Putrescine-Induced Wounding and Its Effects on Membrane Integrity and Ion Transport Processes in Roots of Intact Corn Seedlings

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ABSTRACT

Interactions between putrescine and membrane function were examined with the use of a recently developed microelectrode system that enables us simultaneously to quantify membrane potentials and net K⁺ fluxes associated with individual cells at the root surface of an intact corn (Zea mays L.) seedling. In contrast to the results of others, our analyses indicate that exogenous putrescine (0.5 millimolar), in the absence of calcium, does not maintain membrane stability. In addition, putrescine caused a wound response characterized by a gradual depolarization of the membrane potential and a considerable net efflux of K⁺ from the root. In the presence of calcium, both short term (20 minutes) and long term (24 hours) exposure to a high concentration of exogenous putrescine (5 millimolar) also caused a reduction in the resting membrane potential and a significant K⁺ efflux. However, preincubating corn roots in a solution containing the antioxidant ascorbate ameliorated the wound effects of putrescine and slightly increased potassium uptake. A similar preincubation in the absence of calcium did not protect membranes against putrescine-induced damage. The ameliorating effect of ascorbate on putrescine-induced membrane damage suggests that the wounding response of high putrescine levels in corn roots involves the catabolism of the polyamine by a cell wall diamine oxidase, with the concomitant production of hydrogen peroxide and free radicals resulting in peroxidative damage of the plasmalemma.

Diamines, particularly putrescine, can accumulate in plants as a response to increased growth or to a variety of stress conditions, including potassium deficiency, water, salt, or acid stress (for a review, see ref. 23), and herbicide treatment (5). However, it has been shown that under these conditions the concentration of tri- and tetraamines (such as spermidine and spermine) generally remains unchanged. This stress-mediated increase in diamines has been suggested to be an adaptive advantage to plants, and a number of protective functions for polyamines have been proposed. For example, in studies involving protoplasts and excised cereal leaves, exogenous polyamine applications were effective in preventing senescence by reducing Chl loss (4) and inhibiting the rise in RNAase and protease activities (11). Additionally, it has been suggested (31) that due to its polycationic nature, putrescine could be involved in the ionic regulation of the plant cell symplasm. In response to acid stress, for example, it has been hypothesized (25) that increases in cytoplasmic putrescine levels could help to maintain cytoplasmic pH at a constant value.

It has also been suggested (13) that under stress conditions, polyamines may partially replace calcium in maintaining membrane integrity by binding to phospholipid components of the membrane. The involvement of polyamines in wound-induced membrane permeability has been investigated by several researchers. As a measurement of membrane integrity, Naik and Srivastava (13) spectrophotometrically monitored the leakage of the red pigment, betacyanin, from red beet root discs. They reported that exogenously applied spermidine and spermine decreased betacyanin efflux from the discs of beet (Beta vulgaris L.) roots treated with ethanol. Polyamines were also effective in reducing betacyanin efflux in beet root tissues wounded with ethylene, ammonium sulfate (15), RNAase, or high temperature (1, 26).

However, some confusion and controversy exists concerning the role of polyamines in stabilizing membranes. In the above cited work, Srivastava and Smith (26) reported that exogenously applied putrescine, spermidine, and spermine decreased betacyanin efflux in temperature-stressed beet root discs, while total ion efflux was shown to increase. To explain this discrepancy, they speculated that polyamines may disrupt the plasmalemma but stabilize the tonoplast. Since betacyanin is thought to be retained in the vacuole, its efflux would be reduced. However, Smith and Croker (24) recently demonstrated that this apparent reduction in betacyanin leakage is actually due to a chemical reaction between betacyanin and polyamines in the external solution, which causes a decolorization of the red pigment. These results cast doubt on any hypothesis concerning polyamine stabilization of membranes based on the measurement of betacyanin leakage from plant tissues.

Although a stress-induced increase in putrescine levels may have protective advantages, an excessive accumulation could also be a cause of injury. High levels of exogenous diamines resulted in symptoms of toxicity in broad bean (Vicia faba L.) (18) and in barley (Hordeum vulgare L.) (27), while increased endogenous putrescine may cause a reduction in cell division in osmotically stressed cereal protoplasts (30) and necrosis in salt stressed broad bean (18). Ferrante et al. (7) found that cultures of the protozoan Plasmodium falciparum...
degenerated when exposed to spermine and polynamine oxidase in combination. However, if the polynamine or the polynamine oxidase were added alone, no symptoms were observed. They concluded that polynamine oxidation leads to cellular disruption through the production of toxic aldehydes, hydrogen peroxide, and free radicals. These substances have long been recognized to cause tissue injury through membrane destruction (20).

In plants, diamine oxidase activity has been identified in a number of species of the Leguminosae (for a review, see ref. 23) and in rice (Oryza sativa L.) (3) and oat (Avena sativa L.) (8). The catabolism of putrescine by diamine oxidase occurs almost exclusively within the cell walls (8) and results in the production of pyrroline, hydrogen peroxide, ammonia (28), and possibly free radicals (34). Thus, the toxic effect associated with high levels of exogenous putrescine may be the result of membrane damage caused by diamine oxidase-mediated putrescine catabolism.

In the present work, we investigated the role of putrescine in maintaining and/or altering membrane integrity. The interaction of exogenous putrescine and calcium, in terms of their effects on $E_m$ and net potassium fluxes in roots of intact corn seedlings, was studied. It has been well documented that corn roots are a particularly sensitive tissue, and that various perturbations such as excision, cold shock, etc., can elicit a wound response (10). This response is characterized by a reduction in $K^+$ influx and a stimulation of efflux and by a depolarization of $E_m$. Additionally, we have recently developed a microelectrode system that enables us to simultaneously quantify membrane potentials and net ionic fluxes associated with individual cells at the root surface of an intact corn seedling (14). Thus, we have an experimental system that is well suited for studying the apparently broad spectrum of interactions between putrescine and membrane function. Results of these experiments indicate that putrescine does not replace calcium in maintaining membrane stability. On the contrary, the diamine caused a depolarization of the membrane potential and increased potassium leakage. Additional experiments indicate that hydrogen peroxide and free radical production may be the cause of membrane damage and thus be responsible for the phytotoxic effect of high exogenous putrescine levels.

MATERIALS AND METHODS

Plant Material

Zea mays L. seeds (3377 Pioneer) were surface-sterilized in 0.5% NaOCl, and then were germinated for 2 d in the dark on filter paper saturated with 0.2 mM CaSO$_4$. Subsequently, eight germinated seedlings were selected for uniform growth, transferred to polyethylene cups with polyethylene mesh bottoms (two seedlings per cup), and then covered with black polyethylene beads. Four cups (eight seedlings) were then placed into precut holes in the covers of black polyethylene containers containing 2.4 L of aerated 0.2 mM CaSO$_4$ solution and were grown for 3 d. The seedlings were grown at 22°C under low-light conditions. The primary root of the intact 5-d-old seedlings was used for all $K^+$ flux and electrophysiologically experiments.

Extraction and Quantification of Polyamines

Corn root sections were excised 0 to 0.5, 0.5 to 1.5, 1.5 to 2.5, and 2.5 to 3.5 mm from the tip. Each section was weighed and ground in a glass homogenizer with 5% cold perchloric acid at a ratio of about 25 to 100 mg root tissue/mL perchloric acid. Homogenized tissue samples were extracted in perchloric acid for 1 h in an ice bath. Samples were centrifuged at 24,000 g for 20 min at 4°C, and the supernatant phase, containing the 'free' polyamine fraction, was either immediately benzoylated or stored at -20°C in small cryogenic plastic vials. One mL of 2 N NaOH was mixed with either 500 µL of the free polyamine fraction extracted from root tissue or 50 µL of each 1 mm polyamine standard in 0.01 N HCl. After addition of 10 µL benzoyl chloride, mixtures were vortexed for 10 s and incubated at room temperature for 20 min. Two mL of saturated NaCl and 10 mL cold anhydrous diethyl ether were added to each sample. The tubes were capped, gently mixed, and centrifuged at 1500 g for 5 min at 4°C. Five mL of the ether phase were collected, evaporated under N$_2$ gas at 35°C, and redissolved in 100 µL methanol. Standards for putrescine, cadaverine, spermidine, and spermine were treated similarly to tissue extracts.

The redissolved samples were injected into a fixed 20 µL loop for loading onto a 4.6 mm by 250 mm, 5 µm particle size reverse-phase (C$_{18}$) column. Samples were eluted from the column by a Perkin-Elmer Series 410 pump at room temperature with a flow rate of 1 mL/min. Polyamine peaks were detected by a Perkin-Elmer LC-95 absorbance detector at 254 nm. The isocratic solvent system consisted of 49% acetonitrile (v/v) in deionized water. Results were recorded on a Nelson Analytical 900 Series Intelligent Interface and integrated with Nelson Analytical PC Integrator software from an IBM XT. Results are presented as nmol/g fresh weight.

Measurement of Net $K^+$ Fluxes

Liquid membrane-type neutral carrier-based $K^+$-selective microelectrodes (tip diameter = 0.5 µm) were constructed as previously detailed (12) using Fluka $K^+$-selective cocktail (catalog No. 60031, Fluka Chemical Co.). We have recently developed a technique that enables us to quantify net ionic fluxes associated with individual root epidermal cells, based on the measurement of radial ion activity gradients in the un-stirred layer at the root surface with ion-selective microelectrodes. These steady state gradients are the result of ion transport at the root surface (influx or efflux) and the diffusion of ions either toward or away from the root (see ref. 14 for a detailed description of the experimental procedures). Briefly, the intact seedling was housed in a Plexiglas chamber attached to the stage of an Olympus compound microscope mounted on its back on the surface of a vibration-damped table (Kinetic Systems Inc.). The $K^+$-selective microelectrode was mounted in a pressure-relieved holder on the preamplifier of a high input resistance dual electrometer (model FD 223, WP Instruments, Inc.). The preamplifier was then mounted onto a Narashige hydraulically driven micromanipulator (model...
MO-204, Narashighe USA) that was attached to the microscope stage such that the microelectrode could be lowered vertically into the solution and reach chosen radial distances from the horizontally oriented root (usually 50 and 100 µm from the root surface).

The root and vertically positioned K⁺-selective microelectrode were viewed under moderate magnification (×60–150) with the Olympus microscope. In order to measure net K⁺ fluxes, the appropriate experimental solution was flowed through the chamber until the previous solution was displaced, and then flow was ceased. The Plexiglas chamber was constructed to minimize mixing of the solution surrounding the root due to mechanical vibration and convection; we have found that steady state ion activity gradients are established at the root surface approximately 5 min after flow is stopped. Subsequently, the K⁺ activity in the unstimulated layer was measured at 50 and 100 µm from the root surface and the net K⁺ flux at the root surface was determined from the following equation derived from diffusion analysis of the spatial symmetry of the K⁺ activity gradient:

\[
J_K = \frac{2 \pi D_K (C_1 - C_2)}{\ln(R_1/R_2)}
\]

where \(J_K\) is the net flux of K⁺ (in µmol cm⁻¹ s⁻¹), \(D_K\) is the self-diffusion coefficient for K⁺ (in cm² s⁻¹), \(C_1\) and \(C_2\) are the K⁺ activities at the two positions, and \(R_1\) and \(R_2\) are the respective distances from the positions where the K⁺ activity was measured to the center of the root. The appropriate conversion factors were used to obtain a net flux in terms of µmol g⁻¹ h⁻¹. The net K⁺ fluxes determined in this study were measured approximately 2 cm back from the root apex.

Electrophysiological Studies

The microelectrode system was constructed such that membrane potentials and K⁺ fluxes could be measured simultaneously. Membrane potentials were measured using a WPI model KS-750 amplifier and microelectrodes (tip diameter = 0.5 µm) made from single-barrelled borosilicate glass tubing and filled with 3 M KCl (adjusted to pH 2 to reduce tip potentials). The reference electrodes for both membrane potential and K⁺ flux measurements were also 3 M KCl-filled micropipettes and were placed in the solution bathing the seed in order to minimize contamination of the solution bathing the root with K⁺ diffusing from the reference electrodes. Cells of the root epidermis and cortex were impaled using a separate hydraulically driven Narashighe micromanipulator mounted at a second position on the microscope stage.

RESULTS

Polyamine Levels in Roots of Corn Seedlings

Endogenous free polyamine concentrations were determined in the terminal 0.5 cm and the three subsequent 1-cm segments back from the root apex (Table I). Putrescine content varied little from the root apex to 3.5 cm back from the tip, averaging 289 nmol/g fresh weight. However, the concentrations of spermidine and spermine were significantly higher in the tip section containing the meristem, and decreased dramatically in the other regions. Similar free polyamine levels in corn roots were reported by Dumortier et al. (6), although they measured a gradual increase in putrescine content from the tip to the base of the root.

<table>
<thead>
<tr>
<th>Table I. Polyamine Content in Four Corn Root Sections</th>
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<tbody>
<tr>
<td>Corn Root Section</td>
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<td>-------------------</td>
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<tr>
<td></td>
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<tr>
<td>cm from tip</td>
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<tr>
<td>0–0.5</td>
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<tr>
<td>0.5–1.5</td>
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<tr>
<td>1.5–2.5</td>
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<td>2.5–3.5</td>
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Effects of 0.5 mM Exogenous Putrescine on \(E_m\) and K⁺ Fluxes in the Presence and Absence of Ca²⁺

In our initial experiments, we wished to determine whether a low concentration (approximately equal to putrescine levels in unstressed or mildly stressed plants) of exogenous putrescine could substitute for calcium in maintaining membrane integrity. Hence, both \(E_m\) and net K⁺ fluxes were monitored as measures of membrane integrity, in response to an experimental protocol which would remove Ca²⁺ from the root cell wall and plasmalemma surface. First, the \(E_m\), K⁺-induced depolarization of \(E_m\), and net K⁺ fluxes were determined under control (+Ca²⁺) conditions that would be indicative of an unperturbed root cell plasmalemma. Representative values for these parameters are seen in the initial phase of the time course presented in Figure 1. An initial resting \(E_m\) of −164 mV was measured in a bathing solution of 0.2 mM CaCl₂, which is a typical value for a low salt-grown corn root. The addition of 50 µM K⁺ (as KCl) to this bathing solution elicited a depolarization of 46 mV, and the subsequent net K⁺ influx was 2.15 µmol g⁻¹ h⁻¹ (inset of Fig. 1). From previous work, we have found these to be fairly typical values for a normal, healthy corn root (14). In contrast, a depolarization of \(E_m\), a reduction in the magnitude of the K⁺-induced depolarization of \(E_m\), and an inhibition of K⁺ influx or a stimulation of efflux have been shown to be characteristic of 'wounded' or perturbed roots and are associated with a decrease in membrane integrity (10).

The addition of a low concentration of putrescine (0.5 mM) in the presence of Ca²⁺ elicited a transient 40 mV depolarization of the membrane, but within 6 min the membrane potential was only 8 mV less negative than its initial value (Fig. 1). The magnitude of the K⁺-induced depolarization (48 mV) and the net K⁺ flux (2.34 µmol g⁻¹ h⁻¹) indicate that low levels of exogenous putrescine do not cause immediate membrane injury to corn roots. Subsequently, Ca²⁺ was removed from the cell wall and plasmalemma surface by flowing a Ca²⁺-free solution containing 1.0 mM EGTA through the root chamber for 15 min. Although the value of the \(E_m\) was relatively unaffected by Ca²⁺ removal, a destabilization of the plasmalemma was observed, as indicated by rapid and dra-
matic transient shifts in the resting potential. This destabilization was apparently intensified when the bathing solution was continuously flowed through the chamber. The removal of calcium by EGTA chelation resulted in a marked reduction in both \( K^+ \)-induced depolarization and net \( K^+ \) flux (Fig. 1, inset). This supports the view that calcium not only maintains membrane stability but plays an important role in ion transport processes.

In Figure 2 (continuation of Fig. 1), 0.5 mM putrescine was added to the bathing solution following extended EGTA treatment. After an initial rapid 45 mV depolarization, the membrane potential continued to gradually depolarize to \( -86 \) mV. The addition of external \( K^+ \) had no further effect on the membrane potential, and flux measurements indicate a considerable net \( K^+ \) efflux from the root (\( -2.73 \) μmol g\(^{-1}\) h\(^{-1}\), inset in Fig. 2). These results are characteristic of a wound response in corn roots. This wound response following application of low levels of exogenous putrescine in the absence of extracellular calcium was only partially recoverable, despite three washes in 0.2 mM CaCl\(_2\) solution. After 50 min in the recovery solution, the membrane potential had nearly returned to its initial value presented in Figure 1, while the net flux of \( K^+ \) had only recovered by 39% (Fig. 2, inset).

**Effects of 5 mM Exogenous Putrescine on \( E_m \) and \( K^+ \) Fluxes**

From our initial experiments, the application of 0.5 mM putrescine appeared to injure corn root membranes in the absence of calcium but had no effect in the presence of calcium. We next conducted experiments in which intact roots were exposed to a 10-fold increase in exogenous putrescine (5 mM) in the presence of 0.2 mM CaCl\(_2\). This putrescine concentration is thought to approximate the level found under severe stress conditions (21, 33).

Initial measurements of \( E_m \), the \( K^+ \)-induced depolarization, and net \( K^+ \) flux were representative of a normal, healthy root. The introduction of 5.0 mM putrescine in the presence of calcium caused a rapid and dramatic depolarization of the membrane potential (Fig. 3). The subsequent addition of \( K^+ \) into the bathing medium after 5 min of putrescine treatment caused an initial 42 mV depolarization, followed by a more gradual depolarization, to \( -71 \) mV. It appeared that a significant portion of this depolarization was not associated with \( K^+ \), but is a continued effect of putrescine exposure. This is supported by the lack of a net \( K^+ \) influx measured in the presence of 5 mM putrescine (inset, Fig. 3). Removal of putrescine from the bathing solution resulted in only a partial repolarization of the membrane potential and a small recovery in net \( K^+ \) influx. An additional CaCl\(_2\) recovery treatment almost completely repolarized the membrane potential to its pretreatment value, but the subsequent net \( K^+ \) influx was only 40% of the control value.

These results suggest that high concentrations of putrescine may cause membrane injury. To determine whether putrescine-induced membrane damage is a transient effect, we exposed 4-d-old corn seedlings grown hydroponically in 0.2 mM CaSO\(_4\) to an additional 24 h treatment in CaSO\(_4\) solution with either 0.5 or 5.0 mM putrescine. The corn seedlings grown in 0.5 mM putrescine exhibited normal values for root membrane potential, \( K^+ \)-induced depolarization, and net \( K^+ \) influx (Table II). However, at the higher concentration of...


Figure 3. Time course of the response of the cortical cell membrane potential and net K⁺ flux in intact low salt-grown corn roots to exposure to high concentrations (5 mM) of putrescine in the presence of 0.2 mM CaCl₂, and to the subsequent removal of putrescine from the experimental solution. The net K⁺ fluxes were measured at time points A, B, C, and D on the figure and the flux values are presented in the inset.

Table II. Effect of Two Concentrations of Exogenous Putrescine on Membrane Potential, K⁺-induced Depolarization, and K⁺ Fluxes in Intact Roots of Corn Seedlings

<table>
<thead>
<tr>
<th>Putrescine Treatment</th>
<th>Initial E, mV</th>
<th>K⁺-induced Depolarization, mV</th>
<th>K⁺ Flux, μmol g⁻¹ h⁻¹ ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>-158</td>
<td>-67</td>
<td>1.97 ± 0.29</td>
</tr>
<tr>
<td>5.0</td>
<td>-30</td>
<td>0</td>
<td>-2.00 ± 0.19</td>
</tr>
</tbody>
</table>

Exogenous putrescine, the resting membrane potential was dramatically reduced, and reached a maximum of only -30 mV. The addition of K⁺ resulted in a large net K⁺ efflux (-2.00 μmol g⁻¹ h⁻¹). The phytoxic effect of 5.0 mM putrescine became clearly visible after 48 h of exposure, as the roots appeared flaccid and translucent.

Ameliorating Effects of Ascorbate

In light of the proposed catabolism of putrescine by a cell wall-specific diamine oxidase (8), we considered the possibility that putrescine caused membrane damage as a result of an excessive production of hydrogen peroxide and subsequent formation of free radicals in the cell wall solution adjacent to the root cell plasmalemma. Thus, we hypothesized that a general free radical scavenger, such as ascorbic acid, might protect membranes against putrescine damage.

To test this hypothesis, we pretreated intact corn roots with 0.5 mM ascorbate in addition to 0.2 mM CaCl₂. The subsequent addition of 5.0 mM putrescine in the presence of ascorbate resulted in an initial 48 mV depolarization (Fig. 4). Twelve min of exposure caused no additional depolarization of the membrane potential. Potassium uptake was measured in the presence of putrescine and ascorbate and was found to be slightly higher than the control value measured in the absence of putrescine (inset, Fig. 4). Removal of both K⁺ and putrescine from the bathing solutions resulted in a repolarization of the membrane potential to its initial value and a subsequent reintroduction of K⁺ elicited a depolarization and net K⁺ influx typical of normal, healthy corn roots. These results indicate that a pretreatment with ascorbate protected corn root membranes against wound damage caused by high concentrations of exogenous putrescine.

Influence of Ascorbate on Putrescine-Induced Membrane Injury in the Absence of Ca²⁺

Experiments were performed to determine if ascorbate similarly protected corn root membranes against damage caused by low levels of putrescine in the absence of calcium (Fig. 5; Table III). The initial K⁺-induced depolarization (-51 mV) and net K⁺ influx (1.35 μmol g⁻¹ h⁻¹) values in 0.5 mM ascorbate, 0.2 mM CaCl₂, and 50 μM KCl were similar to those presented in Figure 4. Removal of K⁺ from the medium resulted in a repolarization of the membrane potential to its initial value of -165 mV. This membrane potential represents the starting conditions of Figure 5. The addition of 1 mM EGTA concomitant with the removal of CaCl₂ from the bathing medium elicited only a slight change in the membrane potential after 17 min of EGTA exposure. However, net K⁺ flux measurements following the introduction of KCl indicate a 44% reduction (0.76 μmol g⁻¹ h⁻¹; inset, Fig. 5; Table III) from that of the initial condition. It should be noted that the inhibitory effect of calcium removal on net K⁺ flux was greater (87% inhibition) when corn roots were not pretreated with ascorbate (Fig. 1).

Figure 4. Time course of the influence of 0.5 mM l-ascorbic acid (abbreviated as asc in the figure) on the response of the cortical cell membrane potential and net K⁺ flux in intact low salt-grown corn roots to exposure to 5 mM putrescine in the presence of 0.2 mM CaCl₂, and to the subsequent removal of putrescine from the experimental solution. The net K⁺ fluxes were measured at time points A, B, and C on the figure and the flux values are presented in the inset.
Figure 5. Time course of the influence of 0.5 mM L-ascorbic acid (abbreviated as asc in the figure) on the response of the cortical cell membrane potential and net K⁺ flux in intact low salt-grown corn roots to exposure to 0.5 mM putrescine in the absence of calcium (+1 mM EGTA), and to the subsequent removal of first, putrescine, and second, EGTA (+0.2 mM CaCl₂). The net K⁺ fluxes were measured at time points A, B, C, and D on the figure and the flux values are presented in the inset.

The responses of the membrane potential and net K⁺ flux to 0.5 mM putrescine clearly show that ascorbate does not protect corn root membranes against putrescine-induced injury in the absence of extracellular calcium. During the course of a 24 min exposure to putrescine, the membrane potential gradually depolarized to −86 mV (Fig. 5). Furthermore, K⁺ introduction did not elicit further membrane depolarization and caused a net K⁺ efflux of −2.85 μmol g⁻¹ h⁻¹. Subsequently, putrescine was removed from the chamber and the bathing solution was changed three times to determine whether the wounding effects of putrescine could be reversed. The removal of putrescine, the reintroduction of calcium concurrent with the removal of EGTA, and finally the removal of ascorbate did not cause a significant repolarization of the membrane potential nor a recovery in net K⁺ influx (Fig. 5, inset).

DISCUSSION

Putrescine: A Calcium Substitute?

Polyamines have been frequently reported to have a stabilizing influence on membranes during periods of stress and have even been suggested to act, in part, as a calcium substitute (13). However, there are several points that should be made concerning much of the experimental evidence that has been presented in the literature in support of this role for polyamines. First, it has been well documented that increased levels of putrescine, a diamine, are associated with a wide range of plant stresses, while the same stress conditions are rarely coupled to a concurrent increase in triamines and tetraamines such as spermidine or spermine (for a review, see ref. 23). Yet experimental evidence that has been used in support of a membrane-protective role for polyamines is nearly always associated with the exogenous application of spermidine and spermine, but rarely with putrescine. Second, most of the experimental results demonstrating the stabilizing effects of polyamines have been conducted with wall-less experimental systems such as protoplasts and organelles (2, 17, 29). As we will suggest in the next section, research concerning polyamine interactions with plant cell membranes could yield strikingly different results depending on the nature of the experimental system (intact plant tissue versus plant systems lacking cell walls).

In the research presented here, we exposed intact corn roots to 0.5 mM exogenous putrescine in the presence and absence of calcium. This level of exogenous putrescine should simulate a moderate stress condition, particularly if symplasmically derived polyamines can easily cross the plasmalemma, as has been suggested in the literature (9, 16). In the presence of calcium, 0.5 mM putrescine had a slight stimulatory effect on

Table III. Effect of Putrescine on K⁺-Induced Depolarization and Fluxes in the Presence of Ascorbic Acid and in the Absence of Calcium

Seedlings were grown hydroponically for 5 d in aerated 0.2 mM CaSO₄ solution. All solutions used in chamber contained 5 mM Mes-Tris buffer at pH 6. Solutions with calcium or potassium contained 0.2 mM CaCl₂ or 50 μM KCl, respectively. In treatment II, all solutions contained 0.5 mM ascorbate. Experiments were conducted three times with similar results.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K⁺-Induced Depolarization</th>
<th>K⁺ Flux</th>
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<tbody>
<tr>
<td></td>
<td>% of control</td>
<td></td>
</tr>
<tr>
<td>I.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺ (control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+0.5 mM PUT</td>
<td>96</td>
<td>109</td>
</tr>
<tr>
<td>+5 mM PUT</td>
<td>65</td>
<td>Efflux</td>
</tr>
<tr>
<td>−5 mM PUT (recovery)</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td>II.</td>
<td></td>
<td></td>
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<tr>
<td>Ca²⁺ + 0.5 mM ascorbate (control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+5 mM PUT</td>
<td>92</td>
<td>122</td>
</tr>
<tr>
<td>−PUT</td>
<td>88</td>
<td>116</td>
</tr>
<tr>
<td>+1 mM EGTA − Ca²⁺</td>
<td>65</td>
<td>56</td>
</tr>
<tr>
<td>+1 mM EGTA + 0.5 mM PUT − Ca²⁺</td>
<td>0</td>
<td>Efflux</td>
</tr>
<tr>
<td>+1 mM EGTA − PUT − Ca²⁺ (recovery)</td>
<td>0</td>
<td>Efflux</td>
</tr>
<tr>
<td>−EGTA + Ca²⁺ (recovery)</td>
<td>0</td>
<td>Efflux</td>
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the net flux of K⁺ (Table III). However, when calcium ions were chelated from the root tissue with EGTA, exposure to 0.5 mM external putrescine resulted in a depolarization of the $E_m$, reduction in the K⁺-induced depolarization of $E_m$, and a dramatic alteration in K⁺ uptake from influx to a substantial K⁺ efflux (Fig. 2). This evidence strongly suggests that putrescine does not replace calcium in maintaining membrane stability and, in fact, induces a wound response similar to that observed following other forms of stress.

**Putrescine Phytotoxicity: A Possible Mechanism for Membrane Damage**

Plant tissues exposed to severe stress have been reported to accumulate extremely high concentrations of putrescine. For example, Smith (21) found putrescine concentrations of 3380 nmol/g fresh weight in K⁺-deficient corn leaves. In addition, Young and Galston (33) measured putrescine levels greater than 7000 nmol/g fresh weight in shoots of K⁺-deficient oat seedlings, and in our own studies we have found putrescine levels to be as high as 4000 nmol/g fresh weight (13-fold increase) in roots of herbicide-treated pea seedlings (unpublished data). Because it has been demonstrated that putrescine readily moves across cell membranes (16), an exogenous application of 5 mM putrescine would appear to be a reasonable level to accumulate in the apoplasm under severe stress conditions.

The validity of these exogenous applications of polyamines has been questioned by some researchers. Roberts et al. (19) suggested that the effects of exogenously fed putrescine may not represent the true physiological effects of endogenous accumulation. Also, Smith (22) noted that high concentrations of exogenously applied putrescine might cause phytotoxicity by contacting the external surface of the plasmalemma normally inaccessible to subcellularly localized endogenous putrescine. However, these arguments would be applicable only if the plant cell plasmalemma is relatively impermeable to polyamines. Although Young and Galston (32) provide evidence supporting this hypothesis, in more recent studies, Pistocchi et al. (16) observed that a significant plasmalemma influx and efflux of putrescine could occur in cultured carrot (Daucus carota L.) cells. Additionally, studies by Friedman et al. (9) indicate that putrescine levels increased in the xylem exudate of salt stressed sunflower (Helianthus annuus L.) plants. Although this area awaits further research, it seems reasonable to speculate that a significant increase in the level of endogenous putrescine could cause an efflux of putrescine across the plasmalemma into the cell wall solution, resulting in a significant increase in apoplastic putrescine concentrations. Consequently, it is our contention that exogenously applied putrescine could cause similar plasma membrane effects to those ultimately arising from a high initial concentration of endogenous putrescine.

The results of short (Fig. 3; Table III) and long term (Table II) exposure of intact corn roots to 5 mM putrescine provide evidence for wounding at the plasmalemma. This was seen as a depolarization in the $E_m$ and a reduction in the K⁺-induced depolarization of $E_m$, as well as a stimulation of K⁺ efflux. These results are in contrast to several previous studies demonstrating a stabilizing effect of polyamines, particularly spermidine and spermine, on membranes. However, a large proportion of these studies in plants were conducted with experimental systems lacking cell walls, including protoplasts (2), chloroplasts (17), and phospholipid vesicles (29). Since di- and polyamine oxidase are found exclusively in the cell wall fraction of plants, these wall-less experimental systems would eliminate the possible interaction between the plasma membrane and products of polyamine metabolism. In contrast, much of the early evidence, conducted with intact plant tissues, implicates exogenous putrescine application to symptons of phytotoxicity (1, 18, 27). From these and our own studies we hypothesize that putrescine-mediated membrane wounding results from the production of hydrogen peroxide and free radicals through the activity of diamine oxidase in the cell wall. Although diamine oxidase has yet to be described from corn, its activity was demonstrated in other grass species, including rice (3) and oat (8). If a similar pathway exists in corn roots, it would be expected that preincubating with ascorbate, a nonspecific free radical scavenger, prior to introducing a high concentration of putrescine should protect corn root membranes against damage.

The results of our electrophysiological and flux studies support this hypothesis. The presence of 0.5 mM ascorbate in the bathing medium ameliorated the wounding effects of 5.0 mM putrescine (Fig. 4). Putrescine did not elicit an inhibitory effect on K⁺-induced depolarization of the $E_m$ and, in fact, caused a slight stimulation in the net influx of K⁺ (Table III). The characteristic putrescine-induced depolarization of the $E_m$ appears to be the only significant change caused by the diamine in the presence of ascorbate. The distinct similarities of the kinetics of the putrescine-induced depolarization of $E_m$ to those caused by K⁺ suggests that the polycation is rapidly transported across the plasma membrane of corn root cells.

In view of the ameliorating effects of an antioxidant on putrescine-mediated membrane damage, it might follow that a pretreatment with ascorbate would protect corn root membranes against the phytotoxic effect of low levels of exogenous putrescine in the absence of calcium. Our results, however, indicate that ascorbate does not protect corn root membranes in the absence of calcium (Fig. 5; Table III). In addition, neither $E_m$ nor K⁺ uptake recovered following the removal of putrescine, ascorbate, and EGTA, or after the addition of calcium back to the medium. It is difficult to explain the mechanism of putrescine-mediated wounding under these conditions, but it can be concluded that the role(s) that calcium plays in stabilizing and protecting biological membranes is probably more complex than simply a physical maintenance of membrane integrity.

**CONCLUSION**

Previous hypotheses have assigned calcium-like properties to polyamines that provide membrane protection in response to various plant stresses. However, we have found that in intact corn roots, putrescine does not substitute for calcium in maintaining membrane integrity. Indeed, it appears that the application of low levels of exogenous putrescine in the absence of calcium causes membrane damage, based on the measurements of root cell electrical properties and K⁺ transport as indicators of membrane integrity. Furthermore, simi-
lar wound responses were elicited when roots were exposed to higher levels (5 mM) of putrescine in the presence of calcium. We propose that the mechanism of putrescine-induced wounded involves the catabolism of the polyamine by a cell wall diamine oxidase, with the concomitant production of hydrogen peroxide and free radicals resulting in peroxidative damage of the plasmalemma. Because much of the previous work attributing a membrane stabilizing role to polyamines in plants was based on wall-less experimental systems, the deleterious aspects of cell wall polyamine catabolism to the plant cell plasma membrane would not have been observed and the potential for polyamine-induced membrane damage would have been missed.

LITERATURE CITED
34. Younes M, Weser U (1978) Involvement of superoxide in the catalytic cycle of diamine oxidase. Biochim Biophys Acta 526: 644–647