Reactions of Corn Root Tissue to Calcium

Magaly R. de Quintero* and John B. Hanson
Department of Plant Biology, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Washing corn (Zea mays L.) root tissue in water causes loss of about one-third of the exchangeable Ca⁺⁺ over the first 10 to 15 minutes. Upon transfer to K⁺-containing solutions, the tissue shows a short period of rapid K⁺ influx which subsequently declines. Addition of 0.1 millimolar Ca⁺⁺ decreases the initial rapid K⁺ influx, but increases the sustained rate of K⁺ and Cl⁻ uptake. It was confirmed (Elzam and Hodges 1967 Plant Physiol 42: 1483–1488) that 0.1 millimolar Ca⁺⁺ is more effective than higher concentrations for the initial inhibition, and that Mg²⁺ will substitute.

The inhibition arises from a mild shock effect of restoring Ca⁺⁺. With 0.1 millimolar Ca⁺⁺ net H⁺ efflux is blocked for 10 to 15 minutes and the cells are depolarized by about 30 millivolts. However, 1 millimolar Ca⁺⁺ rapidly produces increased K⁺ influx and blocks net H⁺ efflux for only a few minutes; blockage is preceded by a brief net H⁺ influx which may restore and increase ion transport by reactivating the plasmalemma H⁺-ATPase.

Stimulation of electrogenic H⁺-pumping with fusiconin eliminates the shock responses and minimizes Ca⁺⁺ effects on K⁺ influx. Fusiconin also strongly decreases Ca⁺⁺ influx, but has no effect on Ca⁺⁺ efflux. Ice temperatures and high pH decreased Ca⁺⁺ efflux, but uncoupler and chlorpromazine did not.

It is suggested that the inhibitory and promotive actions of Ca⁺⁺ are manifested through decreases or increases in the proton motive force.

Elzam and Hodges (8) reported that addition of Ca⁺⁺ to the root medium produced a transitory inhibition of K⁺(⁶⁸Rb) influx in corn roots (but not in barley or oat roots). After about 30 min, the inhibition was lost and Ca⁺⁺ promoted K⁺ influx, the well-known 'fusiconin effect' (24). Inhibition was optimal at 0.1 mm Ca⁺⁺, was found with CaSO₄ and CaCl₂, and occurred over the range of 0.005 to 0.20 mm K⁺⁺ showing noncompetitive kinetics. A similar inhibition was produced by Mg²⁺. Inhibition was suggested to arise from reduced plasmalemma permeability in the presence of Ca⁺⁺, with the later promotion possibly due to reduced K⁺ efflux.

Leonard et al. (20) found La⁺⁺ as well as Ca⁺⁺ to produce the initial inhibition and later promotion of K⁺(⁶⁸Rb) influx, but unlike Elzam and Hodges (8) found competitive kinetics over the first 10 min of influx. On the basis of the impermeability of La⁺⁺, they concluded that the inhibition and later promotion of K⁺ influx arise from reaction of Ca⁺⁺ and La⁺⁺ in the apoplast, including the external surface of the plasmalemma.

In connection with a study of Ca⁺⁺ transport in corn roots, we have made similar observations, and have investigated further the nature of the initial inhibition of K⁺ influx. It is primarily a 'shock' response to restoring Ca⁺⁺ removed during tissue washing which stops net H⁺ pumping and partially depolarizes the cell. Recovery from shock begins in 10 to 15 min and gives increased K⁺ influx. Inhibition and recovery appear to reflect the level of the proton motive force.

A portion of this work has been reported in abstract form (22).

MATERIALS AND METHODS

Root Tissue. Three-day-old etiolated corn seedlings (Zea mays L.; Crow's Hybrid 430, Milford, IL) were grown in the dark at 28°C on paper towels saturated with 0.1 mm CaCl₂ (19). Segments taken 0.5 to 2.5 cm from the tip of the primary root were washed 3 to 4 h in aerated 0.2 mm KH₂PO₄ + 0.2 mm CaSO₄ (pH 6.0) or distilled H₂O at 30°C to recover ion transport capability (14, 19).

Net K⁺ Uptake. Two g of tissue were placed in 50 ml of aerated 0.2 mm KH₂PO₄ (pH 6.0 with Tris) at 30°C. At intervals, 1-ml aliquots were taken, dried, redissolved in 1 ml of 1.5 mm CaCl₂, and K⁺ determined by flame photometry. Uptake was calculated from K⁺ concentrations and volumes.

Ion Influx and Efflux. Solutions for determining flux of radiolabeled ions were aerated 0.1 mm K₂SO₄, 2 mm Mes-Tris (pH 5.6) ± 0.1 mm CaSO₄ labeled with ⁸⁶Rb or ⁴¹Ca. For Cl⁻ influx, 0.1 mm ⁸⁶Cl⁻ was added. After the absorption period, the root segments were rinsed in distilled H₂O and exchangeable ⁸⁶Rb⁺⁺ or ⁴¹Cl⁻ removed in ice cold 5 mm KCl + 0.5 mm CaCl₂ for 10 min; for ⁴¹Ca⁺⁺, the exchange solution was 5 mm CaCl₂ + 0.5 mm KCl. The tissue was then weighed and isotope quantified. In ion efflux studies, segments preloaded with ⁸⁶Rb⁺⁺ or ⁴¹Ca⁺⁺ were rinsed, exchanged at 30°C, for 10 min, and transferred to unlabeled absorption solution. At intervals, tissue was removed and weighed and remaining isotope quantified.

Net H⁺ Efflux. Recording of pH was as described (3, 14), incubating 50 segments in 50 ml of aerated 0.1 mm K₂SO₄, 0.1 mm Mes-Tris (pH 6.0) at 30°C with additions as indicated. Stock solutions for additions were adjusted to pH 6.0. Net H⁺ efflux was calculated from back titration with standard KOH.

Membrane Potential. Membrane potential was measured as described (5). The bathing solution was 0.1 mm K₂SO₄ and 0.1 mm Mes-Tris (pH 6.0) at room temperature (−23°C) with additions of 0.1 mm CaSO₄ as indicated.

ATP Determinations. ATP content of the tissue was determined as described (16).

RESULTS

K⁺ Uptake. Figure 1 shows the effect of 0.1 mm Ca⁺⁺ on net K⁺ uptake by root segments washed in 0.2 mm K-phosphate + 0.2 mm CaSO₄ for 4 h, and then in distilled H₂O for 30 min. Although a different root segment was used (0.5–2.5 cm from the tip, rather than 0–1.5 cm), and net uptake determined rather than K⁺(⁶⁸Rb) influx, the result confirms the report of Elzam and Hodges (8). On transfer of the tissue to the absorption solution, there was a rapid initial uptake of K⁺, which settled to
a lower linear rate after 20 to 30 min. Calcium inhibited the rapid uptake for about 10 min, then initiated a linear phase of uptake double that of the control.

**K⁺ Influx**. When assayed by K⁺(⁴⁰Rb) influx with Ca²⁺ or Mg²⁺ present initially, the inhibitory phase is seen as an elimination of a rapid initial K⁺ influx (Figs. 2 and 3). The inhibitions generally lasted 10 min or longer (in five experiments at this time, K⁺(⁴⁰Rb) influx at 10 min in 0.1 mM Ca²⁺ averaged 0.317 μmol·g⁻¹ fresh weight versus 0.358 for control, and these differences were significant at the 99% confidence level). Comparison with net K⁺ uptake (Fig. 1) indicates that the initial phase of K⁺(⁴⁰Rb) influx must include some cation exchange even in the presence of Ca²⁺ or Mg²⁺.

Figure 2 includes a comparison of washing in water and CaSO₄, which shows that washing without Ca²⁺ is required to produce the initial Ca²⁺ inhibition of K⁺ influx. Investigation of how much washing without Ca²⁺ was required showed that sensitivity to added 0.1 mM Ca²⁺ developed rapidly after 10 min of water washing (data not shown), which coincides with the decline in K⁺ influx after 10 min deprivation of Ca²⁺ (Fig. 2). By washing the root segments in ⁴⁰CaSO₄, and observing the loss of Ca²⁺ during further washing in water or CaSO₄, it was found that water-washing produced loss of ⁴⁰Ca²⁺ over the first 10 to 15 min only (Fig. 4). Loss by exchange with external Ca²⁺ was 3-fold more extensive and occurred over a longer period (Fig. 4). Thus, when root tissue previously washed in Ca²⁺ is transferred to water, we observe three events over a 10-min period: (a) there is...
net loss of Ca\(^{2+}\) to the water; (b) there is lowering of the subsequent rate of K\(^{+}\) influx; (c) sensitivity to subsequently added Ca\(^{2+}\) develops. The last two responses show that Ca\(^{2+}\) is lost from sites critical to K\(^{+}\) transport. It is not known what cation(s) displaced Ca\(^{2+}\) from these sites, but the logical candidate would be \(H^+\) which is extruded during water washing. Whatever the sites are, they represent only about one-third of the Ca\(^{2+}\) subject to Ca\(^{2+}\)/Ca\(^{2+}\) exchange.

Figure 3 verifies (8) that Mg\(^{2+}\) can also produce the initial inhibition. We also confirmed that the initial inhibition was independent of the anion (at 10 min K\(^{+}\)(\(^{86}\)Rb) influx using 0.1 mM Ca\(^{2+}\) salt was 0.33 mmol g\(^{-1}\) fresh weight for SO\(_4^{2-}\), 0.32 for Cl\(^{-}\), 0.33 for NO\(_3^{-}\), and 0.39 for no addition).

Elzam and Hodges (8) found 0.1 mM Ca\(^{2+}\) to be optimal for the initial inhibition. In contrast, the promotive Viets effect requires 1 to 5 mM Ca\(^{2+}\) for full expression, and is not diminished by 25 mM (24). We confirmed the effectiveness of 0.1 mM Ca\(^{2+}\), but unlike Elzam and Hodges we found K\(^{+}\) influx promoted in the first 10 min at higher Ca\(^{2+}\) concentrations (Fig. 5). Evidently, with millimolar Ca\(^{2+}\), the promotive mechanism rapidly masks the inhibitory effect. The relative effectiveness of the inhibitory and promotive reactions is best seen in the ratio of K\(^{+}\) influx at 10 and 60 min (Fig. 5).

Cl\(^{-}\) Influx. Water washing did not produce an initial burst of \(^{36}\)Cl\(^{-}\) influx, and Ca\(^{2+}\) addition was not initially inhibitory (Fig. 6). However, Ca\(^{2+}\) gave sustained Cl\(^{-}\) influx, which without Ca\(^{2+}\) declined after 10 min. Promotion of anion uptake by Ca\(^{2+}\) has been widely observed and is often attributed to neutralization of repulsive negative charge at the membrane surface (17). Comparison of K\(^{+}\) and Cl\(^{-}\) influx (Figs. 2 and 3 versus 6) suggests that the initial burst of K\(^{+}\) influx represents a cation exchange reaction with the protoplast (freely exchangeable K\(^{+}\)(\(^{86}\)Rb) has been removed). Calcium (or Mg\(^{2+}\)) prevents this. Cl\(^{-}\) does not participate in an equivalent anion exchange.

H\(^{+}\) Efflux. Addition of 0.1 mM Ca\(^{2+}\) completely blocked net H\(^{+}\) efflux for about 15 min (Fig. 7). After recovery the rates with Ca\(^{2+}\) were always slightly less than without, despite the increase in salt uptake. In two determinations, the net \(H^+\) efflux/K\(^{+}\)(\(^{86}\)Rb) influx ratio at 60 min averaged 2.16, 1.50, and 1.24 for 0, 0.1, and 1 mM Ca\(^{2+}\). However, the decreasing ratio is not necessarily evidence for increased transport efficiency (although it may exist); we see no theoretical reason to expect a stoichiometric relationship between net \(H^+\) efflux and K\(^{+}\) influx. Glass and Siddiqi (12) have discussed the stoichiometry problem in detail.

The kinetics of the Ca\(^{2+}\) inhibition are shown in the pH traces of Figure 8. (Addition of Ca\(^{2+}\), and to a lesser extent Mg\(^{2+}\), to the absorption medium without roots causes an abrupt drop in pH, followed by rapid recovery to the initial pH. Apparently Ca\(^{2+}\) displaces H\(^{+}\) bound to the glass membrane, transiently increasing free H\(^{+}\) at the electrode surface.) Fusicoccin, which can restore shock-inhibited H\(^{+}\)-pumping in these root segments (2, 3), will relieve the Ca\(^{2+}\)-induced block of net \(H^+\) efflux (Fig. 8A), showing that a major component of the inhibition lies with restricted \(H^+\)-pumping. If FC is present initially there is no Ca\(^{2+}\) inhibition (not shown).

Comparison of Figure 8, A and B, discloses why there is so little initial inhibition of K\(^{+}\) influx with 1 mM Ca\(^{2+}\) (Fig. 5). Compared to 0.1 mM Ca\(^{2+}\), there is only a brief inhibition of \(H^+\)-pumping. However, 1 mM Ca\(^{2+}\) produced a marked overshoot in pH recovery after addition, which indicates a transient net H\(^{+}\) influx. When the tissue is washed in CaSO\(_4\), and only briefly exposed to Ca\(^{2+}\)-free medium, there is no comparable inhibition of net H\(^{+}\) efflux (Fig. 8C) or K\(^{+}\) influx (Fig. 2).

Addition of 0.1 mM Mg\(^{2+}\) produces a result similar to Figure 8A (not shown).

Cell Potential. Calcium rapidly initiated a modest depolarization of the cell potential (Fig. 9). The potential fell 25 to 30 mV.
in 10 min, which corresponds reasonably with the period of inhibited K⁺ uptake. However, repolarization was slow, and no correlation could be established with K⁺ influx; perhaps there is no reason to expect one since the increased K⁺ influx after 10 min would tend to depolarize.

**Fusicoccin.** As with other tissues, FC² stimulates electrogenic H⁺ pumping in corn root segments, hyperpolarizing the cells (7, 14). Fusicoccin eliminated the initial inhibitory action of 0.1 mM Ca²⁺ on K⁺ influx, with only a slight promotive effect of Ca²⁺ evident (Fig. 10). In addition, the gradual decline in K⁺ influx without Ca²⁺ was eliminated (Fig. 10).

**Ca²⁺ Fluxes.** Calcium entry into plant cells is passive down a large electrochemical gradient; efflux is active and driven by Ca²⁺-ATPase or H⁺/Ca²⁺ antiport (17). Microsomal membranes from corn roots exhibit both types of efflux transport (27), and root segments show passive Ca²⁺ influx which is greater under shock conditions which increase ion permeability (26).

The initial 10 min of ⁴⁰Ca²⁺ uptake, corresponding to the period of inhibited K⁺ influx, was rapid and not much affected by FC (Fig. 11). Apparently, the initial uptake of Ca²⁺ is either not responsible for the Ca²⁺ inhibition of K⁺ influx, or Ca²⁺ must be ineffectual in the presence of FC (Fig. 10). Subsequently, however, FC strongly inhibited Ca²⁺ influx. Thus, the increased electrogenic H⁺ pumping due to FC acts both to increase K⁺ influx and decrease Ca²⁺ influx. The increased K⁺ uptake at the low K⁺ concentrations used here is against the electrochemical gradient, and is postulated to be carrier-linked with the H⁺-ATPase (6). The inhibitory effect of FC on Ca²⁺ influx has been observed previously (22, 26), and is suggested to arise from hyperpolarization causing closure of voltage-gated ion channels (28).

Fusicoccin had no effect, however, on Ca²⁺ efflux (Fig. 11). This failure led to some exploratory work on factors affecting Ca²⁺ efflux (Table I). Efflux was lowered by ice temperature and high pH, which might indicate dependence on cellular metabo-
lism and/or a pH gradient across the plasmalemma. However, efflux was not significantly altered by the uncoupler FCCP or by chlorpromazine, an antipsychotic drug that inactivates the Ca-calmodulin complex required to activate the Ca^{2+}-ATPase (25). Chlorpromazine and trifluoperazine, which has similar activity, have been used with FC in studying H⁺-pumping and hyperpolarization of corn root tissue (1). At optimal FC concentrations chlorpromazine inhibits H⁺ efflux and depolarizes the cells; Ca^{2+} has a protective effect (1).

In our experiments the combination of FC and chlorpromazine produced a large efflux of Ca^{2+} (Table 1). In the presence of 0.1 mM Ca^{2+}, 50 μM chlorpromazine (no FC) caused a 42% decrease in K⁺(48Rb) influx plus a 26% increase in both H⁺ efflux and Cl⁻ influx; this is reminiscent of the Cl⁻/OH⁻ exchange and inhibition of K⁺ influx produced by triprotylin (2). We concluded that chlorpromazine was damaging cell membranes and/or acting as a lipid-soluble Cl⁻/OH⁻ antipporter, and that it could not be reliably used for in vitro studies of calmodulin-activated Ca^{2+} efflux pumping.

ATP Concentrations. It is characteristic of injury or shock to corn roots that membrane permeability increases, allowing efflux of K⁺ and degradation of ATP (16), as well as inhibiting H⁺-pumping. Since 0.1 mM Ca^{2+} transiently blocked H⁺ pumping, we checked whether there was also increased K⁺ efflux and decreased ATP. Neither occurred. Loss of preloaded K⁺(48Rb) was not significantly altered. In five experiments, the ATP content 10 min after adding 0.1 mM Ca^{2+} averaged 122.3 nmol·g⁻¹ fresh weight versus 115.8 for control; a corresponding cold shock reduced ATP to 74.0 nmol·g⁻¹ fresh wt. The 'shock' of abruptly resupplying Ca^{2+} to water-washed tissue must have a different basis than that of physical injuries or shocks. They do effect in common, however, a transient inhibition of net H⁺ efflux pumping, cell potential, and K⁺ influx.

DISCUSSION

Our intention here was to determine, if possible, the nature and significance of the transient Ca^{2+} inhibition of K⁺ influx. The data indicate that the inhibition arises from a shock reaction to restoring Ca^{2+} previously lost from the cells by washing without Ca^{2+}. The vacated sites appear to be at the external surface of the plasmalemma. We have found no evidence that penetration of Ca^{2+} into the cell is responsible; Mg^{2+} is just as effective as Ca^{2+} in the short term (Fig. 3), and the initial uptake of Ca^{2+} is not affected by FC (Fig. 11) although FC obliterates the Ca^{2+} inhibition (Fig. 10). The shock transiently blocks net H⁺ efflux (Figs. 7 and 8) and depolarizes the cells (Fig. 9). After a delay which is shorter with higher Ca^{2+} concentrations, there is spontaneous recovery characterized by an augmented K⁺ influx. Certain details deserve comment.

(a) To obtain a shock response, the tissue must be exposed to moderately high Ca^{2+}: Elzam and Hodges (8) washed root segments in distilled H₂O for 30 min. We washed in distilled H₂O or dilute Tris buffer up to 3 or 4 h to obtain tissue recovered from excision injury. However, only 10 to 15 min without Ca^{2+} is sufficient to remove a portion of the Ca^{2+}, to reduce K⁺ influx, and induce sensitivity to added Ca^{2+}. It has long been known that the Viets effect requires the continued presence of external Ca^{2+} (10) but it is not always appreciated how quickly influx rates decline in the absence of Ca^{2+}. Harrington et al. (18) report that 5 min without Ca^{2+} eliminates Ca^{2+}-activated lysine transport by tobacco cells. Influx of K⁺(48Rb) in barley roots declines as quickly (9).

About one-third of the exchangeable Ca^{2+} is lost to distilled H₂O in 10 to 15 min (Fig. 4), and none thereafter. The physiological responses show that some portion of the loss is implicated in membrane transport, probably that Ca^{2+} held in the diffuse double layer at the protoplasm surface and/or complexed with surface ligands. In either case, a cation is needed to discharge Ca^{2+}, and in water washing this must be extruded H⁺; this presumes that any Ca^{2+}-binding ligands are weak acids.

(b) Whatever the vacated sites may be, they potentiate a shock reaction when Ca^{2+} is restored. Net H⁺ efflux is blocked (Figs. 7 and 8), depolarization is initiated (Fig. 9), and net K⁺ uptake is inhibited (Fig. 1), all symptoms of a lowered protonmotive force. However, the shock is a mild one as there is no leakage of K⁺ or loss of adenine nucleotides and there is rapid recovery. In agreement with Elzam and Hodges (8), Mg^{2+} addition is also effective (Fig. 3), and 0.1 mM Ca^{2+} is more effective than higher concentrations (Fig. 5).

The data are inadequate to determine why Ca^{2+} initiates a shock. As mentioned above, we have found no evidence for Ca^{2+} influx as a causal agent, unlike the case with cold shock (26). Blocked cation influx may be involved; there is no initial effect of Ca^{2+} on Cl⁻ influx (Fig. 6), but the rapid initial influx of K⁺ is eliminated (Figs. 2 and 3). Perhaps Ca^{2+} has vacated sites which open channels for passive K⁺ influx or exchange, and restoration of Ca^{2+} closes these. Since the sites are not Ca^{2+} specific (Fig. 3) channel control must lie with divalent cations associated with the membrane surface. If H⁺ displaces Ca^{2+} from the surface, the restoration of Ca^{2+} should displace H⁺ with a net reaction similar to that shown by the pH electrode (Fig. 8)—a transient increase in free H⁺. The shock may result from sensitivity of the proton channel to abrupt acidification externally.

An alternative is that externally bound Ca^{2+} has some regulatory role with a K⁺-transporting H⁺-ATPase of the plasmalemma. At these low K concentrations, K⁺ influx is against the electrochemical gradient and transport must be coupled to an exergonic reaction (6). Resupplying Ca^{2+} might initially produce an aberrant conformational change in K⁺/H⁺-ATPase transport channels; later promotion might arise from better linkage of K⁺ influx to H⁺ efflux.

(c) The failure of 1 mm Ca^{2+} to initially inhibit K⁺ influx (Fig. 5) can be attributed to the protective Viets effect overriding the inhibitory shock. Both 0.1 and 1 mm Ca^{2+} block net H⁺ efflux, but the 1 mm inhibition lasts only a few min and is preceded by a brief period of apparent net H⁺ influx (Fig. 8B). We have not investigated the promotive action of Ca^{2+}, but if there is acidification of the cytoplasm an explanation for the rapid recovery exists. Marré et al. (21) have shown that acidification of corn root cytoplasm by weak acids leads to hyperpolarization and increased K⁺ influx, and they propose that this is due to activation of the plasmalemma H⁺-ATPase.

(d) The action of FC in eliminating the shock effect of 0.1 mm Ca^{2+} (Fig. 10) supports the concept that inhibition of K⁺ influx by Ca^{2+} results from diminished H⁺-ATPase activity. Conversely, Ca^{2+} is not needed to promote K⁺ influx if FC is present. Apparently both the inhibitory and promotive responses to Ca^{2+} can be attributed to H⁺-ATPase activity, or perhaps ΔΨ, which reflects not only electrogenic H⁺-pumping but also the extent to which ΔΨ and ΔpH are utilized in salt transport. Smith (23) has
suggested that Ca\(^{2+}\) may activate and maintain serine uptake in tobacco cells by increasing the proton motive force.

After the initial equilibration, FC strongly inhibits Ca\(^{2+}\) influx (Fig. 11), which we attribute to a voltage-sensitive gating reaction. That is, hyperpolarization by FC may close ‘gated’ ion channels, restricting passive influx of Ca\(^{2+}\) and possible other cations. Since at the same time K\(^+\) influx, which is against the thermodynamic gradient (6) is increased, it must be linked to H\(^+\) pumping (e.g. H\(^+\)/K\(^+\)-ATPase), or alternatively an electrogenic K\(^+\) pump (12). As noted above, Ca\(^{2+}\) in the absence of FC promotes sustained K\(^+\) influx in a fashion which likewise suggests increased Δ\(\mu\)H\(^+\). There is thus an interplay between H\(^+\)-ATPase, cell potential, and passive (channel) ion conductance which Ca\(^{2+}\) can modulate. Calcium has some still obscure role in channel gating of excitable membranes of animals (12, 13), and a similar role in excitable corn root cell membranes could produce the effects observed here. (Thermophiles such as corn seem to be more sensitive to cold and other shocks than cool season plants [29], which may explain why Elzam and Hodges did not see Ca\(^{2+}\) inhibition of K\(^+\) influx with oats and barley.)

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LITERATURE CITED

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