J. Membrane Biol. 119, 277–288 (1991)
002226319100026Z

The Journal of
Membrane Biology

Cytosolic Calcium Regulates a Potassium Current in Corn (*Zea mays*) Protoplasts

Karen A. Ketchum and Ronald J. Poole
Department of Biology, McGill University, Montreal, Quebec Canada H3A 1B1

Summary. The voltage- and time-dependent K^+ current, I_{K^+out}, elicited by depolarization of corn protoplasts, was inhibited by the addition of calcium channel antagonists (nitrendipine, nifedipine, verapamil, methoxyverapamil, bepridil, but not La^{3+}) to the extracellular medium. These results suggested that the influx of external Ca^{2+} was necessary for K^+ current activation. The IC_{50}, concentration of inhibitor that caused 50% reduction of the current, for nitrendipine was 1 μM at a test potential of +60 mV following a 20-min incubation period.

In order to test whether intracellular Ca^{2+} actuated the K^+ current, we altered either the Ca^{2+} buffering capacity or the free Ca^{2+} concentration of the intracellular medium (pipette filling solution). By these means, I_{K^+out} could be varied over a 10-fold range. Increasing the free Ca^{2+} concentration from 40 to 400 nM also shifted the activation of the K^+ current toward more negative potentials. Maintaining cytoplasmic Ca^{2+} at 500 nM with 40 nM EGTA resulted in a more rapid activation of the K^+ current. Thus the normal rate of activation of this current may reflect changes in cytoplasmic Ca^{2+} on depolarization. Increasing intracellular Ca^{2+} to 500 nM or 1 μM also led to inactivation of the K^+ current within a few minutes. It is concluded that I_{K^+out} is regulated by cytosolic Ca^{2+}, which is in turn controlled by Ca^{2+} influx through dihydropyridine-, and phenylalkylamine-sensitive channels.

Key Words Ca^{2+}-activated K^+ current · Ca^{2+} channel · *Zea mays* · 1,4-dihydropyridine · phenylalkylamine · patch clamp

Introduction

Transient depolarization of the cell membrane potential in plants is associated with sensory transduction pathways initiated by both hormonal and environmental signals. Examples of stimuli that are known to initiate electrical change include the auxin, indole-3-acetic acid (Felle, 1988), light irradiation in both the red (Racusen & Satter, 1975) and blue (Nishizaki, 1988; Spalding & Cosgrove, 1989) spectral regions, gravistimulation (Behrens, Gradmann, & Sievers, 1985) and rapid cooling (Minorsky & Spanswick, 1989). Although the sequence of steps that connect stimulus perception to alterations in potential are not known, the molecular mechanisms that mediate the depolarizing and subsequent repolarizing currents are likely to involve the coordinated activity of plasma membrane ion channels.

Studies in diverse systems utilizing ion channel blockers indicate that Ca^{2+} influx contributes to the depolarizing current (Weisenseel & Ruppert, 1977; Minorsky & Spanswick, 1989), although concomitant modulation of other transport processes, such as inhibition of the plasma membrane ATPase (Hanson, Rincon, & Rogers, 1986; Felle, 1988) or stimulation of Cl^- efflux through anion channels (Beilby, 1984; Schroeder & Hagiwara, 1989) may also be required. Perpetuation of the signal transduction cascade, however, appears to be primarily dependent upon the elevation of cytosolic free Ca^{2+}, which can actuate the ensuing physiological response through biochemical pathways involving calcium binding proteins (Marmé, 1989), protein phosphorylation (Blowers & Trewavas, 1989) and/or the regulation of gene expression (Guilfoyle, 1989; Braam & Davis, 1990).

Equally important to the signaling event are the currents that facilitate membrane repolarization, for their function is to re-establish electrical homeostasis so that cells may respond again to the same or different stimuli. Two types of transport systems could conceivably assume this role: the H^+-ATPase, which catalyzes the electrogenic extrusion of protons, and ion channels, which accommodate the efflux of K^+ from the cytosol. Evidence from ion flux studies in intact tissue support this premise where repolarization is coincident with acidification of the extracellular medium (Rincon & Hanson, 1986), K^+ release (Tazawa, Shimmen & Mimura, 1987) or the efflux of H^+ and K^+ (Kinraide, Newman, & Etherton, 1984; Komor et al., 1989).

We have been interested in understanding the cellular events that couple these unidirectional currents to produce transient shifts in membrane poten-

tial and the factors that allow their activity to be modulated in a temporal fashion. One mechanism of establishing this connection is through Ca^{2+} regulation of ion transport proteins. Our objective in this study was to determine whether cytosolic Ca^{2+} could influence the whole-cell K^+ currents which have been described in corn protoplasts (Ketchum, Shrier & Poole, 1989).

Here we present evidence that the K^+ current evoked by membrane depolarization is also Ca^{2+}-dependent. This current is a plausible candidate for repolarizing the membrane potential following signaling events that involve membrane depolarization and changes in the cytosolic free Ca^{2+} concentration. Furthermore, we observed that organic Ca^{2+} channel antagonists from the dihydropyridine and phenylalkylamine families attenuate the K^+ current. These results suggest that one pathway for Ca^{2+} influx in corn suspension cells is through a protein that has pharmacological similarities to an L-type voltage-dependent Ca^{2+} channel (Hosey & Lazdunski, 1988).

Materials and Methods

Cell Cultures and Protoplast Isolation

Corn suspension cells (*Zea mays*, Black Mexican sweet corn, University of Illinois #78-002) derived from root tissue were cultured as previously reported (Ketchum et al., 1989). Protoplasts were prepared from cells 3 or 4 days after transfer with the following modifications of our original isolation procedure (Ketchum et al., 1989). Plasmolyzed cells, 0.2 g wet wt, were incubated at 28°C for 4 hr in 5 ml of digestion buffer (Musashige and Skoog growth medium supplemented with 1.5% Cellulase Y-C, 0.1% Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), 0.5% Macerozyme R-10 (Yakult Biochemical, Nishinomiya, Japan), 0.5% BSA[1], 20 mM citric acid, 1 mM DTT, and 300 mM mannitol, pH 5.5). Undigested cells and broken protoplasts were then separated from the digestion mixture by filtering the solution through a 2-cm glass wool plug loosely positioned in the end of a 10-ml syringe. The glass wool was first wetted with 2 ml of rinse medium (digestion buffer minus enzymes) and following the passage of the digestion mixture the glass wool was washed with an additional 3 ml of the same solution. The filtration through glass wool was repeated, and the second flowthrough was then centrifuged at 30 × *g* for 10 min in a clinical centrifuge. The supernatant was aspirated and the pellet, containing the protoplasts, was resuspended in 10 ml of rinse medium. Protoplasts were centrifuged and resuspended in fresh rinse medium three times to completely remove cell wall degrading enzymes. The final pellet was resuspended in extracellular medium (*see Solutions* below) and the protoplasts were then placed on ice in the dark. Protoplasts were used for whole-cell recording 10 to 24 hr after isolation.

[1] *Abbreviations:* BSA, bovine serum albumin; DTT, dithiothreitol; MES, 4-morpholineethansulfonic acid; Tris, tris-(hydroxymethyl)aminomethane; EGTA, ethylene glycol-*bis*(β-aminoethyl ether); BTP-ATP, *bis*-tris-propane-adenosine 5′-triphosphate; HEPES, (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; TEA^+, tetraethylammonium ion.

Tight-Seal Whole-Cell Recording

The whole-cell patch-clamp technique (Hamill et al., 1981) was used to record potentials or currents from corn protoplasts at 24°C with a List EPC-7 patch-clamp amplifier (Medical Systems, Greenvale, NY). Signals were filtered at 10 kHz, digitized at 44 kHz by a pulse code modulation unit (Medical Systems, PCM-2) and recorded on a Beta cassette recorder (Sony, SL-HF860D). Current amplitudes were later analyzed on either an analog (Tektronix, 5103N) or digital (Hameg, HM 208) oscilloscope and traces were plotted on an *X-Y* recorder (Linseis, LY17100). Currents were filtered at 3000 Hz with a low-pass Bessel filter (Frequency Devices, 902LPF). Electrodes with a resistance between 6 and 10 MΩ, measured in extracellular medium, were prepared from borosilicate glass capillaries (Kimble Products, Kimax-51).

Solutions

Extracellular medium used for patch-clamp experiments included 1 mM K-gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM MES, with the osmolarity adjusted to 685 mOsmol with 654 mM mannitol and the pH titrated to 6.25 with concentrated Tris. When used to resuspend protoplasts for storage, this solution was supplemented with 1 mM DTT. Intracellular medium (pipette filling solution) contained 100 mM K-gluconate, 2 mM $MgCl_2$, 4 mM EGTA-Tris, 5 mM BTP-ATP, 10 mM HEPES, and 450 mM mannitol. The final osmolarity was 635 mOsmol, and the pH was brought to 7 with concentrated Tris. For experiments where the concentration of EGTA was elevated to increase the calcium buffering capacity of the intracellular solution, the concentration of HEPES was increased to a molarity equal to that of the EGTA to assure that the pH was maintained at 7. The mannitol and/or gluconate concentration was correspondingly decreased to compensate for the change in osmolarity.

Quin2 (Molecular Probes, Eugene, OR) fluorescence was used to measure the free calcium concentration of the pipette filling solution (Tsien, Pozzan & Rink, 1982). Briefly, 20 μM quin2 was added to a sample of intracellular solution and the fluorescence at 492 nM (excitation wavelength of 339 nm) was recorded on a Perkin-Elmer LS-3 fluorescence spectrometer. Minimum fluorescence (at zero free calcium), was determined by titrating the sample to pH 9 with concentrated Tris and adding excess EGTA to chelate all free calcium as described by Rink and Pozzan (1985). The maximum fluorescence (taken as fluorescence at 100 μM free Ca^{2+}) was established by titrating the solution with either $CaCl_2$ or Ca-gluconate until the signal saturated. The fluorescence change from 0 to 100 μM Ca^{2+} was approximately sixfold. The level of free calcium in each solution was then determined from the calibration curve published for this dye (Tsien, Pozzan & Rink, 1982). Alterations of the free calcium level and assessment of the calcium buffering capacity of the different intracellular solutions were made by adding known amounts of $CaCl_2$ or Ca-gluconate. The final pH was then checked to verify that the change in free calcium concentration did not shift the acidity of the intracellular medium.

Finally, chloride-containing salts were used in some experiments, replacing the impermeant gluconate, so that the chloride reversal potential could be shifted to more positive potentials (*see* text).

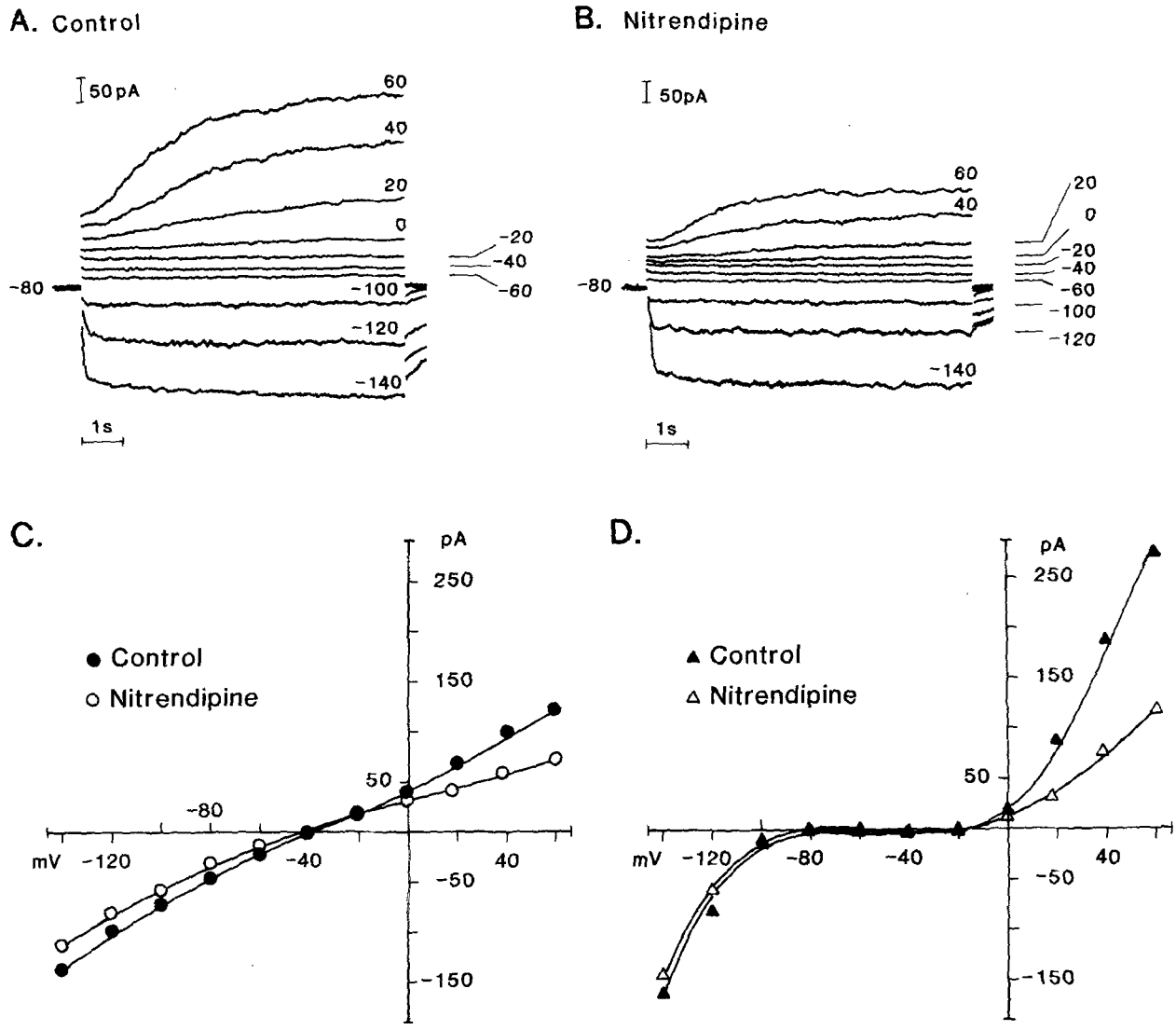

Fig. 1. Effect of nitrendipine on whole-cell currents in corn protoplasts. (*A*) Control: Voltage steps from a holding potential of −80 mV to step potentials between −140 and +60 mV. (*B*) Nitrendipine: Same voltage steps repeated following a 20-min incubation period with 1 μM nitrendipine. (*C*) *I/V* relationship for the time-independent current: (●) control and (○) with 1 μM nitrendipine. (*D*) *I/V* relationship for the time-dependent currents: (▲) Control and (△) with 1 μM nitrendipine. Similar observations were made of five different cells

Results

To investigate the Ca^{2+}-dependence of the whole-cell currents in corn protoplasts we applied reagents that are known to antagonize plasma membrane Ca^{2+} flux. These compounds were of two types, organic Ca^{2+} channel inhibitors (Hosey & Lazdunski, 1988) and ionic Ca^{2+} channel blockers (Hille, 1984). We hypothesized that a reduction in Ca^{2+} influx would decrease the cytosolic free Ca^{2+} concentration and thereby indirectly affect any Ca^{2+}-activated currents.

Organic Ca^{2+} Channel Inhibitors

Nitrendipine, added to a final concentration of 1 μM reduced the voltage-dependent, outward K^+ current (I_{K^+out}) evoked by membrane depolarization (Fig. 1). Complete inhibition of this current was obtained when the nitrendipine level was elevated to 100 μM (Figs. 2 and 3). Currents exhibited a progressive decrease in magnitude following the addition of the drug to the chamber which could not be attributed to either the solvent (Fig. 2) or to the "run down" of currents, typically 5–15%, that occurs during whole-

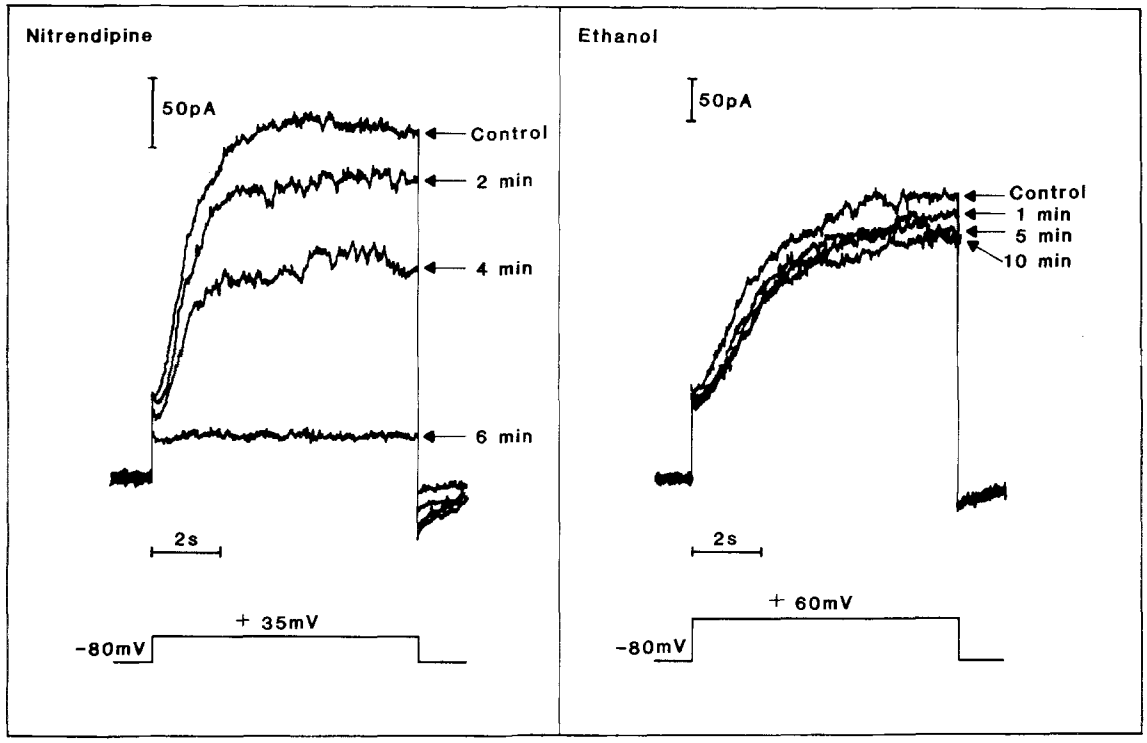

Fig. 2. Complete inhibition of the time-dependent, outward K^+ current by 100 μM nitrendipine. *Left panel:* Whole-cell currents recorded with a depolarizing pulse from −80 to +35 mV. Voltage steps conducted before nitrendipine addition (control), and 2, 4, and 6 min after the inhibitor was added to the chamber. The same response was obtained from three cells. *Right panel:* Whole-cell currents recorded with an equivalent concentration of the solvent, 0.4% ethanol. Voltage steps to +60 mV from a holding potential of −80 mV were performed before (control) and after (1, 5, and 10 min) ethanol addition

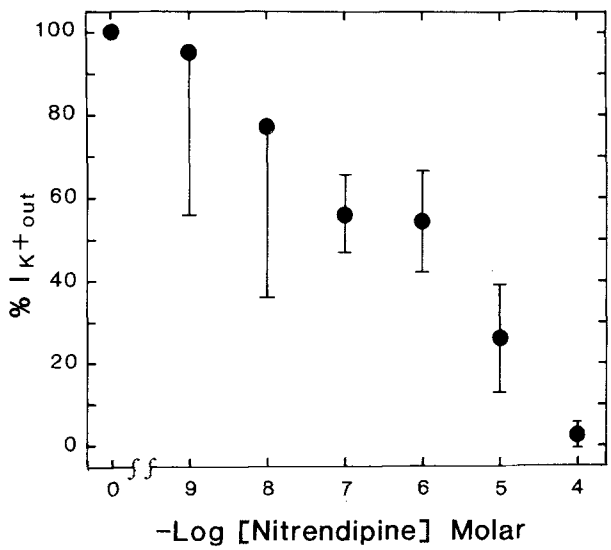

Fig. 3. Influence of nitrendipine concentration on I_{K^+out}. Whole-cell currents were recorded at a test potential of +60 mV following a 20-min incubation with nitrendipine. Points are the average of five cells, except 100 μM where three cells were used, with the SD represented by the vertical lines

cell recording from these protoplasts. The IC_{50} for nitrendipine was approximately 1 μM measured at a test potential of +60 mV following a 20 min incubation period (Fig. 3). I_{K^+out} was partially inhibited by nitrendipine at a concentration of only 10 nM, yet complete inhibition required an inhibitor concentration of 100 μM (Fig. 3). This wide range suggests the possibility of heterogeneity either in the nitrendipine-sensitivity of Ca^{2+} channels, or in the Ca^{2+}-sensitivity of K^+ channels in these cells.

In 19 out of 28 cells tested, the time-independent current (I_i) was also partially reduced in the presence of nitrendipine (Fig. 1*C*). The degree of inhibition averaged 32% in those cells where a decrease in I_i was observed (values included cells treated with 0.001 to 100 μM nitrendipine). We found no correlation between inhibitor concentration and the reduction of I_i. Furthermore, the resting membrane potential did not exhibit any change that directly corresponded to the observed decrease in the instantaneous conductance.

In contrast to the two currents analyzed above, the inward K^+ current (I_{K^+in}), evoked at hyperpolar-

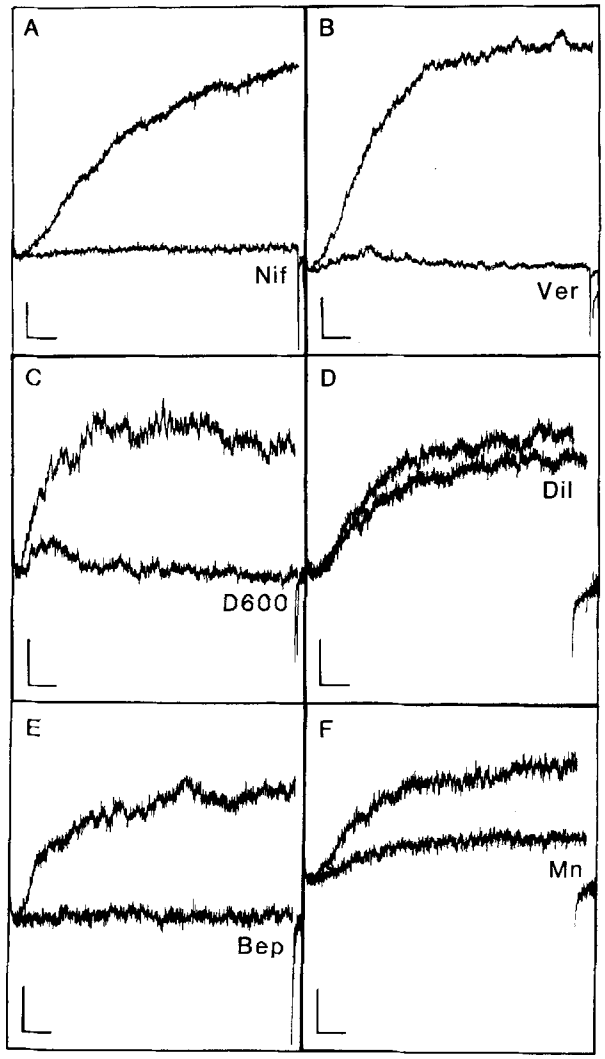

Fig. 4. Effect of different Ca^{2+} channel antagonists on the time-dependent, outward K^+ current. Each current trace was recorded at a step potential of +60 mV from a holding potential of −80 mV. Time-independent currents were subtracted: the upper trace in each case shows the time-dependent current in the absence of inhibitor. (*A*) 100 μM nifedipine; vertical scale, 30 pA. (*B*) 100 μM verapamil; vertical scale, 40 pA. (*C*) 100 μM methoxyverapamil (D600); vertical scale, 20 pA. (*D*) 1 mM diltiazem; vertical scale, 20 pA. (*E*) 100 μM bepridil; vertical scale, 20 pA. (*F*) 20 mM $MnSO_4$; vertical scale, 20 pA. Horizontal scale in all panels is 800 msec. Each inhibitor was tested on three cells

izing potentials, was unaffected by the dihydropyridine reagent (Fig. 1*D*).

Other organic Ca^{2+} channel antagonists from the 1,4-dihydropyridine and phenylalkylamine families as well as the compound bepridil caused similar inhibition of I_{K^+out}, whereas diltiazem, a reagent from the benzothiazepine group, had no effect (Fig. 4). We could not unequivocally determine the sensitivity of this current to the diphenylbutylpiperidine pimozide, since the solvent chloroform (0.8% final concentration) caused transient inhibition of the whole-cell currents in corn cells.

Ionic Ca^{2+} Channel Blockers

The addition of ionic Ca^{2+} channel blockers had relatively little effect on I_{K^+out}. We observed only partial inhibition of the K^+ current with millimolar levels of La^{3+}, Mn^{2+}, Cd^{2+}, or Co^{2+} (Figs. 4, 5 and *data not shown*).

Interestingly, the primary response to La^{3+} was an inhibition of the time-independent current (Fig. 5). The magnitude of I_i was inversely related to the extracellular La^{3+} concentration, and as the membrane conductance was reduced a concomitant hyperpolarization of the resting membrane potential occurred.

The average membrane potential of corn protoplasts was −96 ± 34 mV when whole-cell recordings were conducted in 1 mM external potassium salts (Ketchum et al., 1989). The variability seen in the resting potential appears to be partially attributable to variation in the Cl^- conductance of the plasma membrane. In the most extreme case, observed in a subpopulation of protoplasts, the resting potential corresponded to the Cl^- reversal potential of −10 mV (Poole & Ketchum, 1989). These cells exhibited a large time-independent current, which was reduced by external $LaCl_3$. Addition of $LaCl_3$ also caused dramatic hyperpolarization of $\Delta\Psi$ by −98 ± 22 mV (n = 6) from an initial potential of −10.6 ± 7 mV. All of these protoplasts showed a time-dependent, outward K^+ current when depolarized in the presence of La^{3+}.

Protoplasts with initial resting potentials more negative than −50 mV (i.e., protoplasts with a K^+-selective time-independent current) also hyperpolarized, although to a lesser extent, following La^{3+} addition. Furthermore, protoplasts patched in the presence of 100 μM $LaCl_3$ or 1 mM $LaCl_3$ had average resting potentials of −85.8 ± 24 mV (n = 14) and −110 ± 34 mV (n = 15), respectively. Addition of $LaNO_3$ caused changes in membrane potential and whole-cell currents similar to those observed with $LaCl_3$.

Modulation of the Time-Dependent K^+ Current by Intracellular Ca^{2+}

The inhibition of the time-dependent K^+ current might be interpreted in two ways: (i) I_{K^+out} is Ca^{2+}-dependent; the decrease in this current is caused by

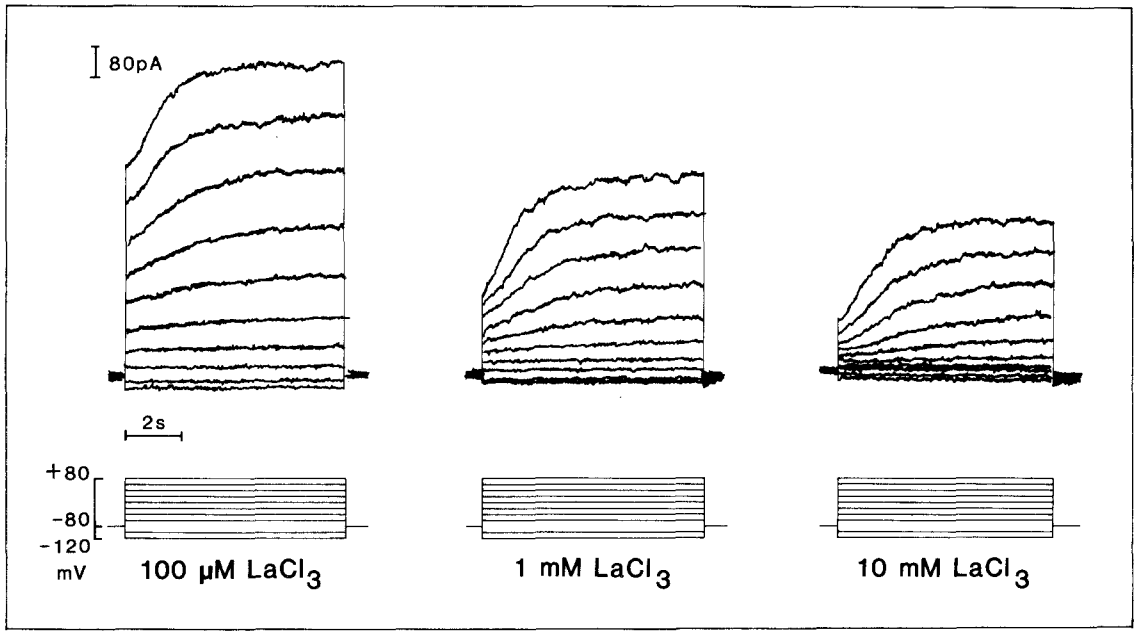

Fig. 5. La^{3+} inhibition of the time-independent current. One cell was assayed with increasing concentrations of $LaCl_3$ in the extracellular medium. The holding potential was −80 mV. Currents recorded at voltage steps ranging from −120 to +80 mV are shown. The membrane potential of the protoplast in 100 μM La^{3+} was −57 mV. When the La^{3+} concentration was elevated the resting potential hyperpolarized to −66 mV at 1 mM and −97 mV at 10 mM $LaCl_3$. All six cells tested showed the same response

the reduced Ca^{2+} flux across the plasma membrane in the presence of the organic Ca^{2+} channel inhibitors, or (ii) the K^+ channels responsible for I_{K^+out} are directly inhibited by the dihydropyridine, phenylalkylamine and bepridil reagents. In other experimental systems high concentrations of organic Ca^{2+} channel blockers have been shown to inhibit voltage-dependent Na^+ and K^+ currents (Hagiwara & Byerly, 1981; Hume, 1985) and Ca^{2+}-dependent K^+ currents (Gola & Ducreux, 1985). To distinguish between these possibilities we performed experiments to test whether a rise in intracellular Ca^{2+} was necessary for the activation of I_{K^+out}.

First, we increased the Ca^{2+} buffering capacity of the intracellular solution by elevating the concentration of EGTA while maintaining free Ca^{2+} at 40 nM. If the influx of Ca^{2+} causes a change in the internal Ca^{2+} concentration, which in turn activates the K^+ conductance, then the development of the time-dependent current would be less likely as the protoplasts are loaded to higher EGTA levels. As seen in Fig. 6 this was indeed the case, where the conductance/surface area due to the time-dependent K^+ current, measured at +60 mV, decreased from 0.36 ± 0.1 S/m² in 4 mM EGTA to 0.11 ± 0.05 S/m² in 40 mM EGTA. Further reduction of I_{K^+out} was obtained in protoplasts loaded with 100 mM EGTA (Fig. 6).

Second, we maintained the Ca^{2+} buffering capacity at a constant level and elevated the free Ca^{2+} concentration. If I_{K^+out} is dependent upon intracellular Ca^{2+}, currents should be enhanced in the higher free Ca^{2+} buffers. Using intracellular media with comparable Ca^{2+} buffering capacities (4 mM EGTA) but containing different free Ca^{2+} concentrations (40 and 400 nM), it was observed that the conductance/surface area increased approximately twofold with the higher Ca^{2+}solution (Fig. 7, inset). In addition, the activation range of the K^+ current shifted towards more negative potentials when recordings were made with the elevated Ca^{2+} buffer (Fig. 7). Thus half maximal current activation was attained at +5 mV with a free Ca^{2+} concentration of 40 nM in contrast to −15 mV with a free Ca^{2+} concentration of 400 nM. The activation kinetics for I_{K^+out} with 400 and 40 nM free Ca^{2+} exhibited a similar voltage dependency (Fig. 8*A*). A slight acceleration in the rate of current development could be observed with the 400 nM solution between −40 and 0 mV step potentials. This difference became negligible with depolarizations more positive than 0 mV.

We attempted to assess the effect of a higher concentration of intracellular free Ca^{2+} (1 μM) on this K^+ current. Unexpectedly, we found that I_{K^+out} rapidly decayed following the establishment of the whole-cell configuration (Fig. 9). Typically, recordings can be made, using standard intracellular solutions (Materials and Methods), for more

than 90 min without a dramatic change in current magnitude. Each of the protoplasts monitored with the 1 μM buffer lost greater than 50% of the original time-dependent current by 6 min after break through. Hence I_{K^+out} has a restricted range of cytosolic Ca^{2+} for maximal activation.

Finally, we examined the sensitivity of the K^+ currents recorded with elevated intracellular free Ca^{2+} (i.e., 400 nM) to the organic Ca^{2+} channel blockers. When the buffering capacity of the intracellular solution was set with 4 mM EGTA, I_{K^+out} was inhibited by the Ca^{2+} antagonists in a similar manner to that previously noted with the 40 nM free Ca^{2+} buffer (*data not shown*). We suspected that the reactions that reduce cytosolic free Ca^{2+} [e.g. export of Ca^{2+} via the plasmalemma Ca^{2+}-ATPase (Robinson, Larsson & Buckhout, 1988) or sequestration of Ca^{2+} into the vacuole by the H^+/Ca^{2+} antiport (Schumaker & Sze, 1985; Blumwald & Poole, 1986)] might be sufficient to overcome the buffering ability of the 4 mM EGTA buffers, so that additional influx of Ca^{2+} through the nitrendipine-sensitive channels would still be required to activate I_{K^+out}. We therefore reassessed the effect of nitrendipine on I_{K^+out} in protoplasts loaded with a 40 mM EGTA, 500 nM free Ca^{2+} pipette solution.

There were a number of distinct properties observed of I_{K^+out} recorded under these conditions. First, as noted with the 1 μM Ca^{2+} cells (Fig. 9), outward currents decayed noticeably with time after establishment of the whole-cell configuration (Fig. 10). Second, although the voltage dependency of the currents (Fig. 10) was not greatly changed from that seen with 4 mM EGTA–400 nM Ca^{2+} (Fig. 7), the time

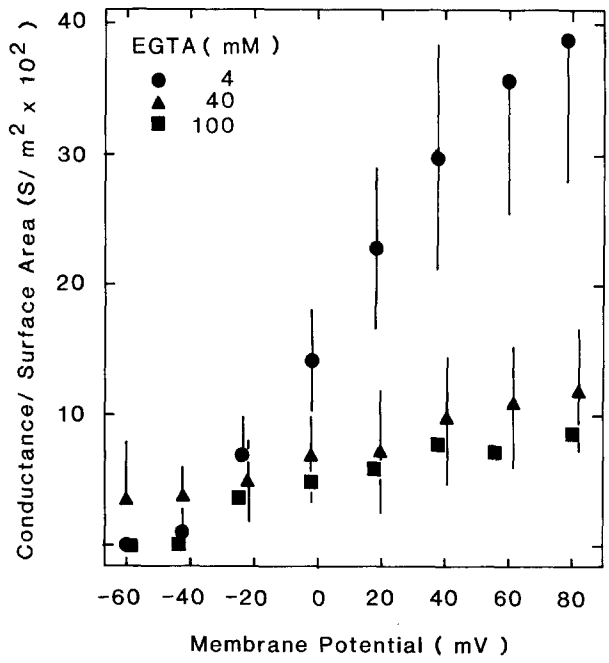

Fig. 6. Increased levels of EGTA in the intracellular medium caused a reduction in I_{K^+out}. Whole-cell currents were recorded with the pipette filling solution containing 4 mM EGTA (●), 40 mM EGTA (▲), and 100 mM EGTA (■). The free calcium concentration of each solution was adjusted to approximately 40 nM. The conductance of the time-dependent, outward K^+ current at each voltage was calculated and normalized to the surface area of the protoplast. Average of four, four and two cells for experiments with 4, 40, and 100 mM EGTA, respectively. SD are represented by the vertical lines. Two cells recorded with 100 mM EGTA had no time-dependent outward current. These were not included in the averages shown

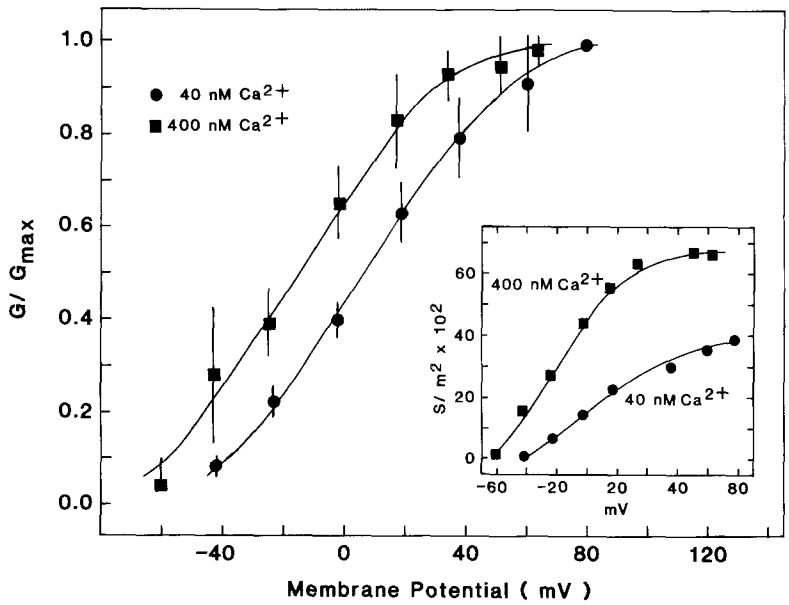

Fig. 7. The activation range of the K^+ current was shifted towards more negative potentials by an increase in the calcium concentration of the intracellular medium. Currents were recorded from protoplasts using intracellular media with identical EGTA levels, 4 mM, but different calcium concentrations of 40 nM (●) and 400 nM (■). The conductance of the voltage-dependent K^+ current at each potential was determined and expressed as the fraction of the maximum current observed (G/G_{max}). Average of four cells with the SD indicated by the vertical line. Cl^- reversal potential in the experiments with 400 nM Ca^{2+} was set to +47 mV. *Inset:* Same data expressed as current normalized to the protoplast surface area. 40 nM calcium trace corresponds to the protoplasts with 4 mM EGTA in Fig. 6

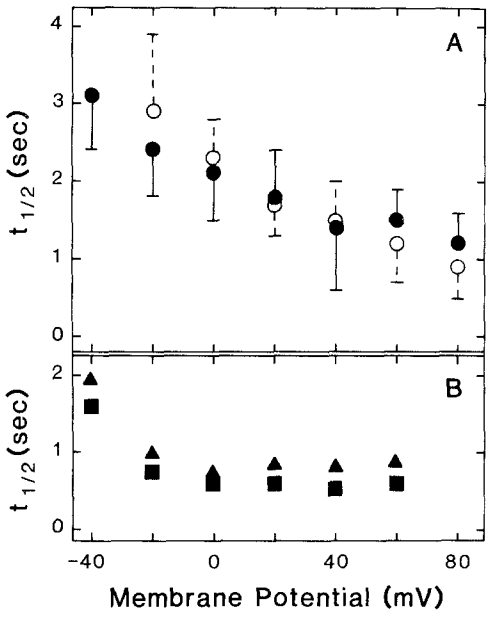

Fig. 8. Half time for activation of I_{K^+out} as a function of membrane potential. (*A*) Protoplasts recorded with 40 nM (○) and 400 nM (●) free calcium. EGTA concentration was 4 mM in each case. Average of four cells, SD marked by the vertical lines (---) 40 nM and (——) 400 nM. (*B*) Activation rate of currents recorded in two protoplasts (■, ▲) with 500 nM free calcium and 40 mM EGTA

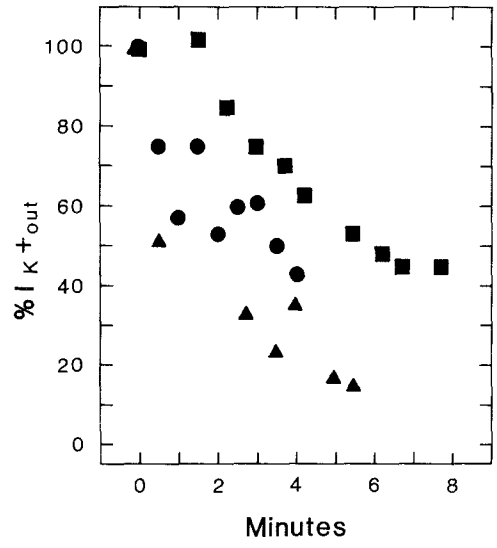

Fig. 9. Decay of I_{K^+out} after establishment of the whole-cell configuration. Three different cells (●, ■, ▲) recorded with a pipette filling solution of 1 μM free calcium and 10 mM EGTA. Zero time is the first step to +60 mV, from a holding potential of −80 mV, approximately 1.5 min after transition from the cell-attached configuration to the whole-cell recording mode. The time-dependent current at this point was taken as 100%, and the current at all subsequent steps was expressed as a percent of this value

course of activation was dramatically altered. The development of I_{K^+out} is usually preceded by a pronounced lag (Figs. 1, 2 and 4), which gives the time course of activation a sigmoidal shape. With the 40 mM EGTA, 500 nM Ca^{2+} buffer the time-dependent current increased in a simple exponential fashion (Fig. 11) and the half-activation time for these currents was markedly reduced (Fig. 8*B*). Most importantly, I_{K^+out} recorded under these conditions was relatively insensitive to nitrendipine (Fig. 12), the decay of current being largely attributable to Ca^{2+} inactivation (*cf.* Fig. 10), while in low Ca^{2+} cells nitrendipine caused total current inhibition (Fig. 12). These data suggest that nitrendipine inhibits the K^+ current through the reduction of Ca^{2+} influx.

Discussion

Ca^{2+}-Activated K^+ Current

The response of plant cells to a variety of physiological and environmental stimuli is accompanied by an efflux of potassium ions from the cytosol (Bange, 1979; Novacky, 1980; Atkinson, Baker & Collmer, 1986; Hanson, Rincon & Rogers, 1986; Schroeder, Raschke & Neher, 1987; Tazawa et al., 1987; Moran et al., 1988). Most notable is the pronounced loss of K^+ in cells that undergo turgor-mediated changes in cell volume. Potassium ion channels, that have been described in guard cells and motor cells of the pulvinus provide the molecular pathway for these K^+ movements (Schroeder, Raschke & Neher, 1987; Moran et al., 1988). In other cell types the role of transient K^+ efflux and the cellular mechanisms which modulate K^+ loss are less well understood.

One function that K^+ currents commonly serve is to maintain electrical homeostasis at the plasma membrane. Thus their role in signal transduction is to provide delayed negative feedback as a means to terminate the electrical transients and ionic fluxes that mediate between stimulus perception and the biochemical reactions of the cytosol. The situations in which K^+ fluxes in animal cells are utilized in this way are varied, and in accordance with this there are a wide range of K^+ channels tailored to respond to each physiological condition (Latorre & Miller, 1983; Hille, 1984; Latorre et al., 1989). A similar function might be expected of the different K^+ currents found in plant tissues.

In this paper we have provided strong evidence for Ca^{2+} activation of K^+ channels at the plasma membrane of higher plant cells. The characteristics of this current (activation upon membrane depolarization, increase in the rate of activation with an increase in cytosolic Ca^{2+}, and a shift in activation range with elevated Ca^{2+}) indicate that its likely

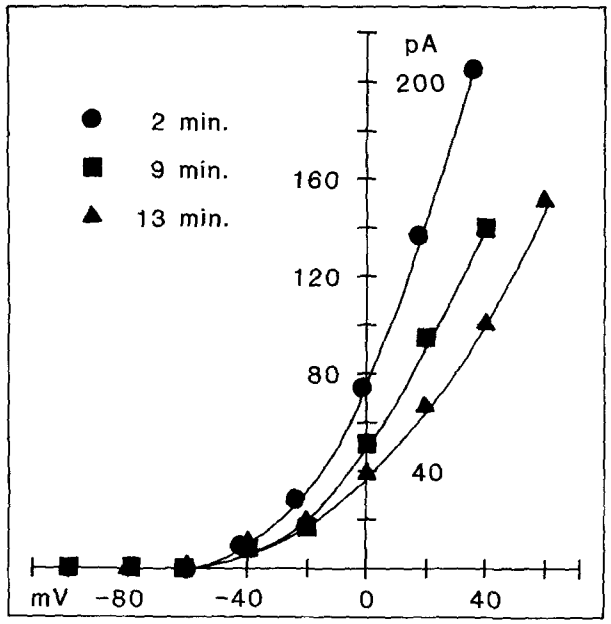

Fig. 10. *I/V* relationship for the time-dependent, outward current recorded with 500 nM free calcium and 40 mM EGTA in the pipette. Cl^- reversal potential was set to +45 mV. Voltage steps conducted 2, 9 and 13 min after establishment of the whole-cell configuration

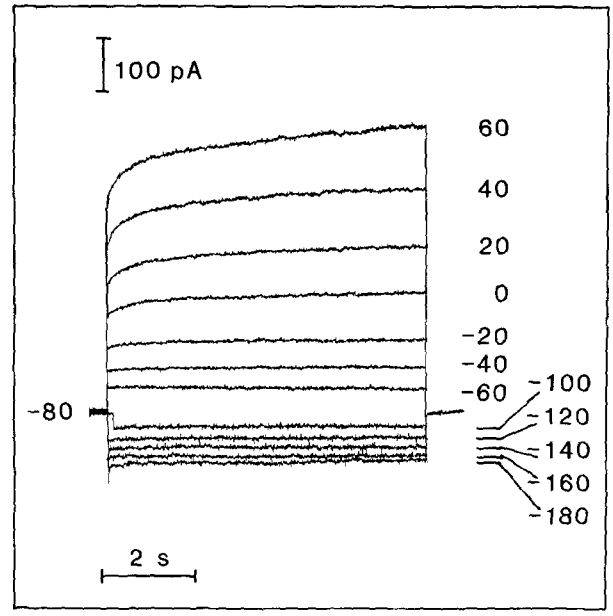

Fig. 11. Rapid activation of the time-dependent, outward currents recorded with a pipette filling solution that contained 500 nM free calcium and 40 mM EGTA. Whole-cell currents were evoked at step potentials between −180 and +60 mV. The holding potential was −80 mV. The chloride reversal potential was set to +45 mV

role is to repolarize the membrane potential during signaling events that combine the elevation of intracellular free calcium with a decrease in the electrical potential of the cell. Moreover, the levels of cytosolic free Ca^{2+} that actuated current development (Ca^{2+} concentration greater than 30 nM and less than 1 μM) are similar to the concentration of Ca^{2+} monitored in corn root cells following IAA addition and observed in guard cells after ABA application (Felle, 1988; McAinsh, Brownlee & Hetherington, 1990). We suspect that this class of ion channels provides the pathway for the passive K^+efflux that has been observed in the response of higher plant cells to stress induced by shifts in temperature (Bange, 1979), wounding (Hanson et al., 1986) or exposure to pathogens (Novacky, 1980; Atkinson et al., 1986).

The Ca^{2+}-activated K^+ current in corn has marked similarities to the K^+ currents described in guard cells from *Vicia faba* (Schauf & Wilson, 1987; Schroeder et al., 1987; Hosoi, Iino & Shimazaki, 1988; Blatt, 1990), motor cells from the pulvini of *Samanea saman* (Moran et al., 1988) and mesophyll cells from trap-lobes of *Dionaea muscipula* (Venus's flytrap, Iijima & Hagiwara, 1987). All of these currents are outward rectifying with similar voltage dependency and activation kinetics. Moreover, each current shows sensitivity to the K^+ channel inhibitor TEA^+ (Iijima & Hagiwara, 1987; Schauf & Wilson, 1987; Moran et al., 1988; Ketchum et al., 1989). It is not known whether the K^+ channels identified in these other cell types are also regulated by intracellular Ca^{2+}.

Ca^{2+}-activated K^+ channels appear to be present at the plasma membrane of other plant species. Tester (1988) has suggested that the K^+ current in *C. corallina* may be Ca^{2+} regulated, based upon pharmacological comparisons with the high conductance Ca^{2+}-dependent K^+ channel described in animal cells. In the alga *Eremosphaera viridis* a Ca^{2+}-dependent K^+ current participates in the transient potentials that follow light-dark environmental transitions (Thaler et al., 1987). In addition, a TEA^+-sensitive, Ca^{2+}-activated channel has been identified in the alga *Mougeotia* (Lew et al., 1990).

The plasma membrane of endosperm cells from *Haemanthus* and *Clivia* (plants of the Amaryllis family) contains Ca^{2+}-activated, nonselective cation channels (Stoeckel & Takeda, 1989). The channels we have described in corn have a distinct pharmacology: sensitivity to TEA^+ (Ketchum et al., 1989) and Ba^{2+} (Ketchum & Poole, 1990), not shared with the channels found in either species. These differences suggest that the K^+ channels described here are not closely related to the cation channels identified in endosperm.

It is of particular interest that cytosolic Ca^{2+} was also a means to inactivate the K^+ current (Figs.

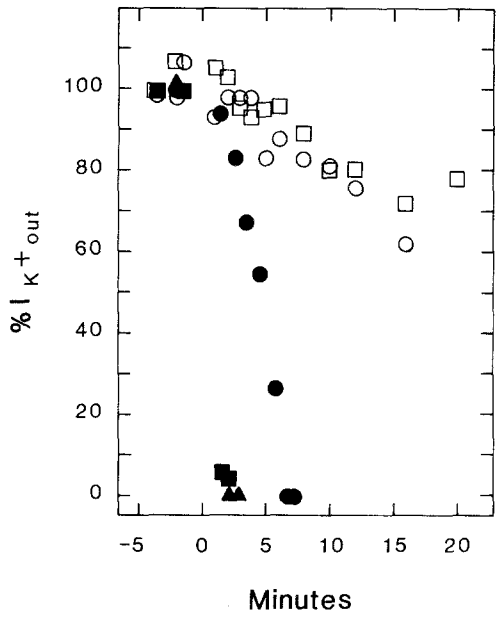

Fig. 12. I_{K^+out} is insensitive to nitrendipine when protoplasts are loaded with a 500 nM Ca^{2+}, 40 mM EGTA buffer. At zero time nitrendipine (100 μM) was added to the chamber. Filled symbols (●, ■, ▲) represent cells in which the pipette filling solution contained 40 nM free calcium and 4 mM EGTA. Open symbols (○, □) indicate cells with a pipette filling solution of 500 nM free calcium and 40 mM EGTA. Currents were expressed as the percent of the time-dependent current recorded on the first voltage step prior to inhibitor addition. In all experiments the holding potential was −80 mV and the test potential was +60 mV

9 and 10). There is precedence for this type of dual regulation of Ca^{2+}-dependent K^+ channels in some animal cells (Alkon et al., 1984; Findlay, Dunne & Petersen, 1985; Hidalgo, 1985) and the challenge here will be to define the regulatory factors that mediate activation and inactivation. Physiologically this inhibition of the outwardly directed K^+current may be crucial in preventing a large-scale loss of potassium under conditions when cytosolic Ca^{2+} reaches a high level.

Other Ca^{2+}-modulated ion channels have been observed at both the tonoplast and plasma membrane in plant cells. At the vacuolar membrane, elevation of cytosolic Ca^{2+} activates Cl^--selective channels in *Chara* (Kikuyama, 1988) and a nonselective channel in sugar beet (Hedrich & Neher, 1987). There is also evidence that Ca^{2+} causes the inactivation of a second, nonselective ion channel at the tonoplast (Hedrich & Neher, 1987). The situation at the plasma membrane is equally complex, where high cytoplasmic Ca^{2+} inhibits inward-rectifying K^+ channels in guard cells (Schroeder & Hagiwara, 1989) and also activates a Cl^- conductance in *Chara* (Shiina & Tazawa, 1987*a*) and in guard cells (Schroeder & Hagiwara, 1989) in addition to the cation channels described above.

It is notable that a wide range of cytosolic Ca^{2+} concentrations have been used in the literature, and it seems likely that the Ca^{2+}-activation ranges of the different ion channels, even on the same membrane, may be quite distinct. At the plasma membrane of guard cells, for example, Schroeder and Hagiwara (1989) promoted Cl^- currents with cytoplasmic Ca^{2+} at 1.5 μM, which in the corn cell system would cause reduction of the Ca^{2+}-activated K^+ current. Evidently both short-term and long-term effects of various levels of cytosolic Ca^{2+} allow considerable versatility in the stimulus-response coupling of plant cells.

Effect of Organic Ca^{2+} Antagonists at the Plasma Membrane

Inhibition of K^+ efflux by the organic Ca^{2+} channel inhibitors (1,4-dihydropyridines, phenylalkylamines and bepridil) gives insight into the mechanism of Ca^{2+} transport at the plasma membrane. These reagents all typify the L-type voltage-dependent Ca^{2+} channel that is found in animal cells (Hosey & Lazdunski, 1988). Their effectiveness in corn suggests that a protein with similar pharmacological sensitivity mediates Ca^{2+} influx in higher plant cells and it is plausible that this transport system is also a voltage-dependent ion channel.

Dihydropyridine-sensitive transport systems appear to be widespread in plant tissues. Direct recordings of ion currents in the giant algal cells of *Nitellopsis obtusa* have demonstrated the presence of a dihydropyridine-sensitive, voltage-dependent Ca^{2+} current at the plasma membrane (Shiina & Tazawa, 1987*b*). Dihydropyridines have also been used to inhibit Ca^{2+}-dependent processes such as mitosis (Wolniak & Bart, 1985), osmoregulation (Okazaki & Tazawa, 1986), cytokinin-induced bud formation (Conrad & Hepler, 1988), as well as $^{45}Ca^{2+}$ transport (MacRobbie & Banfield, 1988). Similar observations have been obtained utilizing compounds from the phenylalkylamine group (Hepler, 1985; Graziana et al., 1988; Brummell & Maclachlan, 1989). However, the receptors for these two classes of antagonists do not coincide in all plant tissues (Graziana et al., 1988) and direct characterization of these Ca^{2+} transport proteins will be needed to distinguish their physiological roles.

La^{3+}-Sensitivity of the Whole-Cell Currents

The observation that the instantaneous current was reduced by La^{3+} (Fig. 5) presents the possibility

that a second, voltage-insensitive Ca^{2+} conductance contributes to the resting membrane potential. The transport of Ca^{2+} in this manner, perhaps combined with activation of chloride channels, could account for the positive shift in potential from E_{K^+} (Ketchum et al., 1989) and the large time-independent currents observed in certain cells (Poole & Ketchum, 1989). Alternatively, La^{3+} inhibition of I_i could result from direct blockage of K^+ channels as has been proposed in *Chara inflata* (Tyerman, Findlay & Paterson, 1986). In support of this latter hypothesis is the observation that the effect of external La^{3+} on I_i is similar to that observed with TEA^+ (Ketchum et al., 1989).

Conclusion

The K^+ channel which facilitates the efflux of K^+ during the depolarization of corn protoplasts was activated by cytosolic Ca^{2+}. This current, originally identified as I_{K^+out}, was inhibited by organic calcium channel antagonists from the dihydropyridine and phenylalkylamine families. Furthermore, K^+currents were enhanced by loading protoplasts with buffers containing moderately elevated concentrations of free Ca^{2+} or, conversely, they were inhibited by increasing the Ca^{2+}-buffering capacity of the cytosol to maintain low levels of free Ca^{2+}. High cytosolic Ca^{2+} also led to inactivation of the K^+ channel. We suggest that this current be called $I_{K(Ca)}$ to reflect the mechanism of channel regulation.

Second, a Ca^{2+} transport system present at the plasma membrane of corn cells was found to have pharmacological similarities to an L-type voltage-dependent Ca^{2+} channel. This channel is sensitive to dihydropyridines, phenylalkylamines and bepridil.

The characteristics of these two transport systems suggest that they may work in concert to facilitate signal transduction at the plasma membrane of higher plant cells.

This research was supported by the Natural Sciences and Engineering Research Council of Canada and by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche of Québec. K.A.K. was supported by a McGill Major Fellowship from The Friends of McGill. The authors would like to thank Dr. Stephen K. Randall and Mr. Vahé Sarafian for critically reading the manuscript.

References

Alkon, D.L., Farley, J., Sakakibara, M., Hay, B. 1984. Voltage-dependent calcium and calcium-activated potassium currents of a molluscan photoreceptor. *Biophys. J.* **46**:605–614

Atkinson, M.M., Baker, C.J., Collmer, A. 1986. Transient activation of plasmalemma K^+ efflux and H^+ influx in tobacco by a pectate lyase isozyme from *Erwinia chrysanthemi*. *Plant Physiol.* **82**:142–146

Bange, G.G.J. 1979. Loss of rubidium and potassium from barley roots on sudden chilling. *Plant Physiol.* **64**:581–584

Behrens, H.M., Gradmann, D., Sievers, A. 1985. Membrane-potential responses following gravistimulation in roots of *Lepidium sativum* L. *Planta* **163**:463–472

Beilby, M.J. 1984. Calcium and plant action potentials. *Plant Cell Environ.* **7**:415–421

Blatt, M.R. 1990. Potassium channel currents in intact stomatal guard cells: Rapid enhancement by abscisic acid. *Planta* **180**:445–455

Blowers, D.P., Trewavas, A.J. 1989. Second messengers: Their existence and relationship to protein kinases. *In:* Second Messengers in Plant Growth and Development. W.F. Boss and D.J.Morré, editors. pp. 1–28. Alan R. Liss, New York

Blumwald, E., Poole, R.J. 1986. Kinetics of Ca^{2+}/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris* L. *Plant Physiol.* **80**:727–731

Braam, J., Davis, R.W. 1990. Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell* **60**:357–364

Brummell, D.A., Maclachlan, G.A. 1989. Calcium antagonist TMB-8 inhibits cell wall formation and growth in pea. *J. Exp. Bot.* **40**:559–565

Conrad, P.A., Hepler, P.K. 1988. The effect of 1,4-dihydropyridines on the initiation and development of gametophore buds in the moss *Funaria*. *Plant Physiol.* **86**:684–687

Felle, H. 1988. Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. *Planta* **174**:495–499

Findlay, I., Dunne, M.J., Petersen, O.H. 1985. High-conductance K^+ channel in pancreatic islet cells can be activated and inactivated by internal calcium. *J.Membrane Biol.* **83**:169–175

Gola, M., Ducreux, C. 1985. D600 as a direct blocker of Ca-dependent K currents in *Helix* neurons. *Eur. J. Pharmacol.* **117**:311–322

Graziana, A., Fosset, M., Ranjeva, R., Hetherington, A.M., Lazdunski, M. 1988. Ca^{2+} channel inhibitors that bind to plant cell membranes block Ca^{2+} entry into protoplasts. *Biochemistry* **27**:764–768

Guilfoyle, T.J. 1989. Second messengers and gene expression. *In:* Second Messengers in Plant Growth and Development. W.F. Boss and D.J. Morré, editors. pp. 315–326. Alan R. Liss, New York

Hagiwara, S., Byerly, L. 1981. Calcium channel. *Annu. Rev. Neurosci.* **4**:69–125

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100

Hanson, J.B., Rincon, M., Rogers, A.S. 1986. Controls on calcium influx in corn root cells. *In:* Molecular and Cellular Aspects of Calcium in Plant Development. A.J. Trewavas, editor. pp. 253–260. Plenum, New York

Hedrich, R., Neher, E. 1987. Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. *Nature* **329**:833–836

Hepler, P.K. 1985. Calcium restriction prolongs metaphase in dividing *Tradescantia* stamen hair cells. *J. Cell Biol.* **100**:1363–1368

Hidalgo, J. 1985. The calcium-activated potassium channel has a biphasic behavior of open probability as function of potential. *Biophys. J.* **47**:137*a*

Hille, B. 1984. Ionic channels of excitable membranes. Sinauer, Sunderland, MA

Hosey, M.M., Lazdunski, M. 1988. Calcium channels: Molecular pharmacology, structure and regulation. *J. Membrane Biol.* **104:**81–105

Hosoi, S., Iino, M., Shimazaki, K. 1988. Outward-rectifying K^+ channels in stomatal guard cell protoplasts. *Plant Cell Physiol.* **29:**907–911

Hume, J.R. 1985. Comparative interactions of organic Ca^{++} channel antagonists with myocardial Ca^{++} and K^+ channels. *J. Pharmacol. Exp. Ther.* **234:**134–140

Iijima, T., Hagiwara, S. 1987. Voltage-dependent K channels in protoplasts of trap-lobe cells of *Dionaea muscipula*. *J. Membrane Biol.* **100:**73–81

Ketchum, K.A., Poole, R.J. 1990. Pharmacology of the Ca^{2+}-dependent K^+ channel in corn protoplasts. *FEBS Lett.* (*in press*)

Ketchum, K.A., Shrier, A., Poole, R.J. 1989. Characterization of potassium-dependent currents in protoplasts of corn suspension cells. *Plant Physiol.* **89:**1184–1192

Kikuyama, M. 1988. Ca^{2+} increases the Cl^- efflux of the permeabilized *Chara*. *Plant Cell Physiol.* **29:**105–108

Kinraide, T.B., Newman, I.A., Etherton, B. 1984. A quantitative simulation model for H^+-amino acid cotransport to interpret the effects of amino acids on membrane potential and extracellular pH. *Plant Physiol.* **76:**806–813

Komor, E., Cho, B., Schricker, S., Schobert, C. 1989. Charge and acidity compensation during proton-sugar symport in *Chlorella:* The H^+-ATPase does not fully compensate for sugar-coupled proton influx. *Planta* **177:**9–17

Latorre, R., Miller, C. 1983. Conduction and selectivity in potassium channels. *J. Membrane Biol.* **71:**11–30

Latorre, R., Oberhauser, A., Labarca, P., Alvarez, O. 1989. Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* **51:**385–399

Lew, R.R., Serlin, B.S., Schauf, C.L., Stockton, M.E. 1990. Red light regulates calcium-activated potassium channels in *Mougeotia* plasma membrane. *Plant Physiol.* **92:**822–830

MacRobbie, E.A.C., Banfield, J. 1988. Calcium influx at the plasmalemma of *Chara corallina*. *Planta* **176:**98–108

Marmé, D. 1989. The role of calcium and calmodulin in signal transduction. *In:* Second Messengers in Plant Growth and Development. W.F. Boss and D.J. Morré, editors. pp. 57–80. Alan R. Liss, New York

McAinsh, M.R., Brownlee, C., Hetherington, A.M. 1990. Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* **343:**186–188

Minorsky, P.V., Spanswick, R.M. 1989. Electrophysiological evidence for a role for calcium in temperature sensing by roots of cucumber seedlings. *Plant Cell Environ.* **12:**137–143

Moran, N., Ehrenstein, G., Iwasa, K., Mischke, C., Bare, C., Satter, R.L. 1988. Potassium channels in motor cells of *Samanea saman*. A patch-clamp study. *Plant Physiol.* **88:**643–648

Nishizaki, Y. 1988. Blue light pulse-induced transient changes of electric potential and turgor pressure in the motor cells of *Phaseolus vulgaris* L. *Plant Cell Physiol.* **29:**1041–1046

Novacky, A. 1980. Disease-related alteration in membrane function. *In:* Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas and J. Dainty, editors. pp. 369–380. Elsevier/North-Holland Biomedical, Amsterdam

Okazaki, Y., Tazawa, M. 1986. Ca^{2+} antagonist nifedipine inhibits turgor regulation upon hypotonic treatment in internodal cells of *Lamprothamnium*. *Protoplasma* **134:**65–66

Poole, R.J., Ketchum, K.A. 1989. Ion channels at the plasma membrane. *In:* Plant Water Relations and Growth Under Stress. M. Tazawa, M. Katsumi, Y. Masuda, and H. Okamoto, editors. pp. 23–30. Yamada Science Foundation, Osaka, Japan

Racusen, R., Satter, R.L. 1975. Rhythmic and phytochrome-regulated changes in transmembrane potential in *Samanea* pulvini. *Nature* **255:**408–410

Rincon, M., Hanson, J.B. 1986. Controls on calcium ion fluxes in injured or shocked corn root cells: Importance of proton pumping and cell membrane potential. *Physiol. Plant.* **67:**576–583

Rink, T.J., Pozzan, T. 1985. Using quin2 in cell suspensions. *Cell Calcium* **6:**133–144

Robinson, C., Larsson, C., Buckhout, T.J. 1988. Identification of a calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase in a plasma membrane fraction isolated from maize (*Zea mays*) leaves. *Physiol. Plant.* **72:**177–184

Schauf, C.L., Wilson, K.J. 1987. Effects of abscisic acid on K^+ channels in *Vicia faba* guard cell protoplasts. *Biochem. Biophys. Res. Commun.* **145:**284–290

Schroeder, J.I., Hagiwara, S. 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338:**427–430

Schroeder, J.I., Raschke, K., Neher, E. 1987. Voltage dependence of K^+ channels in guard-cell protoplasts. *Proc. Natl. Acad. Sci. USA* **84:**4108–4112

Schumaker, K.S., Sze, H. 1985. A Ca^{2+}/H^+ antiport system driven by the proton electrochemical gradient of a tonoplast H^+-ATPase from oat roots. *Plant Physiol.* **79:**1111–1117

Shiina, T., Tazawa, M. 1987*a*. Ca^{2+}-activated Cl^- channel in plasmalemma of *Nitellopsis obtusa*. *J. Membrane Biol.* **99:**137–146

Shiina, T., Tazawa, M. 1987*b*. Demonstration and characterization of Ca^{2+} channel in tonoplast-free cells of *Nitellopsis obtusa*. *J. Membrane Biol.* **96:**263–276

Spalding, E.P., Cosgrove, D.J. 1989. Large plasma-membrane depolarization precedes rapid blue-light-induced growth inhibition in cucumber. *Planta* **178:**407–410

Stoeckel, H., Takeda, K. 1989. Calcium-activated, voltage-dependent, non-selective cation currents in endosperm plasma membrane from higher plants. *Proc. R. Soc. London B* **237:**213–231

Tazawa, M., Shimmen, T., Mimura, T. 1987. Membrane control in the Characeae. *Annu. Rev. Plant Physiol.* **38:**95–117

Tester, M. 1988. Pharmacology of K^+ channels in the plasmalemma of the green alga *Chara corallina*. *J. Membrane Biol.* **103:**159–169

Thaler, M., Steigner, W., Köhler, K., Simonis, W., Urbach, W. 1987. Release of repetitive transient potentials and opening of potassium channels by barium in *Eremosphaera viridis*. *FEBS Lett.* **219:**351–354

Tsien, R.Y., Pozzan, T., Rink, T.J. 1982. Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol.* **94:**325–334

Tyerman, S.D., Findlay, G.P., Paterson, G.J. 1986. Inward membrane current in *Chara inflata:* II. Effects of pH, Cl^--channel blockers and NH_4^+, and significance for the hyperpolarized state. *J. Membrane Biol.* **89:**153–161

Weisenseel, M.H., Ruppert, H.K. 1977. Phytochrome and calcium ions are involved in light-induced membrane depolarization in *Nitella*. *Planta* **137:**225–229

Wolniak, S.M., Bart, K.M. 1985. Nifedipine reversibly arrests mitosis in stamen hair cells of *Tradescantia*. *Eur. J. Cell Biol.* **39:**273–277

Received 13 June 1990; revised 10 September 1990