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Regulation of Cytoplasmic and Vacuolar pH in Maize Root Tips under Different Experimental Conditions[1]

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ABSTRACT

^{31}P-Nuclear magnetic resonance spectra of perfused maize (*Zea mays* L., hybrid WW x Br 38) root tips, obtained at 10-minute intervals over 12 hours or longer, indicate that no cytoplasmic or vacuolar pH changes occur in these cells in the presence of 25 millimolar K_2SO_4, which induces extrusion of 4 to 5 microequivalents H^+ per gram per hour. In contrast, hypoxia causes cytoplasmic acidification (0.3–0.6 pH unit) without a detectable change in vacuolar pH. The cytoplasm quickly returns to its original pH on reoxygenation. Dilute NH_4OH increases the vacuolar pH more than it does the cytoplasmic pH; after NH_4OH is removed, the vacuole recovers its original pH more slowly than does the cytoplasm. The results indicate that regulation of cytoplasmic pH and that of vacuolar pH in plant cells are separate processes.

There is much current interest in intracellular pH and its regulation in plants (22). The investigation of this subject has been hindered by the lack of suitable methods for measuring the pH of the two major intracellular compartments, the cytoplasm and vacuole (22). The most widely used method, using the weak acid [^{14}C]5,5,dimethyloxazolidine-2,4-dione (7, 15, 16, 24), is indirect and fraught with complications (*e.g.* Kurkdjian *et al.* [7–10]). Other methods of intracellular pH measurement, such as those using dyes, microelectrodes (22), and methylamine (9), have not been successfully applied to the cytoplasm of higher plant cells.

We have shown that the ^{31}P-NMR[2] method of intracellular pH determination (14, 18, 20) can be applied to maize root tips (17) and have presented evidence that the cytoplasmic and vacuolar pH of these cells remains essentially constant even during extreme rates of H^+ extrusion (19). Here, we extend these studies by an improved technique involving perfusion of the tissue within the NMR spectrometer, permitting us to obtain adequate NMR spectra within 10 min and to monitor the intracellular pH of maize root tips over periods of more than 12 h, during a sequence of treatments.

MATERIALS AND METHODS

Maize (*Zea mays* L.) hybrid WW x Br 38 (Customaize Research, Decatur, IL) were grown for 2 d in the dark. Root tips, 1.5 to 3 mm long, were cut with a razor blade, washed, and stored at 4°C in 50 mM glucose with 0.5 mM $CaSO_4$ until required. Root tips could be stored in this manner for more than 1 d without any diminution of their capacity for fusicoccin-induced H^+ extrusion or change in the appearance of the ^{31}P-NMR spectra obtained (*e.g.* in ATP levels or cytoplasmic pH). Approximately 8 g of tissue were placed in an 18-mm NMR tube, containing a coaxial capillary of 0.5 M methylene diphosphonic acid in Tris buffer (pH 8.9) as a reference compound for chemical shift measurements. Chemical shifts are expressed in ppm, that is $(\nu_s - \nu_{ref}) \times 10^6/\nu_{ref}$, where ν_s and ν_{ref} are the absolute resonance of a particular sample peak and the reference peak, respectively. After placing the tube in the NMR spectrometer, the tube was connected to reservoirs of O_2- or N_2-bubbled perfusion medium of the composition indicated in "Results" and figure legends; the bottom of the tube was connected to an aspirator pump via a flow meter (Gilmont Instruments, Great Neck, NY). The perfusion rate was 50 to 90 ml min^{-1}, except when indicated otherwise.

Spectra were obtained at room temperature at 40.5 MHz in a XL-100 spectrometer (Varian Associates, Palo Alto, CA) equipped with a specially built probe; a Nicolet Instrument Corporation (Madison, WI) 1180 computer was used. The 18-mm probe has ^{31}P and ^{1}H coils in a cross-coil configuration. The signal from sample water was used for optimizing the magnetic field homogeneity. D_2O, in an additional coil placed near the sample, was used for field-frequency locking.

RESULTS

Figure 1 shows ^{31}P-NMR spectra of maize root tips under perfusion by various media, whose effects wll be described below. Spectra A to E were obtained in 10 min and clearly show resonances assigned (17) to glucose-6-P (peak 1), cytoplasmic P_i (peak 2), and vacuolar P_i (peak 3). If spectral data are accumulated over a 30-min period, (Fig. 1F), other peaks to the right of peak 3 rise above noise levels; these are attributable to ATP, UDPG, and nicotinamide adenine nucleotides on the basis of chemical shifts (1) and from known concentrations of these compounds in plant tissues (17). Figures 2 to 4, in which the chemical shifts of peaks 1 to 3 and corresponding pH values are plotted against time, are derived from spectra such as those in Figure 1, A to E.

Effect of Hypoxia. When root tips are perfused with a deoxygenated (N_2-bubbled) medium, peaks 1 and 2 quickly move upfield, indicating acidification of the cytoplasm to a new stable pH value, 0.3 to 0.5 pH unit lower than control values (Figs. 2–4). Hypoxia has no apparent effect on vacuolar pH. This result was obtained when the perfusion rate was reduced to 5 to 10 ml/min (N_2-bubbled medium) or when the 50 ml/min perfusion rate was maintained using completely anoxic medium (medium kept under vacuum for 2 h prior to use and bubbled with N_2 during perfusion). If perfusion is stopped, the cytoplasmic pH quickly drops even more (a further 0.2–0.3 pH unit) (Fig. 2).

Figures 2 to 4 show that, on reoxygenation, cytoplasmic pH returns to its original value within 20 min. The similar response of

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[2] Abbreviation: NMR, nuclear magnetic resonance.

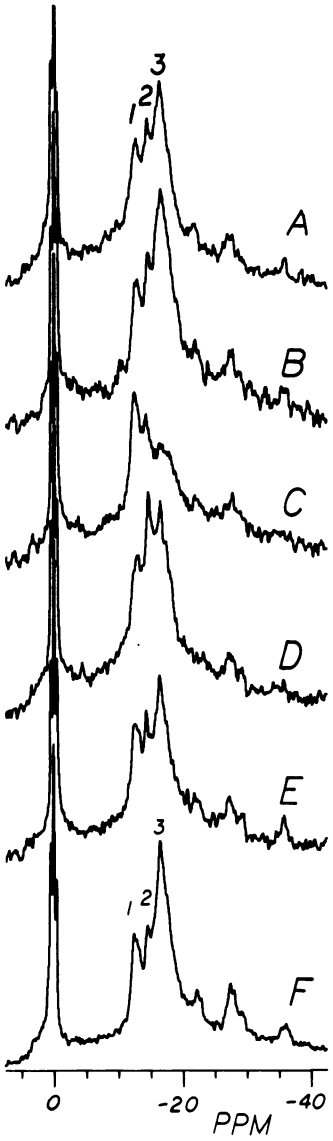

FIG. 1. ^{31}P-NMR spectra (40.5 MHz) of maize root tips perfused with the following: A, 50 mM glucose, 0.1 mM $CaSO_4$, 0.1 mM Mes brought to pH 6.15 with Tris base; B, same as A, except that 25 mM K_2SO_4 was added; C, 5 mM NH_4OH, 0.1 mM $CaSO_4$ (pH 9.95); D, same as A, except that solution was bubbled with N_2; E, same root tip sample as D but after reoxygenation. Spectra A to E were obtained in 10 min. Spectrum F was obtained in 30 min; the sample was perfused as in A.

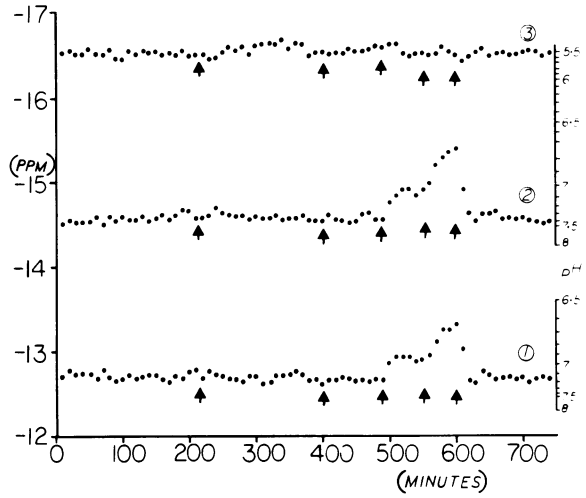

FIG. 2. Changes in ^{31}P-NMR chemical shifts and corresponding pH values under different ionic and oxygenation conditions. Root tips were perfused initially with O_2-bubbled 50 mM glucose, 0.1 mM $CaSO_4$, 0.1 mM Mes brought to pH 6.15 with Tris base, at a rate of 60 to 90 ml min^{-1}. At first arrow, perfusion was changed to include 25 mM K_2SO_4. At second arrow, return to original perfusion buffer. At third arrow, same medium, but bubbled with N_2 instead of O_2; and perfused at 10 ml min^{-1}. At fourth arrow, perfusion was stopped. At fifth arrow, perfusion with oxygenated medium resumed (60 ml min^{-1}). Spectra were each collected over 10 min.

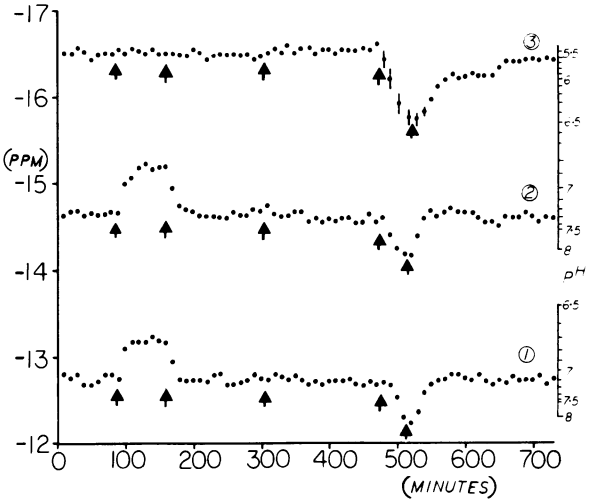

FIG. 3. Comparison of hypoxic and K_2SO_4 perfusions with effect of 10 mM NH_4OH. Root tips were perfused initially as at the start of Figure 2. At first arrow, perfusion with N_2-bubbled medium at 5 ml min^{-1}. At second arrow, original perfusion conditions resumed. At third arrow, same medium but also containing 25 mM K_2SO_4. At fourth arrow, 10 mM NH_4OH and 0.1 mM $CaSO_4$ (pH 10.1); this treatment broadens peak 3, as indicated by bar symbols (see text). At fifth arrow, original perfusion conditions resumed. Spectra were collected over 10 min.

peaks 1 and 2 to the treatment and the lack of shift in peak 3 further support the conclusion that the compounds represented by peaks 1 and 2 are in the same intracellular compartment, whereas the P_i represented by peak 3 is in a separate compartment (17).

Under hypoxia, peak 2 not only changes in chemical shift but also noticeably increases in intensity (Fig. 1D), indicating that cytoplasmic P_i levels increase, a phenomenon observed in bacteria (23) and animal cells (2, 12). This can be attributed, at least in part, to the decrease in ATP levels which occurs in maize root tips during hypoxia (21).

Effect of NH_4OH. As reported previously (17, 19), mM concentrations of NH_4OH increase the pH of boh intracellular compartments (Figs. 3 and 4). At 10 mM external NH_4OH, the cytoplasmic pH eventually increases approximately 0.7 to 0.8 pH unit, whereas the vacuolar pH increases approximately 1 pH unit; vacuolar pH increases much more rapidly than the cytoplasmic pH (Fig. 3). In 5 mM NH_4OH, the cytoplasmic pH increases only slightly (0.2–0.3 pH unit), whereas the vacuolar pH still increases noticeably (Fig. 4). On withdrawal of NH_4OH, the cytoplasm returns to its original pH much more rapidly (approximately 30 min) than the vacuole (approximately 120 min). The bars in Figures 3 and 4 for peak 3 during NH_4OH treatment indicate that this peak is broadened by NH_4OH, as can be seen from Figure 1C. Since a broadened peak is seen even after the vacuolar pH has ceased to change, this broadening means that the pH increase varies among different vacuoles. After removal from NH_4OH, peak 3 narrows toward its normal width, as vacuolar pH is restored to normal in all cells.

Response to H^+ Extrusion. Perfusion of root tips with 25 mM

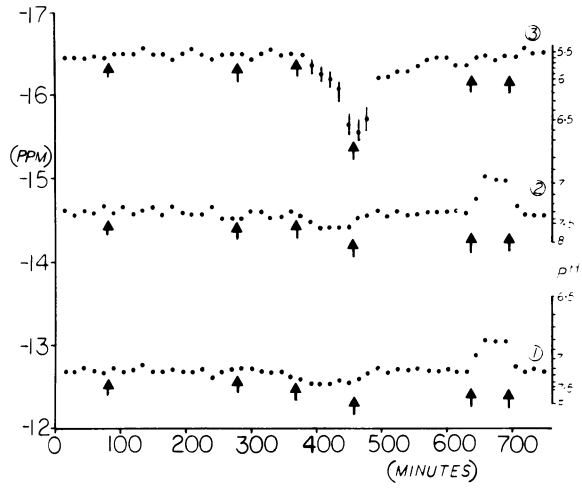

FIG. 4. Comparison of effect of 5 mM NH_4OH with that of hypoxia and of K_2SO_4. Root tips were perfused initially as at start of Figure 2. At first arrow, 25 mM K_2SO_4 was included in the medium. At second arrow, original perfusion buffer. At third arrow, 5 mM NH_4OH and 0.1 mM $CaSO_4$ (pH 9.95); this treatment broadens peak 3, as indicated by bars. At fourth arrow, initial perfusion conditions resumed. At fifth arrow, perfusion with N_2-bubbled buffer (5–10 ml min^{-1}). At sixth arrow, initial perfusion conditions resumed again. Spectra were each collected over 15 min.

Table I. *Effect of 25 mM K_2SO_4 on Chemical Shifts of Maize Root Tip ^{31}P Resonances*

Means and SE were computed from 100 spectra for each treatment from five separate experiments.

Treatment	Peak 1	Peak 2	Peak 3
		ppm	
Control	−12.682 ± 0.0070	−14.581 ± 0.0068	−16.511 ± 0.0069
K_2SO_4	−12.694 ± 0.0067	−14.584 ± 0.0080	−16.529 ± 0.0082

K_2SO_4, which induces strong H^+ extrusion (4–5 μEq g^{-1} h^{-1}) due to unbalanced ion uptake (6), does not change cytoplasmic or vacuolar ^{31}P chemical shifts (Figs. 2–4). This conclusion is reinforced by Table I, showing that the chemical shifts of peaks 1 to 3 are not affected by the presence of 25 mM K_2SO_4, within experimental error. This agrees with our earlier findings (19).

DISCUSSION

Accuracy of ^{31}P-NMR pH Measurements. To measure intracellular pH using ^{31}P-NMR, ^{31}P chemical shifts are compared with a titration curve of the phosphate compound in question (14). Uncertainties about the solute composition of cytoplasm and vacuole, especially total ionic strength and free Mg^{2+} concentration, lead to uncertainties in absolute pH values (18). The titration curve of P_i in undiluted maize root tip homogenates resembles that of P_i in 100 mM KCl and 2 mM $MgCl_2$ (18), but to apply the NMR method rigorously, one would have to know how Mg^{2+} and ionic strength are distributed between cytoplasm and vacuole. However, information on the influence of these factors on the titration curves (18) suggests that the curves for P_i and glucose-6-P obtained in a mixture of 100 mM KCl and 2 mM $MgCl_2$ (and for P_i in homogenates) give cytoplasmic pH measurements accurate to within approximately 0.2 pH unit. This problem of correcting for intracellular conditions other than pH applies to all methods of intracellular pH determination. For example, responses of pH electrodes vary with ionic strength and composition, and electrode design (25).

Because the slopes of phosphate NMR titration curves are similar in solutions of different composition (over the range of chemical shifts observed for peaks 1 and 2 *in vivo*), the ^{31}P-NMR method measures change in intracellular pH with greater accuracy than absolute pH values, assuming the intracellular concentrations of free ions does not change by a large factor. Titration of P_i and glucose-6-P in solutions of physiological ionic strength (50–200 mM KCl) and Mg^{2+} concentration (0–5 mM) indicate that for chemical shifts near −14.6 (P_i) and −12.7 (glucose-6-P), a change of 0.1 ppm corresponds to a change in pH of 0.12 or 0.16 ± 0.02 unit, respectively (19). This precision of ±0.02 pH unit is much greater than the precision of chemical shift measurement from spectra of root tips obtained in 10 min; sets of spectra of a given sample, taken sequentially, yield standard deviations of approximately ±0.05 ppm. Hence, we consider that cytoplasmic pH changes of 0.1 pH unit or more can be reliably detected with our current experimental arrangement. The use of two cytoplasmic indicators (P_i and glucose-6-P) with closely matching behavior (Figs. 2–4) further increases the certainty of the results. The vacuolar P_i resonance, on the other hand, is situated in a part of the P_i titration curve at which chemical shift is less sensitive to pH, as indicated by the pH scales in Figures 2 to 4, so vacuolar pH changes can be measured with less precision.

Features of Intracellular Compartmentation. Besides being able to measure the average pH of different compartments, the ^{31}P-NMR method also gives an indication, from the width of the resonances, of the pH variation among the cells in a sample, for a given type of intracellular compartment. For example, if the pH variation among the vacuoles in a tissue increases, the width of the vacuolar P_i resonance will increase, as we observed during NH_4OH treatment (Fig. 1C). Even under normal conditions there seems to be appreciable pH variation among vacuoles in maize root tips, because the vacuolar P_i resonance is broader than the cytoplasmic resonance (Fig. 1). The vacuolar P_i resonance in more highly vacuolate tissue, such as etiolated pea stems, is no broader than the cytoplasmic P_i resonance (results not shown), indicating that a broad P_i peak is not an inherent property of vacuoles. Perhaps the process of vacuole formation and growth in and behind the root meristem involves a gradual increase in the pH difference between cytoplasm and vacuole. Vacuoles with different internal pH values might correspond to the different classes of vacuoles isolated from maize root tips (13), which were thought to represent different developmental stages of this organelle.

The linewidth of the cytoplasmic P_i resonance (15 Hz) is broader than that of P_i in simple solution. As has been suggested in studies of skeletal muscle (5), that this may reflect variation in cytoplasmic pH between cells in the tissue. However, there are other possible explanations for this feature (20).

Changes in Intracellular pH. Although Kurkdjian *et al.* (10) recently reported evidence that vacuolar pH in sycamore cells increases after more than 1 h of fusicoccin-induced H^+ extrusion, present results confirm our earlier findings (19) that, at least for the first 2 h, neither vacuolar nor cytoplasmic pH increases significantly during rapid H^+ extrusion by maize root tip cells. As previously noted (19), this seems to rule out the conventional 'biochemical pH-stat' explanation of pH regulation (22) according to which H^+ extrusion should cause an increase in cytoplasmic pH which would stimulate organic acid production, compensating for the H^+ efflux and preventing further increase in intracellular pH. The cytoplasmic pH does not increase even transiently, as indicated by NMR spectra recorded during the first 10 min of induced H^+ extrusion; a transient pH increase in the first few min of H^+ extrusion would lead to a broadening of the cytoplasmic resonances, which was not observed.

In contrast to H^+ extrusion, present results reveal certain conditions that cause significant perturbations of maize root tip intracellular pH. During anaerobiosis, the cytoplasm acidifies

(Fig. 2). This may be because lactic acid accumulates; hypoxia-induced lactate accumulation has been found in maize seedlings (11) and many other species (3). Figures 2 to 5 show that when root tips are perfused with hypoxic medium the cytoplasmic pH decreases rapidly, then stabilizes at a new value 0.3 to 0.5 pH unit lower. This agrees with the hypothesis (4) that under anaerobiosis, a transient lactic fermentation occurs until this acidifies the cytoplasm enough to activate pyruvate decarboxylase, thus bringing on a steady-state of mainly ethanol production. When perfusion is stopped, the cytoplasmic pH decreases further. A possible explanation is impaired escape of the CO_2 formed by alcoholic fermentation, H_2CO_3 readily acidifying solutions of pH greater than 6 (such as cytoplasm). During anaerobiosis, vacuolar pH apparently does not change (Fig. 2–5), H^+ accumulating in the cytoplasm evidently not being transferred to the vacuole. However, since our measurements of vacuolar pH are not as precise as those of cytoplasmic pH (discussed above), we cannot rule out small changes in vacuolar pH (<0.2 pH unit); certainly vacuolar pH does not change nearly as much as cytoplasmic pH.

When root tips are perfused with dilute NH_4OH solutions, the vacuolar pH increases much more rapidly and to a greater final extent than the cytoplasmic pH (Figs. 3 and 4). This difference may be due to tighter pH regulation in the cytoplasm than in the vacuole, as suggested previously (17), or may simply result from the vacuole, because it is more acidic than the cytoplasm acting as a better trap for NH_4OH. However, the more rapid return to control values of cytoplasmic compared with vacuolar pH suggests tighter cytoplasmic pH regulation.

Because the experiments with NH_4OH and hypoxia described here show that cytoplasmic and vacuolar pH can vary independently and to different extents, and that they can return to normal values at different rates after perturbation, we conclude that cytoplasmic and vacuolar pH in plant cells are regulated, at least in part, by separate mechanisms.

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