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EFFECT OF POLYAMINES ON OXYGEN AND METABOLITE TRANSPORT ACROSS MITOCHONDRIAL AND SYMBIOTIC MEMBRANES OF *GALEGA ORIENTALIS* NODULES

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Summary. The effect of various polyamines (PA) on O_2 and succinate uptake by mitochondria, bacteroids and symbiosomes isolated from *Galega orientalis* nodules was investigated. Bacteroid respiration was characterized by a low endogenous rate of O_2 uptake which was stimulated by addition of succinate. Maximum O_2 uptake occurred when *Galega* bacteroids and symbiosomes were incubated with various PA at concentration of 50 µM and mitochondria at concentration of 10–50 µM. Diamines, putrescine (Put) and cadaverine (Cad) markedly increased permeability of the mitochondrial and symbiotic membranes to succinate and to O_2, while spermidine (Spd) and spermine (Spm) showed a lower stimulating effect. Addition of 400 µM Ca^{2+} which usually exerted an inhibitory effect on Put and Cad uptake by mitochondria, reduced O_2 uptake to the control level. Oxygen consumption was also inhibited by 100 mM K^+ which decreased Spd and Spm transport. It might be suggested that the physiological role of PA in nitrogen fixation may be to a large extent associated with their effect on metabolite and O_2 transport across mitochondrial and symbiotic membranes.

Key words: bacteroids, mitochondria, oxygen and succinate uptake, polyamines, symbiosomes

Abbreviations: Cad – cadaverine, PA – polyamines, PBM – peribacteroid membrane, PHB – poly-β-hydroxybutyrate, Put – putrescine, Spd – spermidine, Spm – spermine

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Introduction

Most of the biological functions of PA are attributed to their polycationic nature (Takeda et al., 1983). As polycations, they bind noncovalently to negatively charged phospholipids and many types of proteins which directly modulate membrane permeability and play an important role in the maintenance of membrane integrity (Altman et al., 1977; Srivastava and Smith, 1982).

Little is known about the interaction between PA and plant mitochondria (Torrigiani at al., 1986). Pistocchi et al. (1990) demonstrated that PA, in addition to their binding to the membrane, can be actively transported into the mitochondria isolated from tubers of *Helianthus tuberosus*. The same authors clearly indicate that this transport is dependent on both respiration and membrane potential.

In the present study we provide data demonstrating that exogenous PA alter permeability of mitochondrial and symbiotic membranes to dicarboxylates and to O_2 that may serve as the basis for revealing the potential significance of PA in the root nodules.

Materials and Methods

Plant material and bacterial strain

Goat's rue (*Galega orientalis*) seeds were scarified, surface sterilized with 70% ethanol (v/v) for 60 min and germinated for 5 days on buffered water agar plates (pH 7.0) at 22 °C. Sterile seedlings were transferred to plastic growth pots (2 l) 10 plants per pot and cultivated on Hellriegel's solution and micronutrients (Hoagland and Arnon, 1950) with 21 mg.l^{-1} nitrogen in a naturally illuminated greenhouse. *Galega* seedlings were inoculated with *Rhizobium galegae* strain HAMBI 540 (HAMBI = The culture collection at the Department of Microbiology, University of Helsinki) using inoculant dose of 10^8 viable cells. The strain was cultivated and maintained on yeast-mannitol (YM) medium (Vincent, 1970) at 28 °C. Nodules were harvested 40 days after planting and were used immediately.

Isolation procedures

Intact symbiosomes were isolated from *Galega* nodules using a modification of the technique for isolating soybean symbiosomes (Day et al., 1989). Eight to 10 g of nodules were crushed in 30 ml of cold homogenisation buffer (450 mM mannitol, 10 mM EGTA, 10 mM $MgSO_4$, 5 mM dithiothreitol, 1% (w/v) BSA, 1% PVP-40, 20 mM ascorbate, 25 mM MES-KOH buffer, pH 7.0). The homogenate was filtered and centrifuged on a Percoll step gradient (30, 60 and 80%) in a swing-out rotor at 4000×g for 30 min at 4 °C. The symbiosome fraction was located at the interface between 30

and 60% Percoll and a small pellet of free bacteroids was obtained at the bottom of the tube. The symbiosome fraction was collected with Pasteur pipette, diluted with wash buffer (450 mM mannitol, 25 mM MES buffer, pH 7.0, 3 mM $MgSO_4$) and pelleted at 500×g for 5 min at 4°C to exclude Percoll. Symbiosomes were then resuspended in wash buffer to the desired concentration.

Free bacteroids were obtained by vigorously vortexing symbiosome suspension for 4 min to rupture PBM.

Mitochondria were purified from nodules by the method of Day et al. (1986).

The degree of purity of the preparations was regularly monitored by electron microscopy (Price et al., 1987)

Oxygen uptake measurements

Oxygen uptake was measured using a Clark-type electrode DW 1 (Hansatech, England) in 2 ml of reaction medium with 0.7 mg mitochondrial, bacteroid or symbiosome protein at 25 °C.

Protein was estimated by the method of Lowry et al. (1951).

Transport studies

The silicone oil filtration technique (Palmieri and Klingenberg, 1979) was used to measure the uptake of [^{14}C] labelled compounds into symbiosomes and bacteroids. The reaction mixture contained isolated organelles in wash buffer (450 mM mannitol, 25 mM MES buffer, 3 mM $MgSO_4$, 0.5 mM $CaCl_2$, titrated to pH 7.0 with 1 M 1,3-bis[tris-(hydroxymethyl)methylamino]propane), [2,3-^{14}C] succinate (1.55 GBq.mmol^{-1}) or [U-^{14}C] sucrose (20 GBq.mmol^{-1}), and where indicated, Put, Cad, Spd or Spm (hydrochloride form) at concentration of 10, 50 or 100 µM. The reaction mixture (0.5 ml) was layered in 2 ml Pyrex glass tubes on top of a silicone oil layer (0.6 ml) which was layered on 0.4 ml of 1.6 M $HClO_4$. The reactions were initiated by applying labelled substrate to the reaction mixture. After the desired time interval, the reaction was terminated by pelleting isolated organelles through the silicon oil into the acid at 12 000×g. Bacteroids were centrifuged for 15 s and symbiosomes for only 5 s (the shorter time preventing most of any contaminating free bacteroids from pelleting). The organelle pellet was removed by suction, dispersed by shaking and the radioactivity was counted in a scintillation counter. When PA were used, they were added to the reaction medium 1 min prior to addition of the [^{14}C] substrate. Silicone oil AR-200 was used undiluted.

Reagents

Silicone oil AR-200 (density 1.04 g.l^{-1}) was purchased from Wacker Chemical Co. (Munich, Germany). All other chemicals were from Sigma Chemical Co. (St. Louis, Mo, USA).

Results and Discussion

Electron microscopic observations (data not presented) on mitochondria, bacteroids and symbiosomes isolated from *G. orientalis* nodules revealed a very slight (less than 2.5%) contamination by each other.

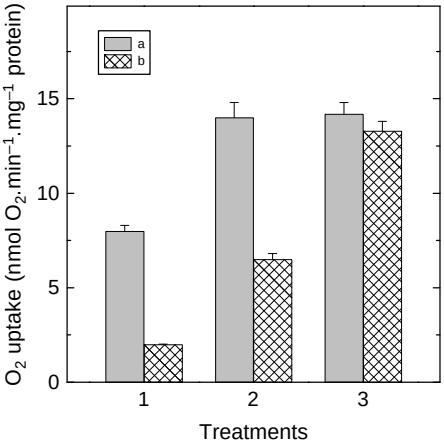

Fig. 1. Oxygen uptake by bacteroids (a) and mitochondria (b) isolated from *Galega orientalis* nodules. The reaction medium contained 0.45 M sorbitol, 5 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.2), 10 mM TES buffer. (1), endogenous O_2 uptake rate. In (2), 10 mM Na-succinate was added; in (3), 10 mM Na-succinate and 1 mM NADH were added. Data are expressed as means (±SE) of four replicates.

Typical O_2 uptake obtained with *Galega* nodule mitochondria, bacteroids and symbiosomes is shown in Fig. 1 and Table 1. Bacteroid respiration was characterized by a low endogenous rate of O_2 uptake which could be stimulated by addition of succinate (Fig. $1a_1$ and $1a_2$). This fact could be related to the low content of endogenous reserves of PHB in *Galega* bacteroids. Intact bacteroids did not respond to added NADH (Fig.$1a_3$), indicating that mitochondrial contamination was negligible; generally, plant mitochondria oxidized exogenous NADH rapidly (Fig. $1b_3$). The endogenous rates of symbiosome O_2 uptake was 28% lower than that occurring in free bacteroids (Table 1). Addition of succinate as respiratory substrate stimulated O_2 consumption of symbiosomes by 89% compared to their endogenous O_2 uptake (Table 1).

Table 1. Oxygen consumption by symbiosomes and bacteroids isolated from *Galega orientalis* nodules in the presence of various polyamines at concentration of 50 µM. Endogenous O_2 uptake rates: symbiosomes, 6.5±0.3 nmol.min^{-1}.mg^{-1} protein; bacteroids, 7.8±0.4 nmol.min^{-1}.mg^{-1} protein. Data are expressed as means ± SE, n=3.

Treatments	O_2 consumption (nmol.min^{-1}.mg^{-1} protein)	
	symbiosomes	bacteroids
Succinate (control)	12.3±0.5	14.1±0.3
Succinate+Put	31.3±0.9	31.1±0.4
Succinate+Cad	29.4±1.0	28.8±0.4
Succinate+Spd	17.4±0.4	18.5±0.2
Succinate+Spm	16.2±0.2	16.9±0.3

Oxygen uptake as function of PA concentrations

Maximum O_2 consumption occurred when bacteroids were incubated with 50 µM of different PA (Fig. 2). Greatest stimulatory effect on the O_2 uptake of mitochondria was observed at lower external PA concentrations (10–50 µM) (Fig 3). It should be noted that the rate of O_2 uptake by the bacteroids and mitochondria after treatment with Spd and Spm was slower than that following Put and Cad (Fig. 2 and 3).

Under our experimental conditions, the PA were most effective in stimulating O_2 uptake of symbiosomes, whose rates reached approximately to those of free bacteroids. This is in contrast with the controls, where O_2 uptake of symbiosomes was 40% lower than that of free bacteroids (Table 1). It should also be noted that the stimulatory effect of diamines, Put and Cad on O_2 uptake by symbiosomes was again greater than that of Spd and Spm.

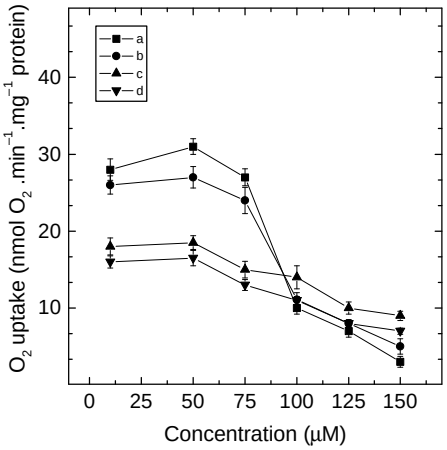

Fig. 2. Effect of increasing concentrations of Put (a); Cad (b); Spd (c); Spm (d) on the O_2 uptake by bacteroids isolated from *Galega orientalis* nodules. The reaction medium contained 0.45 M sorbitol, 5 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.2), 10 mM TES buffer and 10 mM Na-succinate. Each point represents the mean value (±SE) of four replicates.

Polyamine-stimulated oxygen uptake as a function of K^+- and Ca^{2+}-concentrations

Polyamines are positively charged at physiological pH (Takeda et al., 1983). For this reason, positively charged ions are expected to inhibit the PA uptake by mitochondria since they compete for the same binding sites on the membrane. This expectation was verified for Mg^{2+} and K^+ ions, whereas Ca^{2+} did not have any significant effect on Spd uptake (Pistocchi et al., 1990). Notably, the concentrations of cations used were very close to these measured intracellularly (Pistocchi et al., 1990). Relatively rapid inhibition of Put transport in

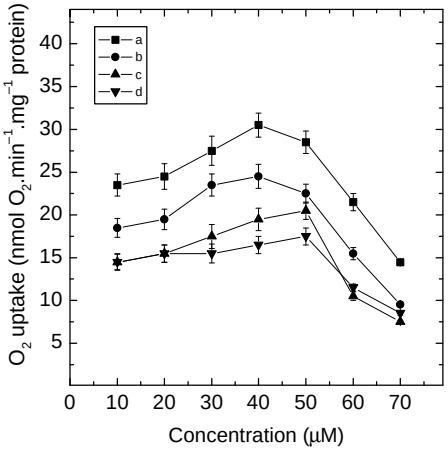

Fig. 3. Effect of increasing concentrations of Put (a); Cad (b); Spd (c); Spm (d) on the O_2 uptake by mitochondria isolated from *Galega orientalis* nodules. The reaction medium was as in Fig. 2. Each point represents the mean value (±SE) of four replicates.

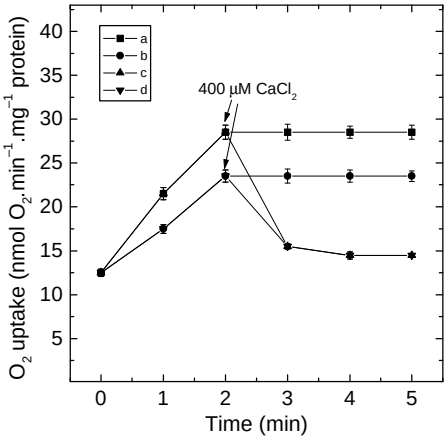

Fig. 4. Effect of Ca^{2+} on the O_2 uptake by mitochondria isolated from *Galega orientalis* nodules. The reaction medium was as in Fig. 2. In (a), 50 µM Put was added; in (b), 50 µM Cad was added; in (c), and (d), at the solid arrow, 400 µM $CaCl_2$ was added. Each point represents the mean value (±SE) of four replicates.

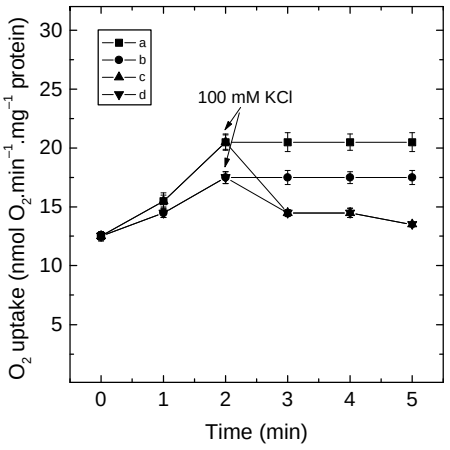

Fig. 5. Effect of K^+ on the O_2 uptake by mitochondria isolated from *Galega orientalis* nodules. The reaction medium was as in Fig. 2. In (a), 50 µM Spd was added; in (b), 50 µM Spm was added; in (c) and (d), at the solid arrow, 100 mM KCl was added. Each point represents the mean value (±SE) of four replicates.

roots of intact maize seedlings by Ca^{2+}, Mg^{2+} and La^{3+} have also been observed (DiTomaso et al., 1992). We have investigated PA-stimulated O_2 uptake of mitochondria in connection with the uptake of K^+ and Ca^{2+}. As shown in Fig. 4, after addition of 400 µM $CaCl_2$ the stimulatory effect of Put and Cad on mitochondrial O_2 uptake was completely inhibited. Stimulatory effect of Spd and Spm was reduced when 100 mM KCl was added (Fig. 5). Therefore, the presence of inorganic cations in reaction medium completely abolished PA-stimulated O_2 uptake that may be due to the inhibiting effect of these ions on the transport of PA into mitochondria (Pistocchi et al., 1990; DiTomaso et al., 1992). Clearly, further studies are needed to identify possible control mechanisms.

Succinate utilization

The rates of [^{14}C] succinate uptake into symbiosomes were increased substantially by addition of various PA to symbiosome suspension (Fig. 6). Diamines, Put and Cad appeared to be the most effective in stimulating succinate uptake (4.0- and 3.6-fold, respectively) while Spd and Spm showed a lower stimulating effect (by 2.3- and 2.0-fold, respectively). Succinate uptake into bacteroids was also stimulated by PA, but to a lesser extent than that into symbiosomes. Addition of Put and Cad to bacteroid suspension resulted in an increase of radioactivity by 2.8- and 2.4-fold, while Spd and Spm enhanced succinate utilization 1.6- and 1.3-fold.

Our data on the high activity of succinate uptake strongly support the general assumption that dicarboxylates are the

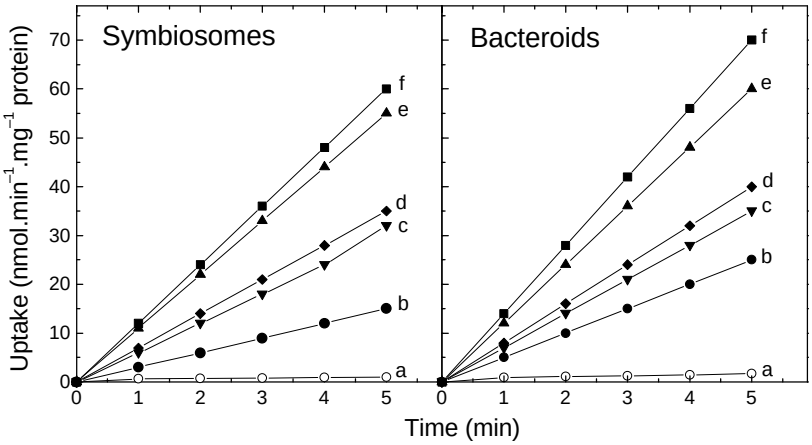

Fig. 6. Time-dependent uptake of [^{14}C] sucrose (a) and [^{14}C] succinate (b) by symbiosomes and bacteroids isolated from *Galega orientalis* nodules. To the reaction medium containing succinate as carbon substrate were supplied spermine (c), spermidine (d), cadaverine (e) and putrescine (f) at concentration of 50 µM.l^{-1}. Succinate and sucrose were added at concentration of 1 and 5 mM, respectively. Symbiosomes and bacteroids were suspended in a reaction medium containing 25 mM MES, pH 7.0, 450 mM mannitol, 3 mM $MgSO_4$, and 0.5 mM $CaCl_2$.

most probable substrates supplied to bacteroids *in vivo* in legume nodules (Udvardi et al., 1997). In contrast to the rapid uptake of succinate, uptake of sucrose was very slow, suggesting that transport of this substance across the symbiotic membranes of *Galega* nodules is via passive diffusion.

The symbiosomes succinate uptake was from 50 to 67% slower than that of bacteroids one, but the uptake of symbiosomes was stimulated to a greater extent by PA. As a result, the rates of succinate uptake in symbiosomes supplied with PA were similar to those obtained in bacteroids. One possible explanation of the stimulant effect of PA is that they may alter membrane permeability and play a role in maintenance of membrane integrity (Srivastava and Smith, 1982). Most of the experimental results demonstrating the stabilizing effect of PA have been conducted with wall-less experimental systems such as protoplasts and organelles (Pistocchi et al., 1990). Polyamines selectively rigidify membrane surface and stabilize protoplasts against post-isolated lysis (Altman et al., 1977). It seem reasonable to assume that exogenously applied PA could cause similar stabilizing effect on the mitochondrial and symbiotic membranes, which improves the properties and increases the capacity of the transporters for succinate on these membranes. On the other hand, it is established that PA were very active in the process of releasing calcium from suspension-cultured *Glycine max* cells (Young and Kauss, 1983) which might also be a reason for increased membrane permeability to succinate. In addition, formation of an electrical potential across the PBM can stimulate the uptake of the dicarboxylate anions (Ou Yang et al., 1990). Roberts

et al. (1986) found that PA raised the polarization value for membranes. Thus, addition of PA to the isolated organelles may be responsible for the higher energization of mitochondrial and symbiotic membranes that may lead to the stimulation in succinate uptake.

The data of Table 1 and Fig. 1–6 show that Put and Cad, which have two amino groups, had significantly greater stimulating effect on succinate and O_2 uptake than Spd and Spm. The reason for this effect might be that the three- and tetraamines are more capable to rigidify the membranes than the diamines (Roberts et al., 1986) which would decrease their stimulating effect on succinate transport and O_2 uptake.

Conclusions

The observed correlation between increased supply of carbon and increased O_2 uptake after PA treatment indicates a specific action of these substances on the permeability of the mitochondrial and symbiotic membranes, which can play an important role in the regulation of nodule nitrogen fixation. It is difficult to explain the mechanism of PA-mediated stimulation of succinate and O_2 uptake into mitochondria, bacteroids and symbiosomes from *G. orientalis* nodules; but it seems appropriate to postulate that the PA physiological role in nitrogen fixation might be associated to a large extent with the changes of metabolite and O_2 transport across mitochondrial and symbiotic membranes.

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References

Altman, A., R. Kaur-Sawhney, A. W. Galston, 1977. Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. Plant Physiol., 60, 570–574.

Day, D. A., G. D. Price, P. M. Gresshoff, 1986. Isolation and oxidative properties of bacteroids and mitochondria from soybean nodules. Protoplasma, 134, 121–129.

Day, D. A., G. D. Price, M. K. Udvardi, 1989. Membrane interface of the *Bradyrhizobium japonicum–Glycine max* symbiosis. Peribacteroid units from soybean nodules. Aust. J. Plant Physiol., 16, 69–84.

DiTomaso, J. M., J. J. Hart, D. L. Linscott, L. V. Kochian, 1992. Effect of inorganic cations and metabolic inhibitors on putrescine transport in roots of intact maize seedlings. Plant Physiol., 99, 508–514.

Hoagland, D. R., D. I. Arnon, 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ., 347, 1–39.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

Ou Yang, L. J., M. K. Udvardi, D. A. Day, 1990. Specificity and regulation of the decarboxylate carrier on the peribacteroid membrane of soybean nodules. Planta, 182, 437–444.

Palmieri, F., M. Klingenberg, 1979. Direct methods for measuring metabolite transport and distribution in mitochondria. Methods Enzymol., 56, 279–301.

Pistocchi, R., F. Antognoni, N. Bagni, D. Zannoni, 1990. Spermidine uptake by mitochondria of *Helianthus tuberosus*. Plant Physiol., 92, 690–695.

Price, G. D., D. A. Day, P. M. Gresshoff, 1987. Rapid isolation of intact peribacteroid envelopes from soybean nodules and demonstration of selective permeability to metabolites. J. Plant Physiol., 130, 157–164.

Roberts, D. R., E. B. Dumbroff, J. E. Thompson, 1986. Exogenous polyamines alter membrane fluidity in bean leaves – a basis for potential misinterpretation of their true physiological role. Planta, 167, 395–401.

Srivastava, S. K., T. A. Smith, 1982. The effect of some oligo-amines and oligo-guanidines on membrane permeability in higher plants. Phytochemistry, 21, 997–1008.

Takeda, Y., K. Samejima, K. Nagano, M. Watanabe, H. Sugeta, Y. Kyogoku, 1983. Determination of protonated sites in thermospermine and in some other polyamines by ^{15}N and ^{13}C nuclear magnetic resonance spectroscopy. Eur. J. Biochem., 130, 383–389.

Torrigiani, P., D. Serafini-Fracassini, S. Biondi, N. Bagni, 1986. Evidence for subcellular localization of polyamines and their biosynthetic enzymes in plant cell. J. Plant Physiol., 124, 23–29.

Udvardi, M. K., D. A. Day, 1997. Metabolite transport across symbiotic membranes of legume nodules. Ann. Rev. Plant Physiol. Plant Mol. Biol., 48, 493–523.

Vincent, J. M., 1970. A Manual for the Practical Study of the Root Nodule Bacteria. IBP Handbook, No. 15, Blackwell Scientific Publications, Oxford.

Young, D. H., H. Kauss, 1983. Release of calcium from suspension-cultured *Glycine max* cells by chitosan, other polycations, and polyamines in relation to effects on membrane permeability. Plant Physiol., 73, 698–702.