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Transport of phosphocholine in higher plant cells: ^{31}P nuclear magnetic resonance studies

(choline transport/phosphate transport/phosphocholine phosphatase/cell wall/plasma membrane)

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ABSTRACT **Phosphocholine (PC) is an abundant primary form of organic phosphate that is transported in plant xylem sap. Addition of PC to the perfusate of compressed P_i-starved sycamore cells monitored by ^{31}P NMR spectroscopy resulted in an accumulation of PC and all the other phosphate esters in the cytoplasmic compartment. Addition of hemicholinium-3, an inhibitor of choline uptake, to the perfusate inhibited PC accumulation but not inorganic phosphate (P_i). When the P_i-starved cells were perfused with a medium containing either P_i or PC, the resulting P_i distribution in the cell was the same. Addition of choline instead of PC to the perfusate of compressed cells resulted in an accumulation of PC in the cytoplasmic compartment from choline kinase activity. In addition, PC phosphatase activity has been discovered associated with the cell wall. These results indicate that PC was rapidly hydrolyzed outside the cell and that choline and P_i entered the cytosolic compartment where choline kinase re-forms PC.**

Phosphocholine (PC) was identified as a major phosphorylated component of xylem exudate of plants (1, 2). The presence of considerable amounts of PC in the xylem vessels of various plants had raised the possibility that it was an organic phosphate ester for phosphate and nitrogen transport and that a variable proportion of this phosphorylated material could be utilized in the developing plant at a later period (2, 3).

The purpose of these investigations was to characterize an efficient mechanism for trapping PC within sycamore (*Acer pseudoplatanus*) cells. These cells were derived from the cambium, a tissue that is in close contact with the xylem vessels. The results demonstrate that PC was first hydrolyzed outside the cell and that choline thus formed entered the cytosolic compartment where choline kinase reconverted it to PC (4).

MATERIALS AND METHODS

Plant Material. Sycamore cells were cultured as a suspension in a liquid medium (5) except that Mn^{2+} was excluded to prevent excessive broadening of the vacuolar ^{31}P orthophosphate resonance. Cell suspensions were maintained in exponential growth by subculture every 3 days. P_i-starved cells were obtained as described (6). When P_i was omitted from the nutrient solution, the endogenous P_i pool decreased to 10% of the control within 72 hr (6).

Protoplasts were prepared from sycamore cells as described (4).

^{31}P NMR Experiments. ^{31}P NMR spectra of sycamore cells were obtained with a Bruker WM 200 WB spectrometer operating in the pulsed Fourier transform mode at 81 MHz. The spectra were obtained with compressed cells (4 cm tall; 9 g fresh weight) placed in a 25-mm tube under constant perfusion. Details of this assembly and its operation have been described (7). The perfusate consisted of the culture medium devoid of P_i and Mn^{2+} and was adjusted to pH 5.5. Sycamore cells slightly compressed between two circular polymer filters (a means of cramming more cells into the NMR chamber) can survive several days, as long as a well aerated nutrient medium is pumped through the system under slight pressure (7). Assignments of the nucleotides, P_i (cytoplasm and vacuole, respectively), and phosphate esters, including PC to specific peaks, were made in accordance with those given previously (4, 7) and from the spectra of the perchloric extracts, which contained the soluble low molecular weight constituents (4, 7).

PC Hydrolysis by Intact Cells. PC phosphatase activity was assayed by measuring the formation of P_i from PC. Midlogarithmic phase cells were collected on a fiberglass filter and rapidly rinsed three times with 50-ml aliquots of P_i-free culture medium. Three rinses were sufficient to remove >99% of the extracellular P_i. Washed cells were harvested by filtration, resuspended in sterile P_i-free culture medium containing either 50 mM Mes/NaOH or 50 mM Mops/NaOH at various pH values (pH 5–7.5) and incubated at 25°C with constant shaking. The cell density was 100 mg fresh weight/ml. After preincubation for 2 hr, PC was added. At various time intervals, 1-ml portions were rapidly removed and centrifuged for 2 min at 7000 rpm (Kubota KN-70; HS-4 rotor). A fraction of the clear supernatant was used for the colorimetric determination of P_i (8). The contribution of free P_i released from broken cells was taken into consideration for determination of rates of PC hydrolysis to choline and P_i. We have verified that identical results were obtained by following formation of free choline from PC. In this case, choline was measured by a spectrophotometric adaptation of a fluorimetric assay (9).

RESULTS

Accumulation of PC in Sycamore Cells. P_i-starved cells were maintained for 26 hr in a continuously oxygenated circulating medium (P_i-free nutrient medium) at pH 5.5. Figs. 1 and 2 illustrate the changes that occurred in the P_i-starved cells when PC (200 μmol) was added to the perfusion medium (final vol, 1 liter). Total acquisition times of 60 min were used. Addition of PC in the circulating medium led to a marked increase in the resonance at 3.3 ppm (peak b), which was almost entirely attributable to PC, as confirmed by ^{31}P NMR studies of perchloric extracts (7). The NMR titration curve of PC indicated that the position of peak b corresponds to PC above pH 7, and it is considered to be intracellular PC in the cytoplasmic compartment (pH 7.5) and not in the vacuole

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Abbreviation: PC, phosphocholine.

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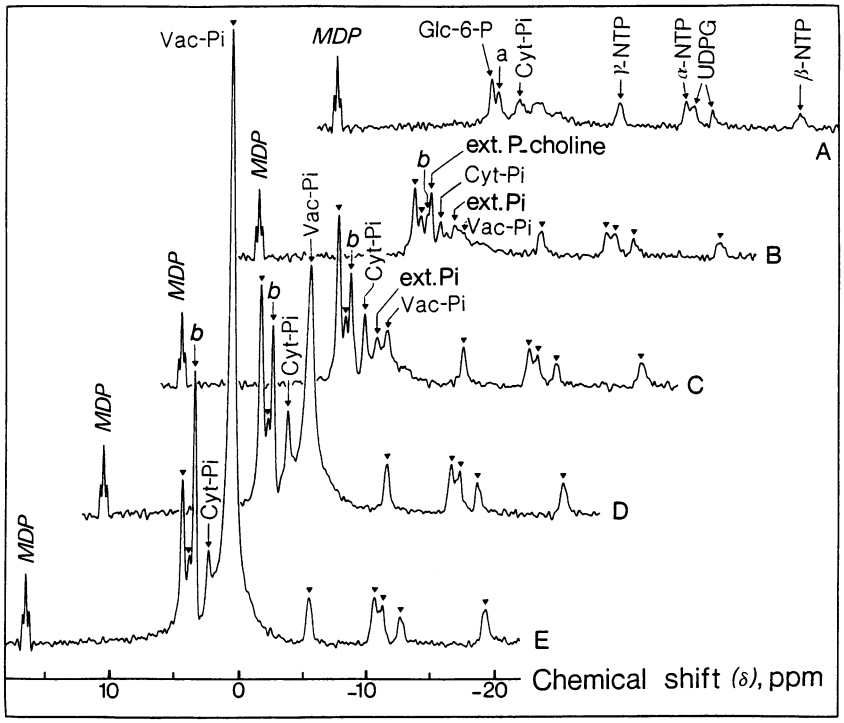

FIG. 1. Representative ^{31}P NMR spectra (81 MHz) of sycamore cells after addition of 200 μmol of PC in the perfusing culture medium. The spectra recorded at 25°C, with a 45° pulse angle, were the result of 6000 transients with a repetition time of 0.6 s (1 hr). Trace A, spectrum of P_i-starved cells; traces B–E, spectra obtained after 1, 4, 6, and 10 hr of perfusion of the compressed cells with a 1-liter culture medium (pH 5.5) devoid of P_i but containing 200 μmol of PC. Glc-6-P, glucose 6-phosphate; Vac-Pi, vacuolar P_i; Cyt-Pi, cytoplasmic P_i; NTP, nucleotide triphosphate (mainly ATP and UTP); UDPG, uridine 5′-diphosphate-D-glucose; b, position of PC. The reference capillary contained ≈1 μmol of methylene diphosphonate (MDP) in ^2H_2O at pH 8.9. Note the steady accumulation of PC and vacuolar P_i with time. Note also the rapid appearance of external P_i (ext.Pi) in the perfusion medium, indicating that PC was rapidly hydrolyzed outside the cell to form choline and P_i.

(pH 5.5). With our experimental conditions and assuming a vacuolar volume to cytoplasmic volume ratio of 5, the final cytoplasmic PC concentration attained exceeded 10 mM. The addition of PC to the circulating medium also caused a sharp increase in the nucleotide, cytoplasmic P_i, and glucose 6-phosphate signals. Furthermore, after a lag phase, P_i accumulated continuously in the vacuole. Thus, it was unclear whether PC was rapidly taken up by the cells or whether it was initially hydrolyzed outside the cell to form choline and P_i. When the P_i-starved cells were perfused with a medium containing either P_i (200 μmol) (Fig. 3) or PC (200 μmol) (Fig. 2), the resulting P_i distribution was the same. Moreover, addition of choline to the perfusate of compressed sycamore cells instead of PC resulted in a marked accumulation of PC in the cytoplasmic compartment probably because of phosphorylation by choline kinase (4). Furthermore, addition of PC to the circulating medium led to the appearance of free P_i outside the cells (Fig. 1, ext.Pi). These data indicate that PC was rapidly hydrolyzed outside the cell by a PC phosphatase and that P_i and choline thus formed entered the cell via specific carriers, exhibiting a high affinity for their respective substrates. When the initial rates of choline uptake were plotted as a double-reciprocal plot, the apparent K_m was ≈2 μM and the V_{max} was 240 nmol of choline incorporated per hr per g fresh weight at 25°C (4).

To verify this hypothesis, we have examined the uptake of PC in sycamore cells in the presence of 1 mM hemicholinium-3, an inhibitor of choline uptake by the cells, and choline kinase (4, 10, 11) (Fig. 4). Hemicholinium-3 blocked the accumulation of PC in the cells. In contrast, under the same conditions, the initial rate of P_i uptake was almost unaffected, indicating that hemicholinium-3 did not prevent PC hydrolysis. Once all the added PC had been depleted (data not shown), the incorporation of P_i into the cells stopped completely. If the cells were carefully washed for 2 hr with a fresh perfusion medium to eliminate hemicholinium-3, a second addition of PC (20 μmol) to the hemicholinium-free perfusate triggered the accumulation of PC in the cells and restored the rate of P_i accumulation (Fig. 4). These experiments confirm that PC was hydrolyzed outside the cell by a phosphatase probably localized in the cell wall.

Characterization of PC Phosphatase. Addition of PC to sycamore cells resulted in a rapid accumulation of P_i outside the cells (see Fig. 1, ext.Pi). In these experiments, PC phosphatase activity of washed sycamore cells (P_i starved) was calculated to be 15–22 μmol of P_i or choline produced per hr per g fresh weight at 25°C and at pH 5.5. The hydrolysis reaction had an apparent K_m of ≈100 μM (average value from five experiments). The activity was also determined as a function of pH by buffering the reaction mixture with either Mes/NaOH or Mops/NaOH from pH 5 to pH 7.5. The optimum pH range was quite sharp from pH 5 to pH 5.5. Accumulated choline in the medium (up to 5 mM choline) did not inhibit PC hydrolysis.

When protoplasts were used instead of intact cells, the rate of PC hydrolysis declined 200-fold (Table 1). Gentle rupture of intact protoplasts by passage through a fine nylon mesh followed by centrifugation produced a supernatant and a pellet that contained almost no PC phosphatase activity (Table 1). These results suggest that sycamore cells have a very powerful PC phosphatase activity associated with the cell wall. We have also observed that the extracellular PC phosphatase activity increased by 2–3 times during development of P_i starvation.

DISCUSSION

These observations indicate that PC was hydrolyzed outside the cell prior to transport through the cell membrane. There are several arguments in favor of this conclusion. First,

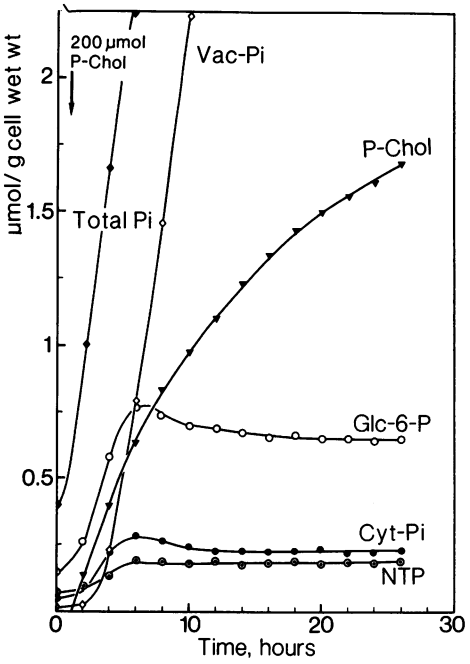

FIG. 2. Time course for appearance of the most abundant ^{31}P NMR detectable phosphorus compounds in P_i-starved cells perfused with a culture medium devoid of P_i and containing 200 μmol of PC. The concentrations of mobile phosphorus compounds (Vac-Pi, vacuolar P_i; P-Chol, PC; Glc-6-P, glucose 6-phosphate; Cyt-Pi, cytoplasmic P_i; NTP, nucleotide triphosphate) in the cell sample (average over the total sample within the detector) were determined as described (4, 7). Note the almost immediate synthesis of glucose 6-phosphate and PC compared to the 5 hr delay in the increase of the vacuolar P_i.

addition of PC to the perfusate of compressed P_i-starved cells resulted in a rapid accumulation of PC in the cytoplasmic

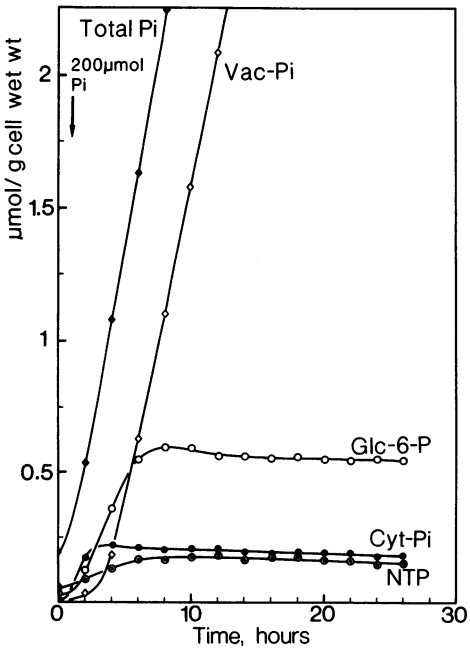

FIG. 3. Time course evolution for appearance of the most abundant ^{31}P NMR-detectable phosphorus compounds in P_i-starved sycamore cells perfused with a culture medium containing 200 μmol of P_i. Experimental conditions were the same as for Fig. 2. Note the almost immediate accumulation of cytoplasmic P_i and glucose 6-phosphate compared to the 3 hr delayed increase of the vacuolar P_i. Abbreviations are the same as in Fig. 2.

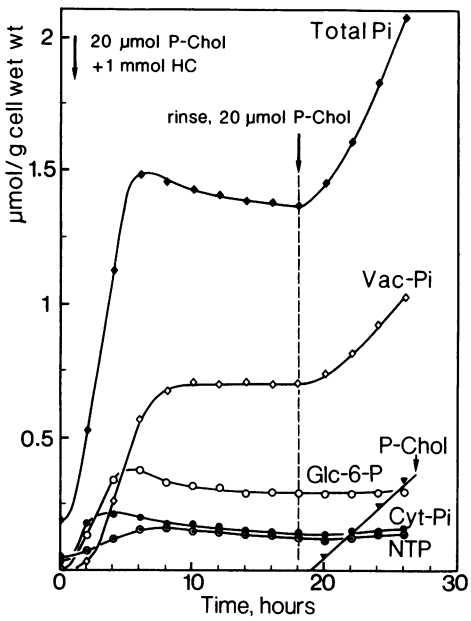

FIG. 4. Effect of hemicholinium-3 on the time course evolution of the most abundant ^{31}P NMR-detectable phosphorus compounds in P_i-starved sycamore cells perfused with a culture medium devoid of P_i and containing a limiting amount (20 μmol) of PC. The concentrations of mobile phosphorus compounds (see Fig. 2) in the cell sample were determined as described (4, 7). At arrow, compressed cells were carefully washed for 2 hr with a fresh perfusion medium to eliminate hemicholinium-3 (rinse) before a second addition of PC (20 μmol). Note that hemicholinium-3 inhibited the accumulation of PC in the cells without affecting that of P_i. Abbreviations are the same as in Fig. 2.

compartment accompanied by an increase in all the other phosphate ester pools. Second, a transport system for choline uptake has been described in plant cells based on the observations that it was saturable (4, 25) and that its activity was inhibited by hemicholinium-3 (4). Third, the addition of hemicholinium-3 to the perfusate strongly inhibited PC accumulation without affecting that of P_i. Fourth, a powerful PC phosphatase activity was found almost exclusively outside the cell, probably associated with the cell wall. Finally, preliminary experiments carried out in this laboratory indicate that when sycamore protoplasts devoid of PC phosphatase are fed with PC instead of choline, no accumulation of PC in the cytoplasm compartment was observed. These results indicate that PC was rapidly hydrolyzed outside the cell and choline thus formed entered the cytosolic compartment where a choline kinase would convert choline to PC within the cell. The V_{max} of PC hydrolysis (22 μmol of P_i produced per hr per g fresh weight) was much higher than that of choline transport into the intact cells (240 nmol of choline incorporated per hr per g fresh weight) (4). This result indicates that at high external PC concentrations there would be no quantitative connection between the activity of exter-

Table 1. PC phosphatase activity in sycamore cells (intact cells) and their protoplasts (intact or broken)

Cell compartment	μmol per hr per g fresh weight
Intact cells	20
Intact protoplasts	0.1
Broken protoplasts	0.2

PC phosphatase was measured. Gentle rupture of protoplasts (broken protoplasts) was obtained after passage of protoplasts through a fine nylon mesh (pore size, 20 μm) (4). These data are from a representative experiment and have been reproduced five times.

nally accessible PC phosphatase and the rate of choline influx. However, at low external PC concentrations (<5 μM), the rate of choline transport would become higher than that of PC hydrolysis. Choline released by the PC phosphatase in the cell wall would be in the right location to be transported into the cell and used for PC synthesis. The low half-saturation of choline transport (the apparent K_m was 2–4 μM) (4) would make possible a very active system, in conjunction with the cytosolic choline kinase, to act as a drain for sequestering choline from its site of formation.

Since choline is not required for the normal growth of sycamore cells (5), the physiological significance of the presence of a system to trap externally added PC within the cell cytoplasm remains an open question. In contrast, several animal tissues or cells are dependent on a supply of choline from the bloodstream (12) or the anaerobic growth of protozoon *Entodinium caudatum* depends on the availability of choline (13). However, many plants accumulate large amounts of PC in their xylem sap (1–3, 14). Martin and Tolbert (3) reported that PC was formed in the roots and transported to the leaves, where it was metabolized. Using sycamore cells after a long period of sucrose starvation, Roby *et al.* (7) and Dorne *et al.* (15) found that the fatty acids derived from membrane polar lipids such as phosphatidylcholine were utilized for respiratory purposes, whereas cytoplasmic PC increased symmetrically and was not further metabolized. After a long period of sucrose starvation, the final cytoplasmic PC concentration attained was considerable and PC did not significantly leak out of the cells. Tolbert's group (14) also suggested that many plants accumulate large amounts of PC in their xylem sap during starvation in long periods of storage in the dark. Thus, during the course of leaf senescence PC molecules, derived from phosphatidylcholine degradation, may escape out of the cell and be exported toward the xylem vessels where they accumulate, as has been shown for various amino acids such as asparagine (16). Under these conditions, PC would be transported in the xylem fluids to meristematic cells or fast-growing tissues where it could be used as a source of choline for membrane proliferation or betaine synthesis (17).

From this study, intact sycamore cells contain a functionally identifiable acid phosphatase involved in the rapid hydrolysis of PC. The question that arises is whether this activity is attributable to the "nonspecific" acid phosphatase present in the cell wall (18–21). Extracellular acid phosphatases have been reported to vary (22–24) and the activity of the extracellular PC phosphatase per unit weight of sycamore cells also increases during the development of P_i deficiency.

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