Amino Acids (1994) 6: 301–309

Amino
Acids

Kinetics and calcium-specificity of polyamine uptake in carrot protoplasts

F. Antognoni, P. Casali, R. Pistocchi, and **N. Bagni**

Dipartimento di Biologia evoluzionistica sperimentale, Università di Bologna, Bologna, Italy

Accepted November 3, 1993

Summary. The kinetics of putrescine and spermidine uptake and the influence of calcium on the kinetic parameters of the transport process were investigated in protoplasts isolated from carrot phloem parenchyma. Spermidine uptake dependence on external concentration was biphasic, both in the absence and in the presence of 1 mM $CaCl_2$. In the first case, saturation was reached at 0.1 to 0.25 mM and the K_m value was 43 μM. When calcium was added, the K_m and V_{max} increased. A similar pattern was found with regard to putrescine uptake. Moreover, in order to clarify the mode of action of calcium on polyamine uptake, lanthanides (lanthanum and gadolinium) were utilised as Ca^{+2}-channel antagonists. When protoplasts were preincubated with these lanthanides, the stimulatory effect exerted by Ca^{+2} on polyamine uptake was almost totally abolished. On the other hand, if lanthanum was supplied instead of calcium, it gave rise to a small enhancement of polyamine transport. These results induce us to suggest that calcium acts on polyamine uptake both by binding to external sites on the plasmalemma and by penetrating into the cell.

Keywords: Amino acids – Calcium – *Daucus carota* – Protoplasts – Putrescine – Spermidine – Uptake

Introduction

The polyamines spermine, spermidine and putrescine are ubiquitous organic polycations implicated in several cellular functions, even though their basic mechanism of action has not yet been clarified.

Since polyamines are regarded as a class of plant hormonal growth regulators, their transport has to be postulated. Both long-distance transport (Rabiti et al., 1989; Caffaro et al., 1993) and uptake at the cellular and subcellular levels of exogenous polyamines has been demonstrated in plants (Pistocchi et al., 1987,

1988). In particular, transport studies have been recently performed using carrot protoplasts in order to eliminate the polyamine interactions with the cell wall, which partly represent an obstacle to polyamine translocation across the plasmalemma. Using this system, the general features of polyamine absorption have already been established: polyamines are taken up very rapidly and their uptake is partly energy-dependent. Calcium is able to activate polyamine transport and this Ca^{+2}-enhanced uptake is also an active mechanism (Pistocchi and Bagni, 1990). The specificity of Ca^{+2} in enhancing polyamine uptake is confirmed by the fact that Mg^{+2} and K^{+} do not affect the process (Antognoni et al., 1993), even though the molecular mechanism by which calcium exerts its effect on the transport process is still unclear.

In this work, the role of calcium on the kinetics of putrescine and spermidine uptake in carrot protoplasts and the mode of action of this inorganic cation in activating polyamine transport was investigated. For this purpose, the effect of lanthanides (lanthanum and gadolinium), which behave as Ca^{+2}-channel blockers was tested with respect to putrescine uptake. Besides, as previous results indicated that auxins stimulate spermidine uptake in the presence of calcium (Kanchanapoom et al., 1991), a putative regulatory effect of other plant hormones (cytokinins, gibberellins and abscisic acid) on polyamine uptake was checked.

Materials and methods

Plant material

Mature taproots of carrot, *Daucus carota* L. cv. Ingrid, grown in fields near Bologna without any addition of growth regulators or antiparasitic agents, were stored unwashed in a cold room (5°C) for up to 3 months.

Isolation of protoplasts

The method described by Keller (1988), partly modified, was used to isolate protoplasts from carrot phloem parenchyma. Briefly, taproots were peeled and 15 g of the phloem parenchyma chopped with a single edge razor blade into cubes of 1 mm^3. The tissue was washed three times with the protoplast medium (0.7 M glycine-betaine, 25 mM Mes-Tris, pH 5.5) and then incubated in a Petri dish with 2% (w/v) Cellulase Y-C and 0.1% (w/v) Pectolyase Y-23 (both from Seishin Pharm. Co. Tokyo, Japan) in the medium cited above, for 3 h at 24°C in the light. Protoplasts were collected by filtering through cheesecloth and washing twice in the protoplast medium by slow centrifugation (twice at 25 × g for 5 min). The pellet contained $7 \times !0^6$ protoplasts ml^{-1}, as counted in a Burker counting chamber.

Polyamine uptake

The procedure was performed as described by Pistocchi et al. (1988) with a slight modification. Protoplasts were incubated in the protoplast medium containing 7.4 KBq in 4 μl [^{14}C] putrescine or [^{14}C] spermidine plus unlabelled polyamine to reach the final concentration indicated in the experiments. When lanthanides were used as Ca^{+2}-channel blockers, protoplasts were preincubated for 5 min in their presence; on the contrary, if lanthanum was used instead of calcium, it was directly added with polyamines. When hormones were used, they were added to the incubation medium at various concentrations.

After a 30 sec incubation, 150 μl of the mixture were transferred to 400 μl microtubes already containing, from bottom to top: 120 μl silicon oil AR 200/AR 20 (1/0.4, v/v)

(Wacker-Chemie GmbH, Germany), 50 μl 100 mM unlabelled polyamine solution and 50 μl silicon oil AR 200/AR 20 (1/0.4, v/v). The uptake time was selected on the basis of a preliminary time course experiment showing that saturation was reached at that time (data not shown). The excess of unlabelled polyamine was inserted between the two silicon oil layers in order to remove all surface-bound polyamine (Pistocchi et al., 1988). The microtubes were then immediately centrifuged at 13,250 × g for 50 sec in a Beckman microfuge E. They were snapfrozen in liquid nitrogen and the tips, containing the protoplasts, were cut off with a razor blade and placed directly in scintillation vials with 500 μl distilled water. After sonication for 1 min, 250 μl aliquots were used for determination of radioactivity in 4 ml scintillation cocktail (Beckman Ready Gel) using a Beckman LS 1800 scintillation counter. Further aliquots were used for protein determination.

In the experiments dealing with [^{45}Ca] uptake, the protoplasts were incubated for 10 min in a mixture containing labelled and unlabelled $CaCl_2$ solution to reach a 1 mM final concentration and then washed with 50 μl 100 mM unlabelled $CaCl_2$ solution inserted between the two silicon oil layers.

Polyamine and protein assay

Polyamines were extracted, separated and detected by the method of direct dansylation described by Smith and Best (1977).

Protein content was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

Results and discussion

Ca^{+2}-specificity of polyamine uptake by carrot protoplasts

Previous studies on polyamine uptake in carrot protoplasts (Pistocchi and Bagni, 1990) indicated that 1 mM $CaCl_2$ gave the maximum stimulation of transport of all three polyamines. Moreover, 1 mM $LaCl_3$ was able to partly

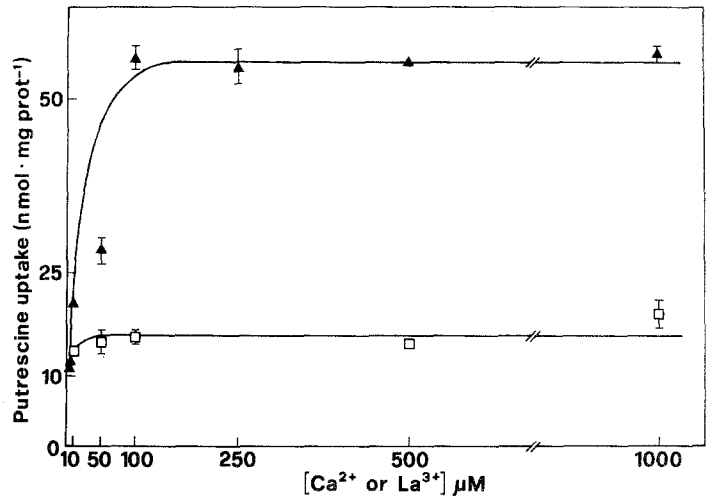

Fig. 1. Effect of increasing concentrations of Ca^{+2} and La^{+3} on putrescine uptake in carrot protoplasts. Uptake was measured 30 sec after incubating the protoplasts in a medium containing a mixture of [^{14}C] and unlabelled putrescine (final concentration 100 μM) in the presence of Ca^{+2} (▲) or La^{+3} (□). Data are the means ± S.E. of two separate experiments each performed in duplicate

mimick the effect of calcium, at least on spermine uptake. In this work, the Ca^{+2} and La^{+3} concentration-dependence over a range of 1 μM to 1 mM was investigated with respect to putrescine uptake. As shown in Fig. 1, the absorption of this polyamine was enhanced five-fold in the presence of Ca^{+2} and only 1.5-fold with La^{+3}, the greatest stimulation being observed in both cases at 100 μM concentration of the cation and thereafter remaining constant up to 1 mM.

In all the experiments on polyamine uptake, the extent of the stimulation exerted by Ca^{+2} varied from 1- to 7.5-fold, although an activation ranging from 2.6 to 5 times was more frequently observed (Fig. 2). This different stimulation by calcium, together with the fact that this cation exerted its maximum effect at concentrations lower than those previously observed (Pistocchi and Bagni, 1990), could be justified by the sensitivity of carrot protoplasts towards calcium, may be related to a different physiological stage of the cells or to a change in the number of Ca^{+2}-regulated receptors located at the plasmalemma level, depending on the age of the carrots.

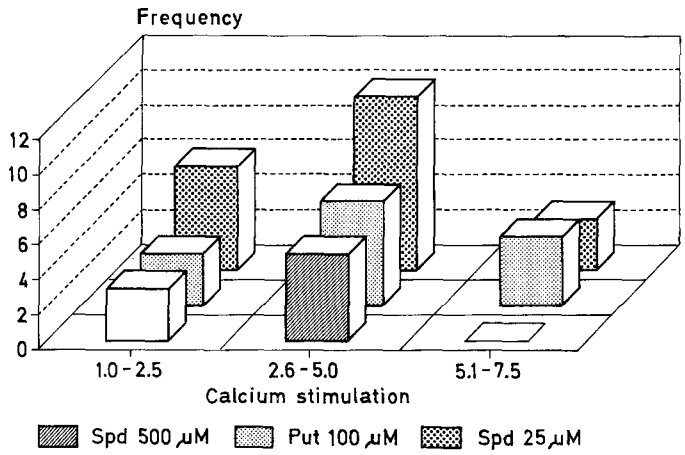

Fig. 2. Ca^{+2}-stimulation of polyamine uptake in carrot protoplasts. Frequency classes of n-fold enhanced uptake (n = values on x axis)

Concentration-dependence of putrescine and spermidine uptake

The concentration-dependence of putrescine and spermidine uptake was investigated both in the absence and in the presence of 1 mM $CaCl_2$. Spermidine uptake dependence on the external concentration was biphasic, both with and without $CaCl_2$ (Fig. 3); in the absence of the cation, saturation was reached at 0.1 to 0.25 mM ($K_m = 43\mu M$, $V_{max} = 1.8$ nmol mg $prot^{-1}$ min^{-1}) and a linear system appeared from 0.25 to 50 mM. When 1 mM $CaCl_2$ was added to the incubation medium, the V_{max} rose from 1.8 to 23.4 nmol mg $prot^{-1}$ min^{-1} and the K_m value also became higher (188 μM). This more detailed analysis of spermidine kinetics at low external concentrations has revealed the existence of a saturable system (system I), not previously observed by Pistocchi et al. (1988).

A similar pattern, though less pronounced, was found with regard to putrescine uptake, K_m values being 250 μM and 330 μM without and with calcium

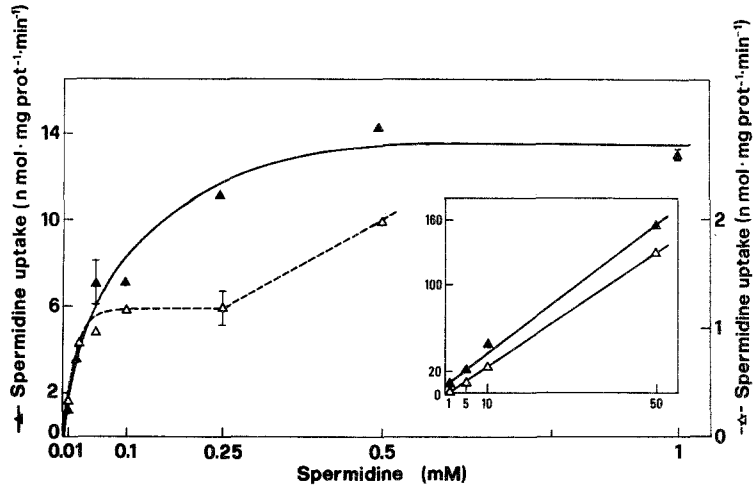

Fig. 3. Spermidine uptake in carrot protoplasts over an external concentration range of 10 μM to 1 mM (system I) and of 1 mM to 50 mM (system II, insert). [^{14}C] spermidine (7.4 kBq) and unlabelled spermidine at various concentrations, in the presence (▲) or absence (△) of 1 mM $CaCl_2$, were added to the incubation medium for 30 sec. Each point represents the mean ± S.E. of three different experiments with duplicate samples; error bars not given when smaller than symbols

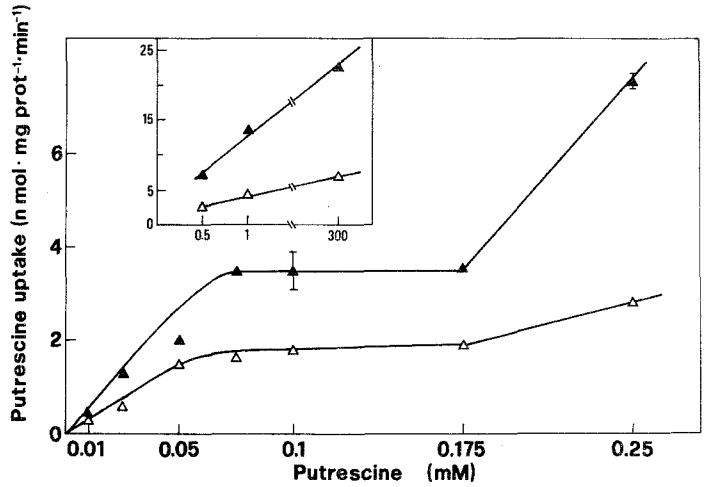

Fig. 4. Putrescine uptake in carrot protoplasts over an external concentration range of 10 μM to 250 μM (system I) and of 500 μM to 300 mM (system II, insert). [^{14}C] putrescine (7.4 kBq) and unlabelled putrescine at various concentrations, in the presence (▲) or absence (△) of 1 mM $CaCl_2$, were added to the incubation medium for 30 sec. Each point represents the mean ± S.E. of three different experiments with duplicate samples; error bars not given when smaller than symbols

respectively (Fig. 4). At high concentrations, the uptake rate of the two polyamines became linear (system II); this may be due either to a loss of plasma membrane selectivity towards the transport substrates when they were present at such concentrations or to the involvement of another transport system such as ion channels (Colombo et al., 1992).

The effects of Ca^{+2} ions on the kinetic parameters of spermidine and putrescine uptake may indicate the existence of two putative transport systems, one of which is activated by Ca^{+2}; this cation may induce an increase in the capacity of the transport system and a decrease in the affinity towards the polyamine.

The effect of low temperatures on the kinetics of spermidine absorption was also investigated by performing the experiments at 4°C. Also in this case, a biphasic system was found, although the rate of uptake was highly reduced (data not shown), dropping from 46 nmol mg $prot^{-1}$ min^{-1} when experiments were carried out at room temperature to 3.8 nmol mg $prot^{-1}$ min^{-1}. As the saturable component (system I) of spermidine uptake was not completely blocked, the results suggest that the polyamine transport system partly works also at 4°C. The lower uptake capacity was even more evident at high external concentrations (data not shown).

The endogenous polyamine content in carrot protoplasts varied slightly depending on the age of the carrots, ranging from 20 μM to 80 μM putrescine and from 6 μM to 12 μM spermine, while spermidine content remained more or less constant at about 75 μM. In our uptake experiments, the total polyamine concentration used (labelled and unlabelled) was of the same order of magnitude as the endogenous content.

In addition, a possible regulation of polyamine transport by plant hormones was checked, since an activating effect of indoleacetic acid on spermidine uptake was previously found (Kanchanapoom et al., 1991). Our attention was focused on the effect of abscisic acid (ABA) on polyamine efflux from carrot protoplasts, as it is known that ABA induces a stimulation of ion efflux at the plasmalemma level of guard cells (MacRobbie, 1991) by stimulating K^+ efflux. Moreover, Colombo et al. (1992) reported the presence of an ion channel permeable to polyamines in *Arabidopsis thaliana* protoplasts, whose features were similar to that of K^+-permeable channels. This piece of information stimulated us to investigate the effect of ABA (from 10^{-7} to 10^{-3} M) on putrescine efflux from protoplasts, but no effect was observed (data not shown). With regard to the uptake process, neither ABA, cytokinins (natural and synthetic, free and conjugated), nor gibberellins (free and conjugated), at various concentrations, were found to affect it, either in the absence or in the presence of $CaCl_2$, apart from a slight inhibitory effect observed at very high concentrations (10^{-3} M) of these hormones (data not shown).

Effect of lanthanides on putrescine uptake

Although calcium seemed to be necessary for polyamine transport in carrot protoplasts, its role in the process remains uncertain and one of the most important questions still debated is whether Ca^{+2} acts at sites on the external surface and/or by penetrating into the cell.

Table 1 shows that the reverse is not true: putrescine did not affect Ca^{+2} uptake, although an inhibition was observed at high polyamine concentrations, as previously found with spermine (Pistocchi and Bagni, 1990). In order to better understand the mode of action of Ca^{+2} on polyamine uptake, an approach

Table 1. [^{45}Ca] uptake in carrot protoplasts in the presence of increasing putrescine concentrations. Protoplasts were supplied with 18.5 kBq in 4 μl of a 20-fold diluted isotope solution plus unlabelled $CaCl_2$ for 10 min. Data are the means $\pm$S.E. of triplicate samples

Putrescince concentration (μM)	^{45}Ca uptake (nmol mg prot^{-1})
0	3.10 $\pm$ 0.38
10	3.23 $\pm$ 0.17
50	2.97 $\pm$ 0.15
100	3.03 $\pm$ 0.17
500	2.83 $\pm$ 0.29
1000	1.37 $\pm$ 0.19

involving the use of Ca^{+2}-channel inhibitors was adopted. The rare earths lanthanum (La^{+3}), a competitive inhibitor of Ca^{+2} uptake into cells (dos Remedios, 1981) and gadolinium, also found to act as a Ca^{+2}-channel antagonist (Millet and Pickard, 1988; Ding and Pickard, 1993), were used in a concentration range of 10 μM to 1 mM during an uptake experiment in the presence of Ca^{+2}. We confirmed that both lanthanum and gadolinium behave as Ca^{+2} uptake antagonists also in our system, although a total inhibition was not observed (about 60% inhibition, data not shown).

When protoplasts were preincubated with these lanthanides the stimulatory effect exerted by Ca^{+2} on polyamine uptake was almost completely abolished (Fig. 5). The higher specificity of gadolinium as a Ca^{+2}-channel blocker with

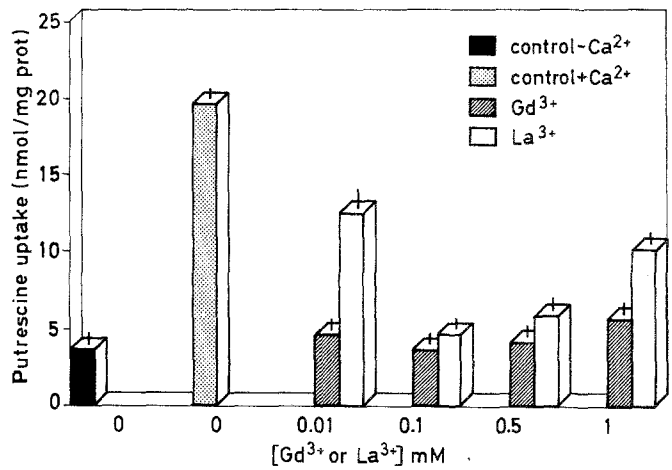

Fig. 5. Effect of increasing concentrations of lanthanides on putrescine uptake into carrot protoplasts. Protoplasts were preincubated 5 min with the lanthanides, then a mixture of labelled and unlabelled putrescine (final concentration 100 μM), containing 1 mM $CaCl_2$, was added and the uptake was measured after 30 sec. Data are the means $\pm$S.E. of two separate experiments each performed in duplicate

respect to lanthanum (Ding and Pickard, 1993) was confirmed by the greater inhibitory effect on putrescine uptake (Fig. 5). It is interesting to notice that an higher inhibition on the uptake was exerted by lower lanthanum concentrations (0.1 and 0.5 mM). As mentioned earlier, when La^{+3} was supplied alone, it was responsible for a small activation of putrescine transport, thus mimicking somehow the effect of Ca^{+2}. Lanthanum does not enter plant cells (Thomson et al., 1973), but it does compete for external Ca^{+2}-binding sites and blocks external Ca^{+2}-dependent reactions. Based on this observation, the idea was previously put forward (Pistocchi and Bagni, 1990) that calcium might act on polyamine uptake at the surface level by binding to external sites. On the contrary, the results obtained with Ca^{+2}-channel antagonists presented here seem to suggest that an influx of Ca^{+2} is also required for this cation to modulate the transport system. In fact, the experiments performed with $^{45}Ca^{+2}$ showed that supplying carrot protoplasts with 1 mM $CaCl_2$, about 19% of the cation was found inside the cell within a short time (40 sec) and then decreased (data not shown). The effect of La^{+3} could be explained either with some effect of cations from extracellular sites or by the displacement of Ca^{+2} from external binding sites or by only a partial block of Ca^{+2} influx into the cell.

Acknowledgements

This work was supported by funds of the National Research Council of Italy, special project R.A.I.S.A., subproject no. 2, paper no. 1173.

References

Antognoni F, Pistocchi R, Bagni N (1993) Uptake competition between polyamines and analogues in carrot protoplasts. Plant Physiol Biochem 31: 693–698

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72: 248–254

Caffaro S, Scaramagli S, Antognoni F, Bagni N (1993) Polyamine content and translocation in soybean plants. J Plant Physiol 141: 563–568

Colombo R, Cerana R, Bagni N (1992) Evidence for polyamine channels in protoplasts and vacuoles of *Arabidopsis thaliana* cells. Biochem Biophys Res Comm 182: 1187–1192

Ding JP, Pickard BG (1993) Mechanosensory calcium-selective cation channels in epidermal cells. Plant J 3: 83–110

Johannes E, Brosnan JM, Sanders D (1992) Parallel pathways for intracellular Ca^{2+} release from the vacuole of higher plants. Plant J 2: 97–102

Kanchanapoom M, Antognoni F, Pistocchi R, Bagni N (1991) Effect of auxins on spermidine uptake into carrot protoplasts. Physiol Plant 82: 19–23

Keller F (1988) A large scale isolation of vacuoles from protoplasts of mature carrot taproots. J Plant Physiol 132: 199–203

Mac Robbie EAC (1991) Effect of ABA on ion transport and stomatal regulation. In: Davies WJ, Jones HG (eds) Abscisic acid, physiology and biochemistry. Bios Scientific Publishers, Oxford, pp 153–168

Millet B, Pickard BG (1988) Gadolinium ion is an inhibitor suitable for testing the putative role of stretch-activated ion channels in geotropism and thigmotropism. Biophys J 53: 155a

Pistocchi R, Bagni N (1990) Effect of calcium on spermine uptake in carrot cell cultures and protoplasts. J Plant Physiol 136: 728–733

Pistocchi R, Bagni N, Creus JA (1987) Polyamine uptake in carrot cell cultures. Plant Physiol 84: 374–380
Pistocchi R, Keller F, Bagni N, Matile P (1988) Transport and subcellular localization of polyamines in carrot protoplasts and vacuoles. Plant Physiol 87: 514–518
Rabiti AL, Pistocchi R, Bagni N (1989) Putrescine uptake and translocation in higher plants. Physiol Plant 77: 225–230
dos Remedios CG (1981) Lanthanide ion probes of calcium-binding sites on cellular membranes. Cell Calcium 2: 29–51
Smith TA, Best GR (1977) Polyamines in barley seedlings. Phytochem 16: 841–843
Thomson WW, Platt KA, Campbell N (1973) The use of lanthanum to delineate the apoplastic continuum in plants. Cytobios 8: 57–62

Authors' address: Dr. N. Bagni, Department of Biology, University of Bologna, Via Irnerio 42, I-40126 Bologna, Italy.

Received August 25, 1993