Brownlee, 1993; for a review see Tazawa *et al.*, 1987).

Results

To test directly whether depolarization-activated Ca^{2+} channels exist in carrot cells, the whole-cell patch–clamp technique was applied to isolated carrot protoplasts (n = 85). The cytosol of cells was perfused using patch electrodes with a K^+-free solution to abolish K^+ channel currents and cells were bathed in a 40 mM $CaCl_2$ solution to enhance the resolution of putative Ca^{2+} currents (Tsien and Tsien, 1988). Furthermore, solutions were composed to distinguish Cl^- currents and Ca^{2+} currents by imposing negative Cl^- equilibrium potentials and positive Ca^{2+} equilibrium potentials. The membrane potential was con-

tinuously held at −140 to −160 mV, which lies in the range of physiological resting potentials in higher plant cells (Spanswick, 1981; Bates and Goldmith, 1983). Many physiological signals trigger early depolarization of the plasma membrane to potentials positive of −120 mV. When a depolarizing voltage ramp was applied to carrot cells, voltage-dependent inward currents became activated at potentials positive to −135 mV (±16 mV, n = 46) (Figure 1A). Maximum currents occurred at −84 mV (±15 mV, n = 46), which was in the range of the equilibrium potential for chloride, showing that these currents were not carried by chloride ions (Figure 1).

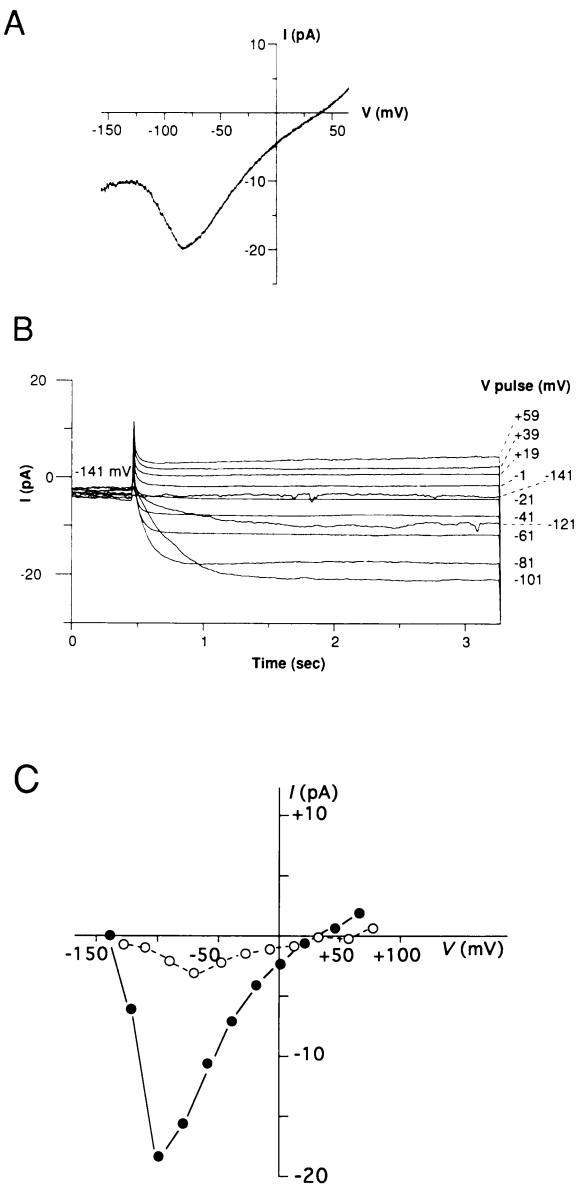

Fig. 1. Voltage-dependent Ca^{2+}-permeable currents in carrot protoplasts. (**A**) Whole-cell currents measured in the presence of 40 mM $CaCl_2$ in the extracellular medium during a 4 s voltage ramp ranging from −161 to +59 mV. Downward deflections of current traces correspond to cation influx in all figures. (**B**) Consecutive voltage steps applied from a holding potential of −141 mV to pulse potentials (V_{pulse}) ranging from −141 to +59 mV, as indicated to the right of currents. (**C**) Currents at the end of voltage pulses shown in (B) after linear leak subtraction, recorded with 40 mM $CaCl_2$ in the bath (●) are plotted as a function of the applied membrane potentials. ○, currents recorded in the presence of 5 mM $CaCl_2$ in the extracellular medium after linear leak subtraction.

The current–voltage relationship, determined by consecutive depolarizing voltage steps, showed that the currents reversed at a membrane potential of approximately +30 (±15 mV, n = 15) (Figure 1B and C). The equilibrium potentials of all ions with the exception of Ca^{2+} and protons were more negative than −60 mV, indicating that voltage-dependent currents were carried primarily by either Ca^{2+} or protons (see Materials and methods). We examined the selectivity of voltage-dependent channels with respect to the relevant physiological cations H^+, Ca^{2+}, K^+ and Mg^{2+}. Changing the extracellular pH from 5.5 to 7.7 resulted in no measurable changes in the time and voltage dependence nor in the reversal potential of voltage-dependent currents, showing that these channels are not proton selective (data not shown, n = 5). Subsequent experiments were performed at more neutral pH values to reduce any contributions of protons to inward currents. Decreasing the extracellular Ca^{2+} concentration to 5 mM resulted in an inward current of smaller magnitude with a similar voltage dependence to that observed with 40 mM $CaCl_2$ in the external medium (Figure 1C). With 5 mM $CaCl_2$ in the bath, the chloride equilibrium potential was −22 mV which significantly differed from the positive reversal potential of whole-cell currents, supporting the findings that voltage-dependent whole-cell currents were not chloride selective. Reversal potentials determined from whole-cell currents were variable (see below). Nevertheless, these data showed that the depolarization-activated currents were produced mainly by an influx of Ca^{2+} across the plasma membrane of carrot cells.

To examine whether the voltage-dependent Ca^{2+}-permeable channels in carrot cells were selective for K^+, 100 mM K^+ was added to the cytosolic solution and 10 mM K^+ was added to the external medium. Under these conditions, when depolarizing voltage ramps were applied to carrot protoplasts, voltage-dependent inward currents were observed with the same voltage-dependent activity recorded in the absence of K^+ (Figure 2A). These inward currents activated at potentials positive to −123 mV (±11 mV; n = 5) with a maximum current in the range of −75 mV. Voltage-dependent Ca^{2+}-permeable channels were selective for Ca^{2+} over K^+, as the peak inward currents occurred in the range of the Nernst potential for K^+ under the imposed experimental conditions (Figure 2A). Depolarization of carrot protoplasts to more positive potentials (n = 5) led to the activation of voltage-dependent outward-rectifying currents. Experiments performed using the same K^+ concentrations, with external Ca^{2+} removed, showed the presence of outward-rectifying currents which activated at potentials positive to approximately −50 to −20 mV, while inward-conducting Ca^{2+} currents were abolished (Figure 2A). These data suggest that voltage-dependent Ca^{2+} channels and outward-rectifying currents in carrot cells were functionally distinct from one another and that the Ca^{2+}-permeable channels were not K^+ selective. A K^+ permeability of the voltage-dependent Ca^{2+} channels identified here was, however, not excluded by these findings.

To investigate the selectivity of voltage-dependent Ca^{2+}-permeable channels with respect to physiologically occurring divalent cations, Ca^{2+} was replaced in the external

medium by Mg^{2+}. When depolarizing voltage ramps were applied to carrot cells perfused with a solution containing 40 mM $MgCl_2$, voltage-dependent inward currents were observed ($n = 5$) (Figure 2B). At the peak current potential, the magnitude of Mg^{2+} currents was 55% (±5%, $n = 5$) that of Ca^{2+} currents, indicating a modest selectivity of these channels for Ca^{2+} over Mg^{2+} (Figure 2B). Furthermore, voltage-dependent currents reversed at approximately +17 mV (±5 mV, $n = 5$) after correction for the linear background membrane resistance of 20 to >40 GΩ. The complete removal of divalent cations from the bath solution suppressed voltage-dependent inward currents (data not shown).

While voltage-dependent properties of the whole-cell currents were consistent from cell to cell, reversal potentials for whole-cell Ca^{2+}-permeable currents were variable within the range of +20 to +50 mV (e.g. compare Figures 1 and 2B). The source of this variability has not been clearly identified, although the low conductance of Ca^{2+} currents in the range of the reversal potential is similar to the background conductance and would produce variability in reversal potentials due to low cytosolic divalent cation concentrations. Also, the activity of other active and passive conductances may contribute to this variability. Similarly, in whole-cell recordings in animal systems, reversal potentials of voltage-dependent Ca^{2+} channel currents can be shifted strongly from the theoretical equilibrium potential for Ca^{2+} because the conductance near the reversal potential approaches that of the background conductance (Fenwick *et al.*, 1982). Therefore, accurate reversal potentials were determined from single-channel recordings as described later.

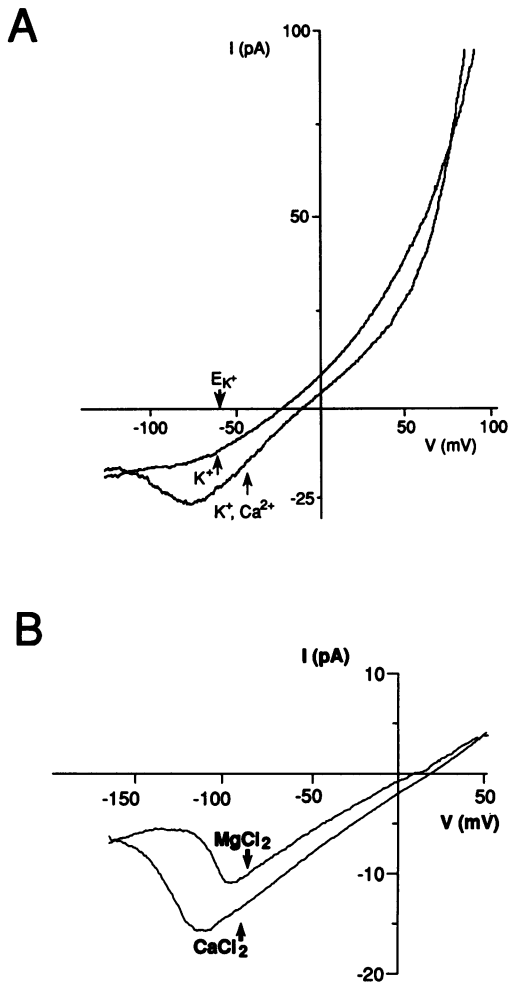

Fig. 2. Selectivity of voltage-dependent Ca^{2+}-permeable channels. (**A**) Whole-cell currents measured during a 4 s voltage ramp between −130 and +90 mV from a holding potential of −127 mV. Carrot protoplasts were bathed in 10 mM K^+ glutamate, 10 mM HEPES with and without 40 mM $CaCl_2$ as indicated by the arrows. The pH was adjusted to 6.7 by the addition of 1.4 mM Tris and the osmolality adjusted with sorbitol to 600 mosmol/kg. The internal solution contained 100 mM K^+ glutamate, 2 mM $MgCl_2$, 10 mM $Tris_2$–EGTA, 10 mM MgATP, 200 μM GTPγS, 10 mM HEPES–Tris (pH 7.2), osmolality 620 mosmol/kg adjusted with sorbitol. The K^+ equilibrium potential is indicated (E_{K+}). (**B**) Whole-cell currents measured in carrot protoplasts perfused with an external solution containing 10 mM HEPES–Tris (pH 6.7), sorbitol, osmolality 600 mosmol/kg and either 40 mM $MgCl_2$ or 40 mM $CaCl_2$ as indicated by the arrows. The internal solution was identical to the one described in Materials and methods.

Time-dependent properties of voltage-dependent Ca^{2+}-permeable channels were studied by stepping the membrane potential from the holding potential of −141 mV to more positive potentials (Figure 3). At −81 mV inward Ca^{2+} currents were activated with a half-time of ~60 ms (Figure 3A). When the potential was stepped back to the holding potential, deactivation of voltage-dependent channels was observed with a half-time of 70 ms at −141 mV (Figure 3A). To determine whether Ca^{2+} channel currents showed time-dependent decay during stimulation (inactivation), the membrane potential was held to −81 mV for extended durations. A slow decay of Ca^{2+} currents was observed, with a half-time of ~60 s (Figure 3B, $n = 2$). Following this decay in Ca^{2+} currents, 80% of initial peak currents could be restored by subsequently holding the membrane at −141 mV for 80 s, showing that Ca^{2+} channels display stimulation-dependent and reversible inactivation.

In addition to stimulus-induced inactivation, voltage-dependent Ca^{2+} currents usually decreased slowly following establishment of whole-cell recordings ('run down'). However, this run down of Ca^{2+} currents differed from voltage-dependent inactivation as it could not be reversed by hyperpolarizations in the range from −120 to −160 mV lasting for up to 12 min. A fraction of current inactivation (~20%) may, therefore, have been attributable to run down (Figure 3B), suggesting slightly longer stimulus-induced inactivation times. The run down of Ca^{2+} currents indicates a role for additional cytosolic regulatory mechanisms. Experiments revealed that GTPγS is not required for the activation of Ca^{2+} currents ($n = 17$) suggesting other, yet to be identified, regulatory components for Ca^{2+} channel modulation. In addition, cells were found that did not show voltage-dependent Ca^{2+} currents which may suggest strong regulation of Ca^{2+} channels by physiological factors.

In additional experiments, calcium in the bath solution was replaced with 30 mM $BaCl_2$. Voltage-dependent currents carried by barium showed a peak current amplitude at −80 mV (±12 mV, $n = 11$) and reversed at +45 ± 13 mV. Barium currents were larger in amplitude than those carried by calcium, although in the presence of barium, the voltage-dependent channels rapidly inactivated. Based on these data, a permeability sequence of $Ba^{2+} > Ca^{2+} > Mg^{2+}$ was determined.

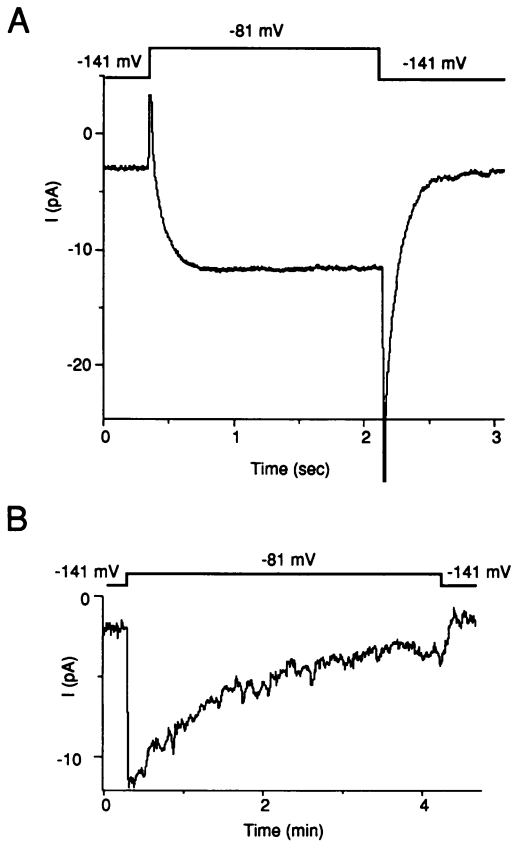

Fig. 3. Time-dependent activity of voltage-dependent Ca^{2+}-permeable channels. (**A**) Inward Ca^{2+} current generated by a 1.7 s voltage pulse to –81 mV from a holding potential of –141 mV. (**B**) Inactivation of voltage-dependent Ca^{2+} channels resulting from a prolonged stimulation of 4.1 min at a holding potential of –81 mV. Solutions were identical to those described in Materials and methods.

The wash-out of Ca^{2+} currents allowed the recording of single voltage-dependent ion channels in whole-cell recordings (Figure 4). At a holding potential of –141 mV, only a few discrete single-channel current steps were observed (Figure 4A). Depolarization of carrot cells led to an increase in opening events of single channels comparable with the depolarization-induced activation of macroscopic Ca^{2+} currents (n = 5, Figure 4A). Single-channel currents were analyzed by detailed Gaussian analysis to determine amplitudes of unitary transitions at various membrane potentials (Figure 4B). Single-channel recordings resulted in estimates of a main ion conducting state with a mean conductance of 13 ± 4 pS.

The resolution of single-channel currents at steady-state membrane potentials was limited because of the activation of multiple voltage-dependent Ca^{2+} channels during depolarization in whole cells. Therefore, the single-channel conductance, as well as reversal potentials of single-channel currents, were determined accurately by depolarizing voltage ramps. The main single-channel conductance derived from voltage ramps was in the range of 13–14 pS (Figure 4C). The reversal potentials of single-channel currents was +41 mV, which lies ~100 mV more positive than the equilibrium potential of all major ions present in the cytosolic and bath solutions, with the exception of Ca^{2+}, showing that the channels are permeable to Ca^{2+} ions. The permeability ratio for Ca^{2+} over Mg^{2+}, derived

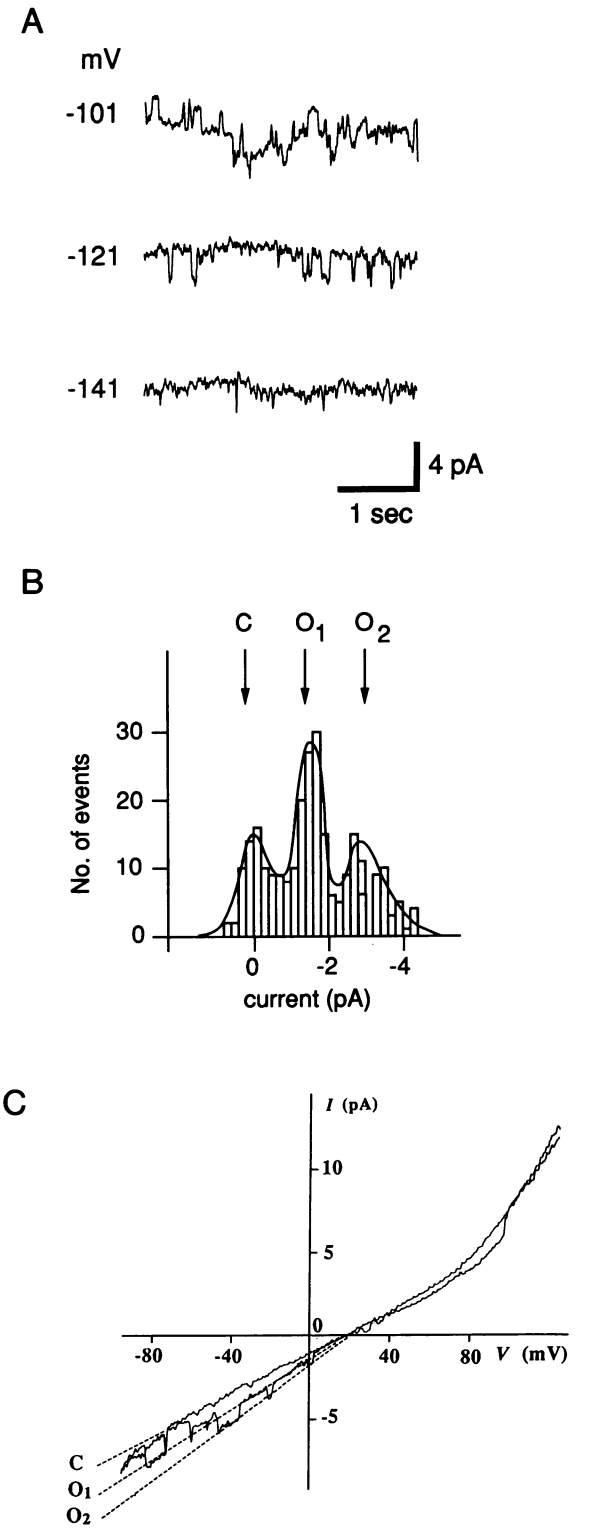

Fig. 4. Single-channel recordings of voltage-dependent Ca^{2+}-permeable channels. (**A**) Single-channel currents recorded at the membrane potentials indicated to the left with solutions corresponding to those in Figure 1 at a bath pH of 7.4 by the addition of 4 mM CaOH. (**B**) Amplitude histogram of single-channel currents measured at –101 mV. C, O_1 and O_2 indicate peak Gaussian current levels of closed, one and two open channel conductances, respectively. (**C**) Single Ca^{2+} channel currents recorded during two consecutive 4 s depolarizing voltage ramps from –101 to +119 mV. C, O_1 and O_2 indicate current levels of closed, one and two open channel conductance states, respectively. The conductance C to O_1 was 14.0 pS and the conductance state O_1 to O_2 was 13.1 pS.

from reversal potentials of single-channel currents and a calculated free cytosolic Mg^{2+} concentration of 2.4 mM, was 1.72:1. For the previously discussed reasons, single-channel reversal potentials provide more accurate ionic selectivity values than whole-cell current reversal potentials (Fenwick *et al.*, 1982). The voltage dependence of single Ca^{2+}-permeable channel currents, as well as reversal potentials, correlate closely with the macroscopic voltage-dependent Ca^{2+}-permeable currents described above, showing that Ca^{2+}-permeable channels produced depolarization-activated Ca^{2+} influx currents in carrot cells.

Discussion

Numerous physiological, pharmacological and Ca^{2+} flux studies have provided indirect evidence suggesting that voltage-dependent Ca^{2+} channels in the plasma membrane of higher plant cells play a central role in the initiation of various signal transduction processes, including plant–pathogen interactions, geotropic responses, and hormone- and light-regulated growth and development (Hepler and Wayne, 1985; Leonard and Hepler, 1990). However, prior to this study, direct voltage clamp-derived evidence for depolarization-activated Ca^{2+} channels in the plasma membrane of higher plant cells had been completely lacking to our knowledge. In a previous reconstitution study, bepridil-sensitive ion channels from carrot cell membranes which are permeable to Ca^{2+} were identified (Thuleau *et al.*, 1993). Note that bepredil sensitivity alone cannot be taken as direct evidence for Ca^{2+} channel function because potent bepridil block of K^+ channels in plants has recently been reported (Terry *et al.*, 1992; Thomine *et al.*, 1994).

As an early event in higher plant signaling, many physiological stimuli induce membrane depolarization from the normal range of resting potentials (approximately −120 to −180 mV) (Spanswick, 1981; Bates and Goldsmith, 1983) to potentials more positive than −120 mV (Racusen and Satter, 1975; Davies and Schuster, 1981; Bates and Goldsmith, 1983; Felle, 1988; Spalding and Cosgrove, 1989; Ullrich and Novacky, 1991; Ehrhardt *et al.*, 1992; Wildon *et al.*, 1992). Plasma membrane depolarizations can be produced by H^+ pump inhibition, anion channel activation, non-selective ion channel activation or K^+ channel modulation (Schroeder and Hedrich, 1989; Schroeder and Hagiwara, 1990; Tester, 1990). The activation of voltage-dependent Ca^{2+} channels by depolarizations positive to −135 mV reported here shows that Ca^{2+} channels can be activated in response to physiological depolarizations in higher plants.

Assuming a Ca^{2+} influx current of −5 pA (Figures 1–3) and reported cellular buffering capacities in the range of 95% of incoming Ca^{2+} (Becker *et al.*, 1988), depolarization-activated Ca^{2+} channels would produce a rate of increase in the cytosolic free Ca^{2+} concentration in carrot cells of 0.3 μM/s. This rate of increase in cytosolic Ca^{2+} can account for typical signal-induced cytosolic Ca^{2+} elevations observed in plant cells (Hepler and Callaham, 1987; Miller and Sanders, 1987; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990; Gilroy and Jones, 1992; Shacklock *et al.*, 1992; Messiaen *et al.*, 1993). More rapid depolarization-activated rises in cytosolic Ca^{2+} have been observed in algae (Williamson and Ashley, 1982). Many mechanisms can produce more rapid rises in the cytosolic Ca^{2+} concentration, including higher Ca^{2+} channel density, more rapid activation of Ca^{2+} channels and concomitant intracellular Ca^{2+} release (for a review see Tsien and Tsien, 1988).

The plant Ca^{2+} channels reported here were activated by membrane depolarization (Figures 1 and 3), which is a typical characteristic of all voltage-dependent Ca^{2+} channels in animal cells (Fenwick *et al.*, 1982; Nowycky *et al.*, 1985; Hess, 1990). However, voltage-dependent Ca^{2+}-permeable channels in carrot cells display several striking and important differences when compared with all types of voltage-dependent Ca^{2+} channels in animal systems. The threshold potential for activation of voltage-dependent Ca^{2+} channels in carrot cells lies ~50–90 mV more negative (Figure 1) than that of any of the Ca^{2+} channel types described in animal cells (Fenwick *et al.*, 1982; Nowycky *et al.*, 1985; Tsien and Tsien, 1988; Hess, 1990) and ~100 mV more negative than those described in brown algae (Taylor and Brownlee, 1993). This negative threshold potential of Ca^{2+} channels in carrot cells appears to be optimally suited for higher plant cell resting potentials (Spanswick, 1981; Bates and Goldsmith, 1983) and signal-induced depolarizations.

In addition, the Mg^{2+} permeability of these voltage-dependent channels (Figure 2B) differs markedly from ion-selectivity properties of animal Ca^{2+} channels (Nowycky *et al.*, 1985; Hess, 1990). The physiological significance of the Mg^{2+} permeability revealed here remains unknown. However, Mg^{2+} has been shown to compete with Ca^{2+} influx in higher plant cells (Huang *et al.*, 1992). Non-selective Ca^{2+}-permeable channels have been previously reported in plant cells (Schroeder and Hagiwara, 1990; Cosgrove and Hedrich, 1991). The lack of strong selectivity of higher plant plasma membrane Ca^{2+} channels, initially described in guard cells (Schroeder and Hagiwara, 1990) and reported here, may seem unexpected. However, cell wall Ca^{2+} concentrations vary considerably in higher plants and a non-selective conductance may produce increased depolarization under conditions of low cell wall Ca^{2+} concentrations, which in turn could enhance the open probability of depolarization-activated Ca^{2+}-permeable channels. More detailed knowledge of the ion-selectivity properties of Ca^{2+}-permeable channels in the plasma membrane of higher plant cells is required to assess this possibility.

In conclusion, our findings provide direct evidence for the existence of voltage-dependent Ca^{2+}-permeable channels in a cultured higher plant cell which can be activated by physiologically occurring depolarizations during signal transduction. Further studies of these plant Ca^{2+} channels should increase our understanding of Ca^{2+}-dependent signal transduction in higher plants. Determination of conditions which stabilize the voltage-dependent Ca^{2+} currents will provide further insight into the regulation of these ion channels, as well as the opportunity for a detailed pharmacological analysis. Additional experimentation will be required to determine whether voltage-dependent Ca^{2+} channels, with similar properties to those revealed here, exist in other higher plant cells suggested to utilize Ca^{2+} channels for cellular signal transduction.

Materials and methods

Protoplasts were isolated from carrot cells (*Daucus carota* L.) as previously described (Thuleau *et al.*, 1988, 1990). During recordings, protoplasts were bathed in an external solution containing 40 mM $CaCl_2$ (or 5 mM $CaCl_2$) and 10 mM HEPES. The pH was adjusted to pH 6.7 by the addition of 1.6 mM CaOH. The osmolality was adjusted to 600 mosmol/kg with sorbitol. The pipette solution which equilibrated with the cytoplasm contained 2 mM $MgCl_2$, 10 mM $Tris_2$–EGTA, 10 mM MgATP, 200 μM GTPγS, 10 mM HEPES–Tris (pH 7.2) and sorbitol, osmolality 620 mosmol/kg. The same nucleotide concentrations were used as those applied to study voltage-dependent anion channels in higher plant guard cells (Hedrich *et al.*, 1990). Differences in the composition of external and internal solutions utilized for ionic substitution experiments are indicated in the figure legends. Experiments were performed at room temperature (22°C). Data are presented as means ± SD.

Patch–clamp studies (Hamill *et al.*, 1981) were performed with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Data were low-pass filtered with an eight-pole Bessel filter and digitized on-line (TL-125 interface, Axon Instruments), stored and analyzed on a 386-based 33 MHz microcomputer. Filter frequencies were set at ±1/5 of the sampling rate for all experiments (Sigworth, 1983). Recordings were corrected for liquid junction potential (LJP) (Schroeder *et al.*, 1987; Neher, 1992; Ward and Schroeder, 1994) which ranged from +3 to −21 mV. Correct membrane potentials (V_m) were determined by the equation $V_m = V_{experiment} + LJP$, where $V_{experiment}$ is the uncorrected membrane potential recorded during experiments. Equilibrium potentials were calculated after correction for ionic activities in solution (Robinson and Stokes, 1965).

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