Polyamine Uptake, Kinetics, and Competition among Polyamines and between Polyamines and Inorganic Cations

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ABSTRACT

Polyamine uptake, the kinetics of this uptake, and the competition among polyamines and between polyamines and inorganic cations were studied in petals of Saintpaulia ionantha Wendel. Uptake experiments using 14C-labeled polyamines were carried out on single petals, at room temperature (20°C) and in the light. The results show that putrescine, spermidine, and spermine uptake was dependent on the external pH and occurred up to high external polyamine concentrations with Ke values of 8.6, 1.2, and 2.1 millimolar, respectively, with spermidine being the most absorbed at low concentration (17 micromolar). Putrescine and spermidine did not seem to compete for the same site of absorption. Furthermore, putrescine and spermidine uptake was not inhibited by Ca2+, Mg2+, and K+ at the same concentrations (17 micromolar), whereas 1.7 millimolar Ca2+ inhibited and K+ enhanced spermidine uptake. The intracellular localization of the absorbed putrescine was determined using two different methods. Very little label was found in the apoplast, while most of it was localized in the 98,500 g supernatant. According to our data the vacuole, which represents a substantial part of Saintpaulia parenchyma cells, could be a site of putrescine accumulation. 2,4-Dinitrophenol and diethylosilbestrol did not inhibit uptake; however, at 0°C there was a 35% inhibition of spermidine uptake, compared with the controls kept at 20°C as well as a 68% inhibition with 20 millimolar NaSCN.

In previous work (3) putrescine uptake and the kinetics of this uptake were studied in petals of Saintpaulia ionantha which have the advantage, with respect to other plant systems (4), of having a thin epidermis without cuticle and waxes, thus favoring a rapid uptake. The results showed that putrescine uptake occurred against a concentration gradient at low external putrescine concentration (0.5–100 μM) and followed a concentration gradient at higher external putrescine concentrations (100 μM to 100 mM). In addition it was shown that putrescine uptake was dependent on the external pH and two maxima, at pH 5 to 5.5 and at pH 8, were observed.

At present, there is increasing interest in the relationship between ion transport and the effect of plant growth substances and in the influence that polyamine binding to phospholipids can exert on membrane fluidity and on the activity of enzymes associated with membranes (8). Because nothing was known about the mechanism of polyamine uptake and transport in higher plants except for the data reported in our previous papers (1, 3), we have further investigated putrescine uptake in connection with the uptake of different cations and its localization within the cell, a topic only touched upon in the previous paper (3). In addition, spermidine and spermine uptake, the effects of the external concentration, pH, and metabolic inhibitors were also investigated.

MATERIALS AND METHODS

Plant Material. Plants of Saintpaulia ionantha Wendel were grown in the Botanical Garden. The experiments were performed at room temperature (20°C) and in continuous light (2000 lux). The petal epidermis was thin, without stomata and cuticle. Petals chosen were of the same size and age.

Uptake Experiments. Uptake experiments were done with single petals (weight, 50–60 mg; surface area, 2.0 ± 0.3 cm²). Each petal was floated in a watchglass with the whole upper surface in contact with the incubation medium. This consisted of 7.4 Krbq in 20 μl [14C]putrescine or [14C]spermidine or [14C] spermine (4.4 GBq/mmol) in 100 μl of distilled H2O at the pH value ranging from 4 to 11.5, as indicated in the tables and figures. Unless otherwise indicated, the Ca2+ content in distilled H2O was 1 μM as determined by atomic absorption. At the end of the incubation time, the petals were washed several times with H2O containing unlabeled polyamine. They were then ground in a mortar in 2 ml 0.1 N HCl and centrifuged for 10 min at 3000g in a Sorvall Superspeed Centrifuge (rotor SS 34). After centrifugation, 0.1 ml of the supernatant was mixed with 4 ml of scintillation fluid and radioactivity was determined in a Beckman scintillation counter with automatic quench compensation. The radioactivity in the pellet after 3000g centrifugation ranged between 4 and 8% of total radioactivity according to the time course of the uptake experiment and was not considered in later experiments.

Determination of Radioactivity in the Apoplast. Petals exposed to 7.4 Krbq of [14C]putrescine (final concentration 17 μM), after weighing, were completely immersed in 0.1 N NaCl, put under vacuum for 5 min, and then brought back to normal pressure so that the NaCl solution replaced the air in the intercellular space and Na+ exchange with the putrescine bound to the cell walls could occur. The petals were dried and weighed to determine how much solution was absorbed. They were then centrifuged at low speed (500g) in a MSE centrifuge to recover the solution present in the free space and to determine its radioactivity. The weight of this solution and the appearance of anthocyanins (purple color) allowed us to determine whether or not the cells had been damaged.

Fractionation. The method used was specific for cell wall isolation (6). Petals exposed to putrescine as in the above-cited experiment were ground in a mortar in 6 ml of 1 mM Na phosphate-citrate buffer at pH 5.8. The homogenate was centrifuged at 1000g for 15 min and the pellet was resuspended in the grinding medium and recentrifuged. The pellet, containing the cell wall fraction, was washed with distilled H2O on cheese cloth.
POLYamine UPTAKE IN SAINTPAULIA PETALS

was...effect...was or the membrane were scraped and then in 4 ml of scintillation fluid to determine the radioactivity.

Polyamine Analysis. Polyamines were extracted, separated, and detected by the method of direct dansylation described by Smith and Best (14) using precoated Silica Gel 60 TLC plates with concentrating zone with ethyl acetate:cyclohexane (2:3, v/v) or chloroform:triethylamine (10:1, v/v) as the solvents for polyamines and 1,3-diaminopropane analysis, respectively. Spots were scraped from the plates, extracted with acetone on a Vortex mixer, and centrifuged. Fluorescence was measured with a spectrofluorimeter (excitation 360 nm, emission 505.5 nm) and results compared with dansylated standards. The radioactivity present in the spots was determined by placing them in 0.5 ml of methanol and then in 4 ml of scintillation fluid.

pH-Dependent Spermine Partitioning in H2O-butanol. [14C]Spermine (37 kBq in 100 μl) was mixed with 5 ml of H2O and 5 ml of n-butanol, both brought to pH values between 4.5 and 11.5 with dilute HCl or NaOH. The mixtures were vigorously shaken for 10 min. After complete separation of the two phases, the radioactivity was measured by placing 0.1 ml of each phase and of the interface in scintillation fluid. A similar experiment was performed with n-hexane instead of n-butanol, but in this case only the pH of the water was adjusted.

RESULTS

General Properties of Polyamine Uptake. An initial experiment showed that 17 μM putrescine, spermidine, or spermine were all absorbed in Saintpaulia petals with a rate of uptake of 6.7 ± 1.3, 13.9 ± 0.4, and 10.4 ± 1.6 nmol/g fresh weight/h, respectively. The rate of spermine uptake from the upper surface, determined every 10 to 20 min, was constant for 1 h, showing a decrease at 2 h. Like putrescine (3), the spermidine absorbed was also metabolized to spermine (2% of labeled polyamine uptake) and partly (6%) was degraded to 1,3-diaminopropane as shown for other plants (13).

As with putrescine (3), spermidine and spermine uptake depended on the external pH. Spermidine uptake at low external concentration showed a maximum at pH 8, but there was a fair amount of uptake at acidic pH values too with the exception of pH 5 (Fig. 1); at high external concentration the maximum was at pH 4.5 with a peak at pH 8 (Fig. 2). Spermine uptake at low external concentration had an optimum at pH 4.5 with the characteristic peak at pH 8 (Fig. 1).

The effect of concentration on spermidine and spermine uptake is shown in Figures 3 and 4. With increasing mol wt of the polyamines, the maximum concentration used was progressively lower since at the higher values plasmolysis occurred. The $K_m$ and $V_{max}$ values for the two polyamines are given in Table I. They were calculated using a linear regression program which gave best fit estimates for $K_m$ and $V_{max}$.

Effect of Polyamines and Cations on Uptake. The presence of one polyamine did not seem to inhibit the uptake of the others. This was seen in two different experiments: in the first, the uptake of 17 μM [14C]putrescine was measured in the presence of the same concentration of unlabeled spermidine and vice versa; in the other, 925 kBq of [14C]putrescine and 7.4 kBq of [14C]spermidine were given together at the same concentration.

Similar experiments were carried out with labeled putrescine or spermidine in the presence of 17 μM or 1.7 mM Ca2+ and Mg2+. As shown in Table II, these cations exerted no inhibitory effect on uptake when given at the same concentration as the amine (17 μM), while at the higher concentration only spermidine was inhibited by Ca2+. The effect of increasing concentrations of KCl was also investigated. The results are shown in Figure 5

where it can be seen that in this case 1.7 mM K+ at pH 5.5 stimulated spermidine uptake. This experiment was also performed at pH 8 and gave the same results.

Intracellular Localization of Absorbed Putrescine. Maximum putrescine uptake by Saintpaulia petals occurs at two different pH values (3). As putrescine is protonated at these values, we examined the interactions between the positive charges of the polyamine and the negative charges of the cell wall. A specific method for cell wall isolation (6) was used which showed that only a small amount of label was bound to this cell wall fraction (Table III). This was confirmed by experiments performed to determine the amount of putrescine localized in the apoplasm. In this compartment the radioactivity found was 0.4 to 4.5% of the total absorbed. Most of the absorbed putrescine (80–95%) of the...
Factors Influencing Putrescine Transport. The driving forces for polyamine uptake are not clearly identified. Previously, putrescine uptake was shown to be not inhibited by uncoupler factors (3). Similarly, spermidine uptake was not affected by 2,4-dinitrophenol added at various concentrations (0.1, 0.5, and 1 mM) to the incubation medium without or with 0.5 mM CaCl₂ at pH 4.5 or 8 containing 17 μM spermidine, so that a H⁺ gradient seemed not to be necessary for uptake to occur (Table IV). The same effect was noted when polyamine uptake was determined after petals had been preincubated for 1 h with 2,4-dinitrophenol and then washed. On the contrary, [³⁵S]arginine uptake in *Saintpaulia* petals was markedly affected by 1 mM 2,4-dinitrophenol in the presence and in the absence of Ca²⁺ in the incubation medium (Table IV). The lower arginine uptake respect to the spermidine uptake may be related to internal arginine pool.

Nevertheless, in other plant systems such as carrot cells cultured in vitro, 2,4-dinitrophenol inhibited putrescine uptake (2) and this is in agreement with previous results obtained with *Esherichia coli* (16). Inhibitors of oxidative phosphorylation were recently shown to inhibit polyamine uptake in plant protoplasts (9) and human platelets (11). Diethylstilbestrol (0.1 mM), a proton pump inhibitor, at pH 5 also had no effect on spermidine uptake in *Saintpaulia* petals.

Possible evidence for an energy requirement in polyamine uptake by *Saintpaulia* petals was obtained from experiments performed at 0°C in which the uptake of spermidine was inhibited by 35.3% with respect to the control kept at 20°C. This low temperature inhibition of putrescine uptake has been observed in *E. coli* (16) and in platelets (11), and it has been attributed to an energy requirement. Alternatively it could be due to the altered functions of membrane transport proteins caused by freeze injury.

An energy-dependent mechanism for polyamine uptake was also shown by experiments in which 20 mM NaSCN was supplied with 17 μM spermidine to *Saintpaulia* petals. In platelets (11) this concentration was seen to enhance putrescine uptake, but in our system it resulted in a 68% inhibition of spermidine uptake, while lower concentrations (below 10 mM) had no significant effect.

### DISCUSSION

Although the parenchyma cell of *Saintpaulia* petals is mature and differentiated, it still has the ability to absorb polyamines. Our data on the rate of spermidine uptake were in agreement with those observed in tobacco and cowpea leaf tissue (9) in which spermidine uptake was rapid, reaching a maximum within 1 h. However, in cowpea protoplasts there was a lag period of 20 h before the onset of rapid spermidine uptake. Spermidine in *Saintpaulia* petals seemed to be the most readily absorbed of the three polyamines; the *Kₐₚ* value for spermidine was lower than for putrescine and spermine, while putrescine showed a higher *Vₘₐₓ* value (3).

Based on the endogenous polyamine content previously determined and on the procedure utilized to calculate the intracellular...
Table II. Effect of Ca\(^{2+}\) and Mg\(^{2+}\) on Putrescine and Spermidine Uptake (Expressed as mmol/g fresh wt-h)

Saintpaulia petals were floated on 1.7 nmol in 20 \(\mu l\) (7.4 kBq) of \([^{14}C]\)putrescine or spermidine added to 100 \(\mu l\) incubation medium containing two different concentrations of Ca acetate and Mg acetate for 1 h. Data presented are the means \(\pm\) SD of 2 to 5 separate experiments, each of them performed with 5 samples.

<table>
<thead>
<tr>
<th>Polyamine 17 (\mu M)</th>
<th>Rate of Putrescine and Spermidine Uptake</th>
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<tbody>
<tr>
<td></td>
<td>Control 17 (\mu M) Ca(^{2+}) 1.7 mM Ca(^{2+}) 17 (\mu M) Mg(^{2+}) 1.7 mM Mg(^{2+})</td>
</tr>
<tr>
<td>Putrescine</td>
<td>5.4 (\pm) 1.2 9.9 (\pm) 0.1 6.9 (\pm) 1.3</td>
</tr>
<tr>
<td>Spermidine</td>
<td>9.9 (\pm) 3.3 12.2 (\pm) 3.5 4.6 (\pm) 0.6</td>
</tr>
<tr>
<td></td>
<td>17 (\mu M) Mg(^{2+}) 1.7 mM Mg(^{2+}) 17 (\mu M) Mg(^{2+}) 1.7 mM Mg(^{2+})</td>
</tr>
<tr>
<td>Putrescine</td>
<td>9.6 (\pm) 2.9 7.4 (\pm) 1.2 7.3 (\pm) 0.9</td>
</tr>
<tr>
<td>Spermidine</td>
<td>10.8 (\pm) 1.6 12.3 (\pm) 1.4 9.7 (\pm) 1.5</td>
</tr>
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</table>

Fig. 5. Spermidine uptake in the presence of increasing concentrations of KCl. \([^{14}C]\)Spermidine (1.7 nmol in 20 \(\mu l\) [7.4 kBq]) was added to 100 \(\mu l\) of a KCl solution at pH 5.5 and at various concentrations for 1 h.

Table III. Distribution of Supplied Putrescine in Differential Centrifugation Fraction of Saintpaulia Petals

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Putrescine</th>
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<tbody>
<tr>
<td></td>
<td>mmol/g fresh wt %</td>
</tr>
<tr>
<td>1,000g pellet (cell walls)</td>
<td>0.08 1.1</td>
</tr>
<tr>
<td>98,500g pellet</td>
<td>0.20 2.7</td>
</tr>
<tr>
<td>98,500g supernatant</td>
<td>7.01 96.2</td>
</tr>
</tbody>
</table>

putrescine concentration (3), spermidine and spermine at the lowest concentration added to the medium (17 \(\mu M\)) were absorbed against a concentration gradient while at higher external concentrations uptake occurred following a concentration gradient (Figs. 3 and 4).

With regard to the intracellular localization of the labeled putrescine absorbed, our results showed that most of the label was localized in the 98,500g supernatant while very little label was found in the apoplast, which, in Saintpaulia parenchyma cells, corresponds to the cell walls and very small intercellular spaces (4). As the vacuole (4) represents a substantial part of Saintpaulia parenchyma cells, it could be considered as a site of putrescine accumulation.

The biphasic effect of pH in polyamine absorption cannot be completely explained by the pK values since even at pH 8 most of the polyamine is protonated. In particular, in the case of putrescine nine-tenths of the amino groups are protonated at pH 8. To understand this pH effect, experiments were performed to determine the partitioning of spermine in relation to its protonation in a nonbiological system such as H\(_2\)O-butanol. At acidic pH values, we found little radioactivity in both the H\(_2\)O and butanol phase (Fig. 6); most of the label was localized in the interface between the two phases. At basic pH values most of the polyamine was found in the butanol, but a considerable fraction was also present in H\(_2\)O. The results obtained with the partitioning of spermine in H\(_2\)O-butanol showed that the interface is a site of polyamine concentration at the most acidic pH values. This could be due to the fact that the positive charges of the protonated polyamine normally interact with butanol via hydrogen bonds (5); in the interface the presence of H\(_2\)O facilitates this interaction. The need for a hydrogen bond interaction between the hydroxyl group of the alcohol and the protonated amino group of the polyamine was also confirmed by a similar experiment performed with a hydrocarbon such as n-hexane instead of butanol. In this case no label was found in the solvent. The behavior of spermine in the H\(_2\)O-butanol system suggests that in biological systems, at least at acidic pH values, the interface between aqueous solutions and the membrane represents a concentration site for polyamines. These can interact with the polar groups of the phospholipids and thus facilitate the uptake mechanism. The peak in polyamine uptake observed at basic pH values could be correlated to the pH optimum of tonoplast ATPase (10). The low amount of label found in the membrane fraction of Saintpaulia petals (Table III) could be due to a
possible exchange of the polyamine with the Na⁺ buffer solution during fractionation.

Competition experiments between different polyamines and between polyamines and inorganic cations such as Mg²⁺ and Ca²⁺ suggest that, in *Saintpaulia* petals, the latter compounds utilize different channels for transport. In human platelets (11) putrescine uptake is not inhibited by Ca²⁺ and only slightly by spermidine and spermine at a concentration 100-fold higher than that of putrescine. In human fibroblasts (12) high concentrations of Ca²⁺ and Mg²⁺ increase the rate of putrescine transport at the same concentration. Furthermore the rate of putrescine uptake in *E. coli* (16) and in *Aspergillus nidulans* (15) is not inhibited by spermidine and spermine, while spermidine uptake is inhibited by putrescine. In *Saintpaulia* petals only K⁺, at a concentration of 1.7 mM, stimulated spermidine uptake and this fact may suggest a cotransport mechanism. High concentrations of KCl, known to depolarize the membrane, exerted an inhibitory effect on polyamine uptake (Fig. 5). Thus, the negative internal membrane potential may be a driving force for spermidine uptake, while a pH gradient does not seem to be necessary unlike that observed for arginine uptake.

The evidence presented here suggests that the uptake process is complex and may be dependent upon membrane potential, other unidentified factors and only partially on an energy-dependent mechanism.

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LITERATURE CITED

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