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Furosemide: a Specific Inhibitor of P_i Transport across the Plasma Membrane of Plant Cells

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A search was made for inhibitors of P_i uptake that act directly on the P_i transporter in the plasma membranes of *Catharanthus roseus* cells to inhibit P_i uptake without inhibition of proton pumping. Using standard electrodes, we monitored changes in pH and in the concentration of K^+ ions, as well as the rate of P_i uptake, when an inhibitor to be tested was applied to the cells in unbuffered medium. A9C (28 μM), a blocker of anion channels, inhibited P_i uptake but it also inhibited the proton pump. However, a structurally similar inhibitor, furosemide, inhibited P_i uptake without inhibiting proton pumping.

It is suggested that the carboxylic group of these inhibitors interacts with the P_i-binding site (probably an amino group) of the P_i transporter in the plasma membrane and that the hydrophobic structure of these inhibitors facilitates their accumulation in the plasma membrane.

Key words: A9C — *Catharanthus roseus* — Furosemide — P_i transport — Proton pump.

In plants, the energy for the uptake of P_i is provided by a proton-cotransport mechanism whereby a proton-motive force is generated by the proton pump in the plasma membrane (Ullrich-Eberius et al. 1981, 1984, Sentenac and Grignon 1985, Sakano 1990). To understand the mechanism of such transport and its genetic regulation, the transporter protein(s) must be isolated and characterized. Among the P_i transporters in plants, only the P_i translocator of chloroplasts has been isolated and characterized, with cloning of its cDNA from two plant species (Flügge et al. 1989, Willey et al. 1991). In these earlier studies, an inhibitor of the chloroplast translocator, DIDS, played an important role as a molecular probe that labeled the translocator protein.

In the case of other P_i transporters of plants, such as P_i transporter of plasma membranes, there are no reports of either the isolation or the characterization of such proteins, probably because no specific molecular probe (inhibitor, activator, etc) has been identified with which to label the P_i transporter. Among compounds that inhibit the uptake of P_i in plant tissues and cells (Lin 1981, Furihata et al. 1992), none has been characterized in its mode of action. It remains to be determined whether such compounds act directly on the P_i transporter or indirectly via inhibition of other systems, such as the proton pump. DIDS, an effective inhibitor of the P_i translocator chloroplsts (Rumpho and Edward 1985, Gross et al. 1990) did not inhibit the P_i transporter of the plasma membrane (Lin 1981). Clearly, a specific probe for each transporter protein is essential for the isolation and characterization of P_i transporters.

Sakano (1990) demonstrated that P_i uptake by *Catharanthus roseus* cells proceeds through a proton-cotransport mechanism: addition of small amounts of P_i to a suspension of cells in an unbuffered medium induced rapid alkalinization of the medium. As soon as the P_i in the external medium was exhausted, the alkalinization ceased and an even more rapid reacidification occurred. Thus, by monitoring the pH of the medium, we can easily determine (1) the time required to exhaust the external P_i (i.e., the rate

Abbreviations: A9C, anthracene 9-carboxylic acid; DCCD, dicyclohexylcarbo-diimide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, disodium salt; DPC, diphenylamine-2-carboxylate; NAA, α-naphthyl acetic acid; NEM, *N*-ethylmaleimide.

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of P_i uptake) and (2) the activity of the proton pump in the plasma membrane.

In this study, we extended Sakano's method to allow convenient discrimination of direct inhibition of P_i transport from indirect inhibition caused by the collapse of the electrochemical potential gradient of protons across the plasma membrane, such as that which would occur as a result of inhibition of the proton pump. Since the gradient (proton-motive force) that provides the energy for the P_i-transport system is composed of two compounds, the pH gradient and the membrane potential, it should be possible to characterize indirect inhibitors by measuring their effects on these components. Although the collapse of the pH gradient is easily detectable as the alkalinization of the medium, measurements of membrane potential by conventional methods, for example with a micro electrode, is not practical in our system. Using our newly developed method, we monitored, in addition to the pH of the medium, the change in the concentration of K^+ ions in the medium using a K^+ electrode. If the membrane is depolarized by the action of an inhibitor, K^+ ions should be released from the cell. However, if the inhibitor does not affect the membrane potential, K^+ ions should be taken up continuously by the cell. Thus, a compound that inhibits the P_i transporter specifically and directly should cause (1) neither alkalinization of the medium nor the efflux of K^+ ions; and (2) prolongation of the time required to attain maximum alkalinization upon addition of P_i (i.e., reduction of the rate of uptake of P_i). Applying these criteria, we identified several specific inhibitors of the P_i transporter that did not inhibit the proton pump in the plasma membranes of *Catharanthus roseus* cells.

Materials and Methods

Plant materials—Catharanthus roseus cells were cultured in Murashige-Skoog medium as described previously (Sakano 1990). Cells that had been cultured for 7 days after transfer to fresh medium were used for all experiments unless otherwise indicated.

Cells were collected on filter paper under suction and washed with 10 mM $CaCl_2$. They were weighed and suspended at a density of 5 g fresh weight per 50 ml in 50 ml of an unbuffered medium that contained 10 mM $CaCl_2$, 1 mM KCl and 2% glucose. The pH and concentration of K^+ ions in the medium were monitored with pH and K^+ electrodes (GC-5011C and K-135; TOA Electronics, Tokyo, Japan), respectively. The electrodes were connected to a pH meter and an ion meter (HM-16S and IM-5S; TOA Electronics), respectively, and the data were recorded with a pen recorder (INR-6000; TOA Electronics). During incubation, air was passed continuously at a rate of 20 ml min^{-1}. The suspension was shaken at 150 rpm at room temperature (25–26°C). Under these conditions, the concentration of K^+ ions in the medium increased temporarily (up to about 2 mM) after addition of cells to the medium and then it decreased continuously. The pH of the medium also increased to about 7.0 when cells were put into the medium. However, within a few hours, pH of the medium dropped and stabilized at around 4 (data not shown). Then, the uptake of P_i was monitored.

Measurement of the uptake of P_i—For the addition of P_i to the suspension of cells, we used a solution of 1.0 M Na_2HPO_4, the pH of which had been adjusted to the pH nearest to that of the suspension, chosen from a series of solutions with pH values that ranged from 3.0 to 7.0 at 0.5-unit intervals.

The uptake of P_i could be followed by monitoring changes in the pH of the medium. After addition of P_i to the medium, the pH of the medium increased continuously until the added P_i had been almost completely absorbed by the cells (Sakano 1990; see also Fig. 1).

The uptake of P_i was also monitored by measuring the decrease in the concentration of P_i in the medium by the method of Bencini et al. (1983), as described previously (Sakano 1990).

In this study, the size of the added aliquot of P_i was always 10 μmol (final conc. 0.2 mM).

Reagents—DIDS was purchased from Dojin Co. (Kumamoto, Japan); A9C, DCCD, DPC and ICH_2COOH were from Wako (Osaka, Japan); and furosemide, NEM and phosphonoformic acid were from Sigma (St. Louis, MO, U.S.A.).

Results and Discussion

Monitoring of the uptake of P_i by measuring pH and the concentration of K^+ ions—Figure 1 shows an example of the time courses of changes in the pH of and in the concentration of K^+ ions and P_i in the extracellular medium after addition of P_i. Before addition, the pH of the medium was low (around 3.6) as a result of the efflux of protons from cells via proton pumps in the plasma membrane. As soon as the uptake of P_i has been initiated by the addition of P_i to the medium, the pH began to increase (Fig. 1A). After 7.5 min, the pH has risen to about 4.3 and then it started to decrease abruptly when the P_i in the medium was almost exhausted (about 10 μM), as shown in Fig. 1B. This result clearly indicated that P_i was cotransported with protons into the cells. Rate of uptake of P_i was 0.27 μmol g^{-1} min^{-1}. Note that the rate was constant throughout the uptake of P_i although the pH of the medium changed from 3.5 to 4.3, as also occurred when the amount of P_i added was increased 10-fold (initial concentration of P_i: about 2 mM). These results are consistent with those reported previously (Sakano 1990).

Before addition of P_i, the concentration of K^+ ions in the medium decreased steadily during incubation of the

cells (Fig. 1A), indicating that the electrogenic efflux of protons due to proton pumping was balanced by an influx of positive charge that was due, at least in part, to K^+ ions (and/or an efflux of anions). In this way, electrical neutrality was maintained, as K^+ ions moved passively into cells along the electrochemical gradient.

When P_i was added to the medium, the concentration of K^+ ions in the medium increased in a similar manner to the pH. However, the increase was temporarily: the uptake of K^+ ions by the cells soon resumed and the rate of uptake was much higher than that before the addition of P_i. The temporary increase in the concentration of K^+ ions in the medium, (that is, the increase in the efflux of K^+ ions from the cells) can be explained in terms of depolarization of the membrane induced by the uptake of P_i, as reported by Ullrich-Eberius et al. (1981). The P_i-induced depolarization of the membrane usually lasts only a short time because the electrogenic proton pump is subsequently stimulated by cytoplasmic acidification that is mediated by the proton-cotransport mechanism (Ullrich-Eberius et al. 1981, Sakano et al. 1992). In our system, this series of events resulted in a resumption of the uptake of K^+ ions, as shown in Fig. 1A. Thus, by monitoring the changes in both pH and the concentration of K^+ ions in the medium, we gained some insight into the function of the proton pump in relation to the transport of P_i. In addition, we were able to determine the activity of the P_i transporter by measuring the time (t_p) required to achieve maximum alkalinization after the addition of P_i (Fig. 1A, B). As noted by Sakano (1990), t_p is inversely proportional to the rate of uptake of P_i by the cells.

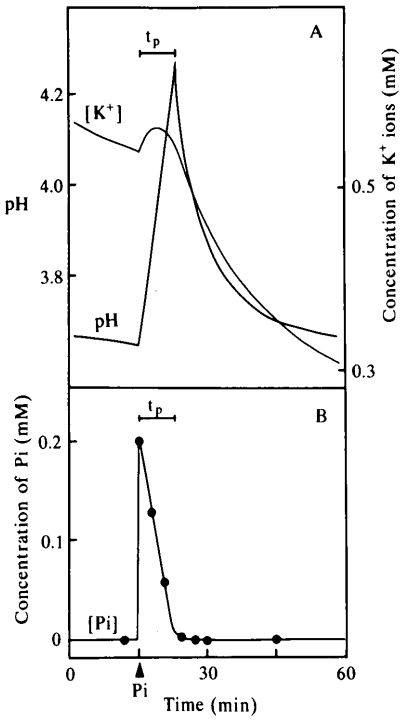

Fig. 1 Time courses of changes in (A) pH and the concentration of K^+ ions and (B) the concentration of P_i. Arrowhead indicates the time at which 10 μmoles of P_i were added into the suspension of cells (5 g fresh weight of cells in 50 ml of a solution of 10 mM $CaCl_2$, 1 mM KCl and 2% glucose). t_p (length of bar) indicates the time required to achieve maximum alkalinization after addition of P_i. In this experiment, t_p was 7.5 min. Note that, in (A), the time at which maximum alkalinization occurred corresponds to the time at which the medium was depleted completely of P_i (B).

Figure 2 shows a typical example of inhibition of the H^+ pump. When 5 μmoles of DCCD, which is a potent inhibitor of the proton pump in the plasma membrane (Kishimoto et al. 1984, Marre and Ballarin-Dentoni 1985), were added to the medium, alkalinization and the concomitant increase in the concentration of K^+ ions in the medium occurred immediately, even in the absence of P_i. When such simultaneous increases in pH and the efflux of K^+ ions were observed upon addition of an inhibitor to the cells, prior to the addition of P_i, we eliminated the inhibitor from the list of candidates for direct inhibitors (see below).

In our search for direct inhibitors, we first examined inhibitors of anion transport that are known as modifiers of sulfhydryl groups, because the P_i transporter of mitochondria has been shown to be sensitive to such reagents (Wohlrab 1986).

An inhibitor that induced an influx of protons and a concomitant efflux of K^+ ions—Figure 3 shows the effects of ICH_2COOH, a potent sulfhydryl reagent, on the time courses of changes in pH and the concentration of K^+ ions. Addition of 50 nmoles of ICH_2COOH (final conc., 1 μM) affected neither the time courses of the changes in pH and

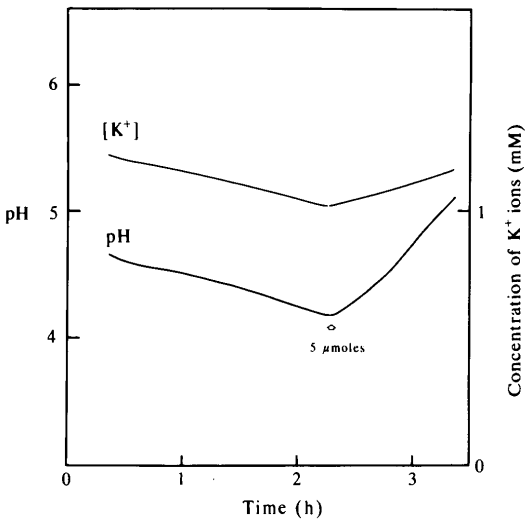

Fig. 2 Effects of DCCD, an inhibitor of the proton pump, on changes in pH and the flux of K^+ ions. DCCD (5 μmol) was added to the suspension of cells at the time indicated (⇧).

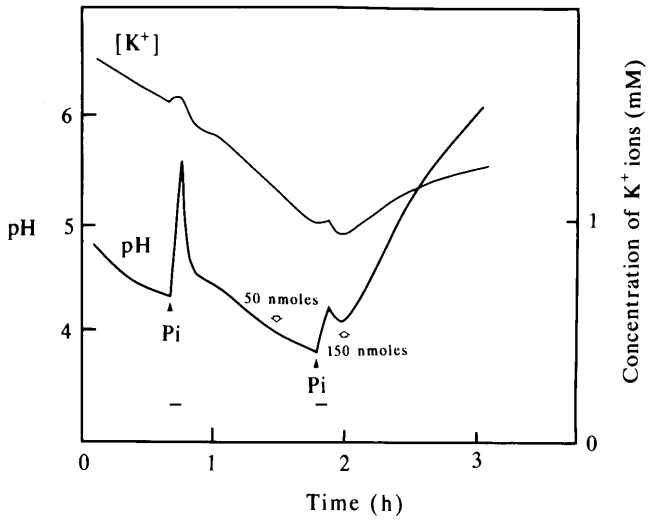

Fig. 3 Effects of ICH_2COOH on changes in pH and the flux of K^+ ions. P_i (10 μmoles) and ICH_2COOH (50 and 150 nmoles) were added at the times indicated by (▲) and (⇧), respectively. The lengths of the bars below peaks indicate t_p.

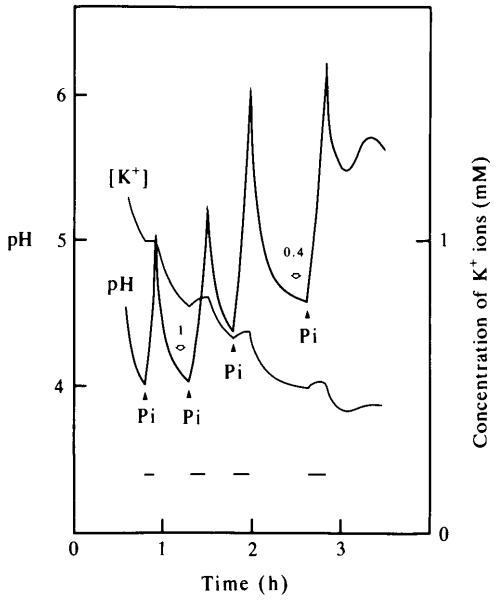

Fig. 4 Effects of A9C on changes in pH and the flux of K^+ ions. At the times indicated by (▲) and (⇧), P_i (10 μmoles) and A9C (1 and 0.4 μmoles), respectively, were added. The lengths of bars below peaks indicate t_p.

the concentration of K^+ ions nor t_p. However, further addition of 150 nmoles of ICH_2COOH (final conc., 4 μM) resulted in increases in both the influx of protons and the efflux of K^+ ions, an indication that the proton pump in the plasma membrane was inhibited by ICH_2COOH. Under these conditions, the electrochemical potential gradient of proton across the plasma membrane appeared to have collapsed. Therefore, we excluded ICH_2COOH as a possible direct inhibitor of P_i transport. NEM (30 μM), another sulfhydryl reagent, did not affect the uptake of P_i (data not shown) even though it has been shown to be a potent inhibitor of the P_i transporter of mitochondria (Wohlrab 1986).

Direct inhibitors of the P_i transporter—Figure 4 shows the effects of A9C, a blocker of anion channels (Shiina and Tazawa 1987), which were quite different from those of ICH_2COOH (Fig. 3). Addition of A9C (1 and 0.4 μmol) did not induce any influx of protons or efflux of K^+ ions, as would have been expected if the proton pump had been inhibited. By contrast, the uptake of P_i was inhibited: the uptake of 10 μmol of P_i required 7.5 min in the absence of A9C, while in the presence A9C (final conc., 28 μM), 15 min were required. Unfortunately, since increases in pH and in the concentration of K^+ ions were also observed after the exhaustion of P_i (see the time courses of changes in pH and in the concentration of K^+ ions after 3 h in Fig. 4), we could not exclude the possibility that the proton pump had been partially inhibited by A9C. In addition, we found that DPC, another blocker of anion channels (McCarty et al. 1993), also inhibited P_i transport in a similar manner (data not shown).

A9C and DPC, both of which inhibited P_i transport, are similar to each other in having a hydrophobic molecular structure and a carboxylic group. We speculate that, if these compounds interact directly with the P_i transporter, the carboxyl moiety should interact with the P_i-binding site (probably an amino group) of the P_i transporter so that binding of P_i would be prevented. It also seems likely that the hydrophobic nature of these inhibitors promotes their accumulation in the plasma membrane.

To test the above hypothesis, we examined several additional compounds that are hydrophobic and include one carboxyl group. Among these compounds, furosemide (Benet 1979) caused the specific inhibition of P_i transport (Fig. 5). When added in successive aliquots to the assay medium, furosemide prolonged t_p in a concentration-dependent manner (see below), but no efflux of K^+ ions was induced at all. These results strongly suggested that furosemide at the range of concentrations tested (up to 100 μM) inhibited the P_i transporter specifically without inhibiting the proton pump. At high concentrations (500 μM or higher), furosemide caused an influx of protons and an efflux of K^+ ions, an indication that some non-specific inhibition occurred (data not shown). Figure 6 shows the effects of furosemide on the activity of the P_i transporter. Inhibition of P_i uptake was directly related to the concentration of furosemide. The concentration of furosemide required to inhibit activity by 50% was approximately 50 μM.

Other inhibitors—DIDS (final conc., 500 μM), known as an inhibitor of the P_i translocator of chloroplast (Rum-

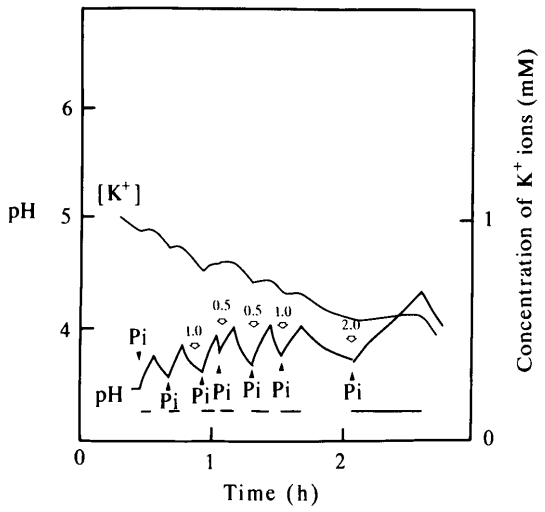

Fig. 5 Inhibition of the uptake of P_i by furosemide. At the times indicated by (▲) and (⇧), P_i (10 μmol on each occasion) and furosemide (amounts as indicated, in μmol), respectively, were added. In every case, P_i was added 30 s after the addition of furosemide. Note that the addition of furosemide induced no increase in the influx of protons or the efflux of K^+ ions. The lengths of bars below peaks indicate t_p.

pho and Edward 1985, Gross et al. 1990), did not affect the uptake of P_i (data not shown). A similar result was reported by Lin (1981) who worked with corn root protoplasts. Phosphonoformic acid (up to 0.5 mM), reported to be an inhibitor of P_i transport in animal cells (Sczezepanska-Konkel et al. 1986) and in protoplasts of *Vicia faba* (Denis and Delrot 1993), did not inhibit uptake of P_i by cultured cells of *Catharanthus roseus* (data not shown).

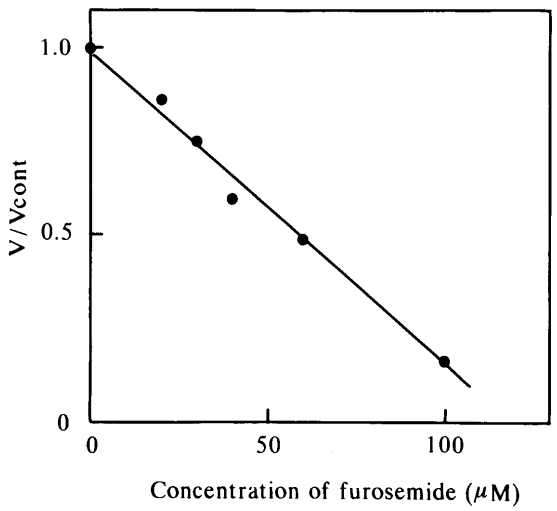

Fig. 6 Inhibition of the transport of P_i as a function of the concentration of furosemide. Ordinate (V/V_{cont}) indicates the ratio of $1/t_p$ ($=V$) measured at a given concentration of furosemide to $1/t_{p\,cont}$ ($=V_{cont}$; cont, control) measured in the absence of furosemide. Data are from the experiment for which results are shown in Fig. 5.

It is of interest to note here that auxins, which are plant hormones, are structurally very similar to the inhibitors of the P_i transporter. They have one carboxyl group each and a hydrophobic structure. In fact, the synthetic auxins 2,4-D (50 μM) and NAA (60 μM) did inhibit P_i transport in a similar manner to A9C and DPC (data not shown). While IAA and NAA at low concentrations (1–10 μM) induce the stimulation of the proton pump and hyperpolarization of the plasma membrane, opposite effects have been reported at higher concentrations (Ephritikhine et al. 1987, Santoni et al. 1990). Bentrup et al. (1973) reported that IAA (10 μM) inhibited the influx of Cl^- ions into cells of *Petroselinum sativum*. Marten et al. (1991) reported that auxin at higher concentrations (5–100 μM) controlled the voltage-dependent activity of anion channels in the plasma membrane.

We hope to find a useful probe for labeling the P_i transporter among the structural analogues of the direct inhibitors of the P_i transporter that we have identified. An extended search for relevalent compounds is in progress in our laboratory.

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