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Glycine Uptake into Barley Mesophyll Vacuoles Is Regulated but Not Energized by ATP[1]

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ABSTRACT

[U-^{14}C]glycine uptake into barley (*Hordeum vulgare* cv Hasso) vacuoles was investigated. Glycine (2 millimolar) transport was stimulated two- to fourfold by NaATP. Stimulation was saturable with respect to ATP (1 millimolar) and linear up to 20 millimolar glycine. Stimulation by NaATP was suppressed by Mg^{2+} in equimolar amounts. Neither MgATP nor Mg-inorganic pyrophosphate had any effect on basal transport rate. Thus, the proton motive force can be excluded as the driving force. Uncouplers (valinomycine/carbonylcyanide-*m*-chlorophenylhydrazone) inhibited the basal rate up to 30% but had no influence on NaATP-stimulated uptake. Vanadate had no effect on either basal or NaATP-stimulated uptake. Nonhydrolyzable ATP analogs (adenylyl(β, γ-methylen)-diphosphate or adenylyl-imidodiphosphate) stimulated comparable to NaATP. Other nucleotides (UTP, ADP) had no effect. Some evidence exists that other amino acids (arginine, alanine, isoleucine, phenylalanine) are transported to a certain extent by a similar mechanism. The results indicate a high capacity channel-like translocator that is regulated but not energized by ATP.

In higher plants, the vacuole functions as an intermediate storage space for ions, sugars, and amino acids. Because intact vacuoles can be isolated from monocot plants (17), a number of transport processes across the tonoplast have been investigated, *e.g.* the malate-carrier, which plays an important role in CO_2 fixation in CAM plants (22) and during opening and closing of guard cells (1), the chloride-transport as an example for ion uptake into the vacuole (18) and transport of amino acids. Homeyer *et al.* (13) characterized the Phe uptake into barley vacuoles as a pmf[4]-dependent process specific for aromatic and other lipophilic amino acids (Leu, Ile). Dietz *et al.* (7) found a different mechanism for influx of Ala, Leu, and Glu: uptake was stimulated by MgATP but uncouplers had no effect. Transport was activated as well by addition of thiol reagents in the absence of ATP. Therefore, a pmf-dependent mechanism appeared to be unlikely. NaATP stimulated Ala uptake comparable to MgATP.

Glycine plays a dominant role in photorespiration (20) and high levels of glycine can be found in green plant cells. Barley mutants can accumulate up to 4.7 μmol g^{-1} fresh weight glycine by photorespiration—about 10 times the concentration found in wild types (5). Although much information exists about transformation of glycine into serine in mitochondria (21), not much is known about its transport between the different organelles, peroxisomes and mitochondria. Diffusion of glycine is limited because it is the most polar of all amino acids. It only possesses a hydrogen atom as side chain. Carrier systems for glycine are known from yeast cells (16). So far, little information exists about glycine transport systems in higher plant cells but they can be expected because of its localization in several organelles and its central role in plant metabolism. Fischer and Lüttge (9) found a light-dependent glycine uptake in *Lemna gibba* due to a combination of diffusion and carrier-mediated transport.

This work shows translocation of glycine into the vacuole by a carrier-mediated mechanism. Because we found characteristics different from the Phe and the Ala carrier—ATP in the presence of Mg^{2+} had no effect although two- to fourfold stimulation could be found with NaATP—we postulate a third amino acid transport system at the tonoplast.

MATERIALS AND METHODS

Chemicals and Radiochemicals

If not stated otherwise, all chemicals were from Sigma (Deisenhofen, FRG), Aldrich (Steinheim, FRG), Merck (Darmstadt, FRG), or Boehringer (Mannheim, FRG) and are of the highest purity available. Cellulase TC and Pectinase Boerozym M5 were from Serva (Heidelberg, FRG). Silicon oil (AR 200) was a gift from Wacker Chemie (Munich, FRG). [U-^{14}C]glycine (4.1 GBq $mmol^{-1}$) and ^3H_2O (185 MBq mL^{-1}) were from NEN/Dupont (Dreieich, FRG).

Plant Material

Barley (*Hordeum vulgare* cv Hasso) was a gift from Lochow-Petkus (Bergen, FRG) and was grown in a 14 h light (25°C) and a 10 h dark (14°C) cycle and 70 to 80% humidity.

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[4] Abbreviations: pmf, proton motive force; AMP-PCP, adenylyl(β,γ-methylen)-diphosphate; AMP-PNP, adenylyl-imidodiphosphate; CCCP, carbonylcyanide-*m*-chlorphenylhydrazone.

Preparation of Vacuoles

Vacuoles were isolated from barley mesophyll protoplasts according to the method of Kaiser *et al.* (17) with slight modifications (14). Incubation time with the enzyme solution (2.5% cellulase/0.5% pectinase) varied between 120 and 30 min with time of the year.

Uptake Experiments

Uptake experiments were carried out at room temperature. First, we used the method described by Homeyer and Schultz (14). Later, this method was modified as follows: 330 μL of the incubation medium (30% Percoll, 5 mM DTT, 0.2% BSA, 45 mM K^+-gluconate, 2 mM [U-^{14}C]glycine (40 kBq mL^{-1}), 60 kBq mL^{-1} ^3H_2O, and effectors as indicated in the legends were mixed with 220 μL of a suspension of purified vacuoles, adjusted to 20% Percoll, in 1.5 mL reaction vials (Eppendorf, Hamburg, FRG). At five different time points, 100 μL aliquots were transferred into 400 μL vials (Hartenstein, Würzburg, FRG) and overlayered by 250 μL silicon oil and 90 μL H_2O. Incubation was stopped at given time intervals (between 4 and 40 min) by centrifugation for 20 s at 10,000*g* (Microfuge E; Beckman, Munich, FRG). Seventy-five microliter aliquots were used for counting of radioactivity with a dual label program $^{14}C/^3H$ in a scintillation counter (Beckmann LS 80). Volume of floated vacuoles was determined individually in each assay using rapid equilibration of ^3H_2O between the assay medium and the vacuolar space. Transport rates were calculated by linear regression. This method was superior to the procedure used before because all assays were started in parallel, thus avoiding aging of vacuoles between experiments and at the same time guaranteeing higher homogeneity for all time points.

Enzyme Assays

Activities of marker enzymes were determined as described in the following references: vacuolar marker, α-mannosidase (EC 3.2.1.24) (6); Cyt *c* oxidase (EC 1.9.3.1.) as mitochondrial marker, Homeyer and Schultz (14) with slight modifications; peroxisomal marker enzyme, hydroxypyruvate-reductase (EC 1.1.1.8) (4); cytosolic marker, phosphoglycerate-mutase (EC 2.7.5.3.) (3). Chl content was determined according to Arnon (2).

RESULTS

Characterization of Isolated Vacuoles

Because a new barley species (cv Hasso) was used, purity of the isolated vacuoles was examined with several marker enzymes. As shown in Table I, contaminations were very low and comparable to preparations from other groups. We used the ratio published by Homeyer and Schultz (14) of 1 mg Chl/5.2 $\times$ 10^6 protoplasts and of 1 vacuole/protoplast, as can be assumed for mature cells, to relate all measurements to number of protoplasts. Vacuole numbers were estimated by α-mannosidase because this enzyme is located exclusively in the vacuole. Volume of vacuoles was determined to 100 μL (10^7 vacuoles)$^{-1}$ by measuring the vacuolar diameter using a light microscope under standard conditions. Volume of vacuoles was calculated by integration. Because the relation between volume and number of vacuoles differs depending on time of the year and even minor experimental modifications, *e.g.* presence of uncouplers, all transport rates are based on volume and are given in nmol [U-^{14}C]glycine · 100 μL^{-1} · min^{-1}. Volume was determined individually in each measurement (see "Materials and Methods").

Characteristics of Glycine Uptake

Figure 1 shows the ATP dependence of glycine uptake. Neither MgATP (A) nor MgPPi (B) had any effect on transport rates. NaATP in the absence of Mg^{2+} salts stimulated 2.5-fold (C). In single experiments, we found up to fourfold stimulation with NaATP (data not shown). ATP-stimulated transport was inhibited by neither uncouplers (valinomycin/CCCP) (C) nor vanadate (D). Thus, the pmf as the driving force and a direct phosphorylation mechanism can be ruled out. Interestingly, uncoupling by CCCP/valinomycin suppressed the basal rate up to 40%. We will discuss this result later in context with other experiments. In all experiments, glycine uptake was linear with time up to 30 or 40 min. An example for glycine uptake in the presence of NaATP is shown in Figure 2. Only five different time points were taken in standard experiments.

Effect of Cations

A number of ionic transport processes are known, *e.g.* the Na^+/H^+ antiport (10), the Na^+/amino acid antiport in bacteria (24), and the vacuolar Ca^{2+}/H^+ antiport (23). To make sure that ATP and not Na^+ was the effector in the experiment

Table I. *Purity of Isolated Vacuoles*

All values are given in percentage of activity in protoplasts. Numbers of vacuoles were determined by α-mannosidase. Contamination of isolated vacuoles by chloroplasts were determined by mg Chl; cytosolic, mitochondrial, and peroxisomal contaminations were measured with marker enzymes phosphoglycerate mutase, Cyt *c* oxidase, and hydroxypyruvate reductase, respectively.

Source of Data	Chloroplasts	Cytosol	Mitochondria	Peroxisomes
Our measurements	0.47		0.0	0.09
Kaiser *et al.* (17)	0.40	0.50		
Martinoia *et al.* (18)	0.20	0.90	0.0	
Homeyer and Schultz (14)	0.22	0.53	0.0	0.26

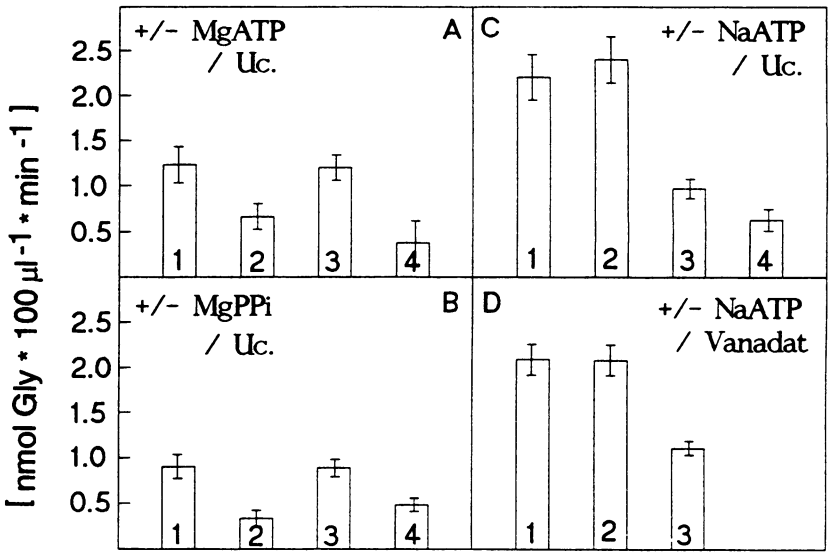

Figure 1. Comparison of the effect of MgATP, MgPPi, and NaATP on glycine (2 mM) uptake. The assay medium was as described in "Materials and Methods." Mg-gluconate (5 mM) was present in all experiments of Figure 1, A and B. Where indicated either a combination of 6 µM CCCP and 5 µM valinomycin (A–C) or 0.4 mM vanadate (D) was used as effectors. Bars 1, 5 mM MgATP, MgPPi, and NaATP, respectively; Bars 2, (1) plus CCCP/Val or vanadate. Bars 3, standard = no additions; Bars 4, (3) plus CCCP/Val. All data represent the mean value of four different experiments with five time points each. Uc., Uncouplers.

shown in Figure 1 (C and D), we investigated the influence of Na^+ on transport rates in the absence and presence of ATP (Fig. 3). From these experiments, we conclude that ATP, not Na^+, is responsible for stimulation. Other cations (K^+, Ca^{2+}) were investigated with the same result (data not shown). From the results shown in Figure 1 (A and C), it was obvious that the presence of Mg^{2+} altered the interaction between the translocator and ATP. Mg^{2+}/ATP complexes are formed with high affinity. In a large number of membrane transport systems—V-ATPase (27), F_1-ATPase (15), Na/K^+-ATPase (25), and PPase (26)—the Mg-substrate complex is the exclusive substrate. In Figure 4, the Mg^{2+} dependence of NaATP-driven glycine uptake is presented. Obviously, Mg^{2+} strongly inhibits ATP-dependent glycine transport. The ATP dependence of glycine uptake (Fig. 5) is basically the reciprocal of Figure 4. Stimulation occurs at concentrations higher than 1 or 2 mM ATP. From Figures 4 and 5, we conclude that small contaminations of Mg^{2+} (1–2 mM) are still present in the incubation medium, *e.g.* caused by bursted vacuoles. From earlier tests (data not shown), we knew that complete removal of mono- or bivalent cations from the incubation medium leads to destabilization of the tonoplast and presumably of its protein complexes as well. Therefore, EDTA titration of Mg^{2+} was not indicated. Assuming a residual Mg^{2+} concentration of approximately 2 mM forming a strong 1:1 MgATP complex, both figures show that stimulation of ATP occurs exclusively with ATP noncomplexed with Mg^{2+}.

Concentration Dependence of Basal and ATP-Dependent Glycine Uptake

Figure 6A shows the concentration dependence of glycine transport in the absence and presence of NaATP. Fischer and Lüttge (9) found a comparable biphasic characteristic for

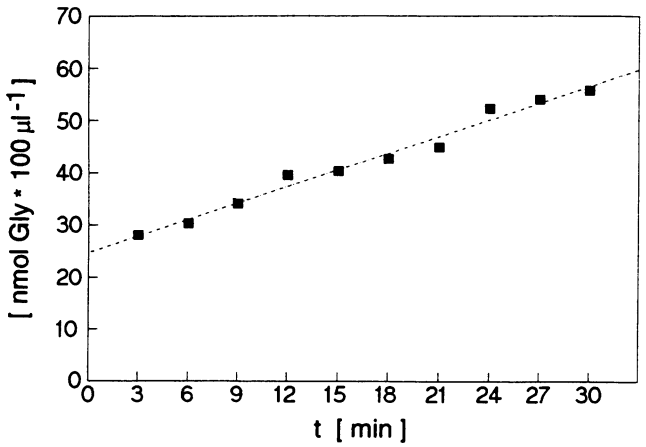

Figure 2. Time course of NaATP-stimulated glycine uptake. Shown are the original data of one single experiment. NaATP concentration was 5 mM.

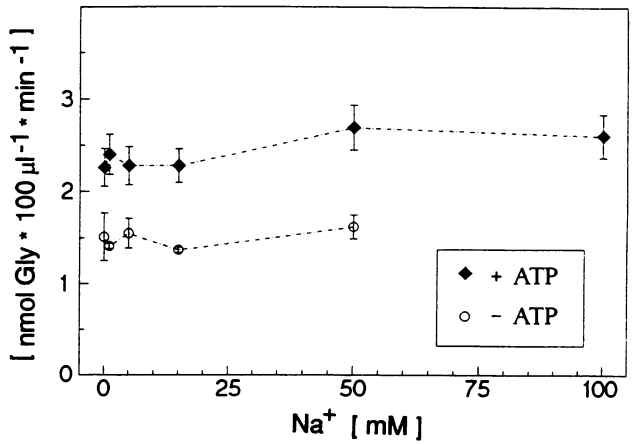

Figure 3. Influence of Na^+ on ATP-stimulated glycine transport. The assay was carried out as described for Figure 1. Where indicated, KATP was present at a final concentration of 10 mM. Mg-gluconate (1 mM) was added to the assay in the presence of ATP. Data represent the mean value of two different experiments with five time points and two replicates per experiment.

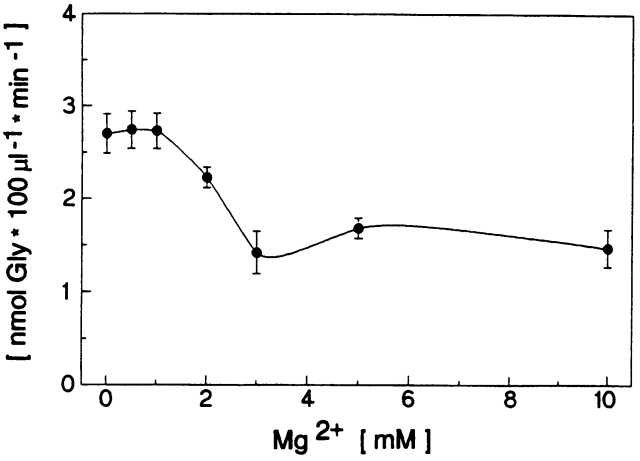

Figure 4. Effect of Mg^{2+} on ATP-stimulated glycine uptake. The experiment was repeated as described for Figure 3. Mg^{2+} was added as Mg-gluconate. NaATP was present at a final concentration of 5 mM.

glycine uptake in *Lemna gibba*. There, glycine uptake was stimulated by light. The linear part of the curve in the absence of ATP in Figure 6A (from 5–20 mM glycine) was assumed to be due to diffusion and was subtracted from both curves. Figure 6B represents the corrected data. Similar corrections were used by Grimm *et al.* (11) for sucrose influx into the phloem and Martinoia *et al.* (18) for chloride transport into barley vacuoles. Glycine uptake in the absence of ATP occurs with a low rate and is saturable at about 5 mM glycine with a K_m of approximately 0.5 mM. ATP-stimulated transport, however, is linear up to 20 mM glycine. A similar characteristic was found for arginine uptake into barley vacuoles (19).

Nucleotide Specificity

Activation is highly nucleotide-specific (Fig. 7). Neither UTP nor ADP had any influence compared with the basal rate. Nonhydrolyzable ATP-analogs (AMP-PCP and AMP-PNP) stimulated transport in the same range as ATP, although presumably with lower affinity to the carrier; full stimulation was reached only at a final concentration of 10 mM of the analog whereas ATP stimulation was saturated at about 2 mM ATP (Fig. 5).

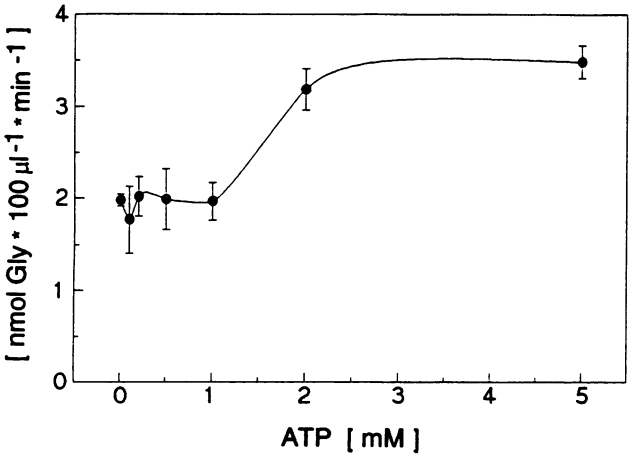

Figure 5. ATP dependence of glycine uptake. Two different experiments were carried out as described for Figure 3. ATP was given as NaATP.

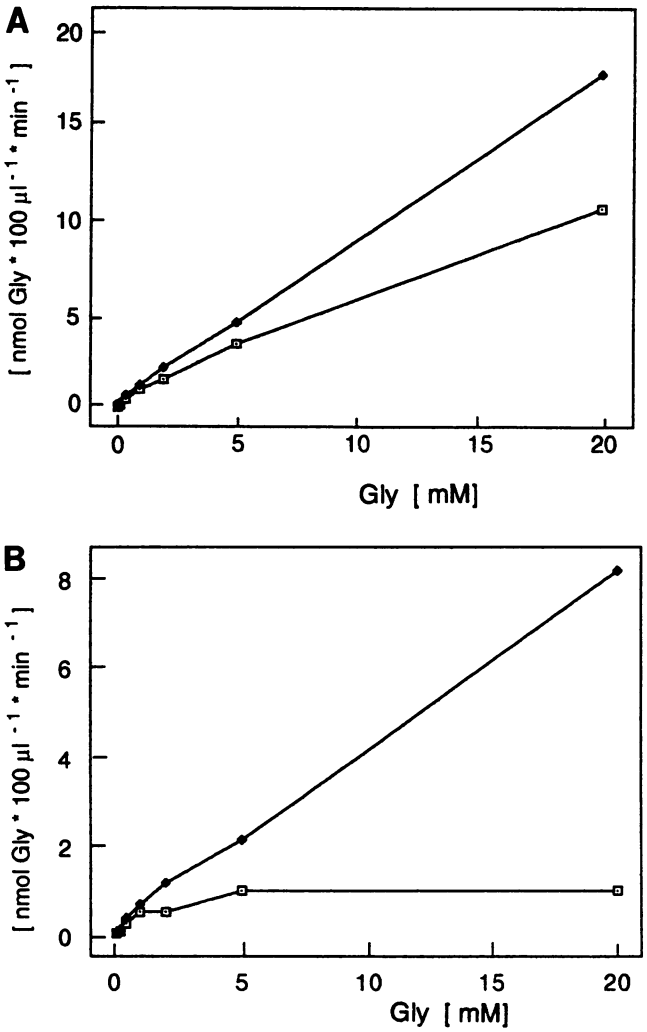

Figure 6. A, Concentration dependence of glycine transport in the absence and presence of ATP. Data are based on two different experiments as listed in the legends of Figure 3. Where indicated, 5 mM NaATP was present. B, Replot of the data of A after subtraction of a diffusion component, the linear part of the curve in the absence of ATP (from 5–20 mM glycine). For further explanations, see text.

Amino Acid Specificity

Table II shows the effect of amino acids on glycine (1 mM) uptake. We chose these specific amino acids because they are representatives for different transport systems characterized by us and other groups. Only 10 mM Phe has a significant effect on glycine uptake. Because NaATP-stimulated transport is not saturable up to 20 mM glycine, a transport of Ala and Arg by the same system is not excluded by these results.

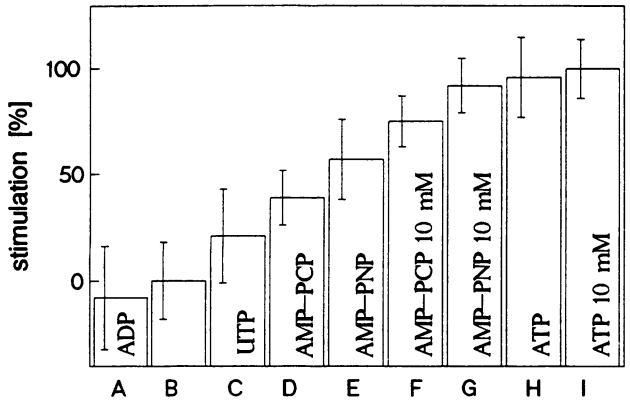

Figure 7. Nucleotide specificity of glycine uptake. Two different sets of experiments were carried out as described for Figure 3. B stands for the standard assay without any further additions. Nucleotides were present at a final concentration of 5 mM at A, C, D, and E and at 10 mM at F, G, and I. One hundred percent corresponds to the maximal stimulation in the presence of 10 mM NaATP and was 2.09 ± 0.37 nmol Gly·100 $\mu L^{-1} \cdot min^{-1}$.

Phe presumably blocks the low capacity saturable component dominant at low glycine concentrations (see Fig. 6B).

DISCUSSION

From our results, we conclude that glycine uptake into the vacuoles is accomplished by a combination of three different mechanisms: (a) a low diffusion rate, (b) a low-capacity saturable component, and (c) a high-capacity nonsaturable transport system dependent on NaATP. Because glycine possesses basically no side chain and the carboxy and the amino group are charged at physiological pH, the membrane permeability for glycine is low and diffusion is limited. This was shown by Driessen *et al.* (8) for lactic acid bacteria. The second transport system can possibly be assigned to side activities of other amino acid transport systems with transport dependent on the pmf. In this case, the constitutive ΔpH after isolation of vacuoles is sufficient to drive glycine influx without further addition of MgATP or MgPPi. Both, more than 50% inhibition of glycine uptake by 10 mM Phe (Table II) and the effect of uncouplers on the basal rate (Fig. 1), favor this explanation.

The most important transport system for glycine is the NaATP-dependent process. Influx can be stimulated up to fourfold compared with the basal rate. In contrast with the Ala system characterized by Dietz *et al.* (7), MgATP had no effect. As already mentioned in "Results," a direct influence of the pmf beside a stabilization of vacuoles can be ruled out because neither MgATP nor MgPPi had any effect. No uncoupling of the NaATP-driven transport could be observed. From the influence of Mg^{2+} shown in Figure 4 and the ATP saturation curve in Figure 5, we conclude that only ATP not complexed with Mg^{2+} functions as effector. Because vanadate had no effect and nonhydrolyzable ATP-analogs (AMP-PCP and AMP-PNP) stimulated comparable to ATP, a direct phosphorylation mechanism can be excluded as well. Direct energization of the carrier system through hydrolysis of ATP to ADP and Pi is ruled out because neither AMP-PNP nor AMP-PCP can be hydrolyzed in the β,γ-position. A possible hydrolysis to AMP and PPi could not be tested because no corresponding ATP analogs are available. Arg uptake into barley vacuoles follows similar characteristics (19). Because of the nonsaturability up to 20 mM amino acid and the ATP effect, we presume that our, and possibly the Arg-system, represent a channel-like translocator with high capacity where ATP only functions as an allosteric effector, not as energy source.

Table II. *Influence of Other Amino Acids on ATP-Driven Glycine Uptake*

Glycine concentration was 1 mM. NaATP was present throughout at a final concentration of 10 mM. Data represent the mean value of two different experiments with five time points and two replicates per experiment.

Additions	Concentration	Rate of Glycine Uptake	% of Control
	mM	*nmol Gly·100 $\mu L^{-1} \cdot min^{-1}$*	
Control		1.19 ± 0.11	100
+L-Phe	1	1.20 ± 0.14	100, 5
	10	0.53 ± 0.18	44, 5
+L-Ala	1	1.15 ± 0.06	96, 9
	10	0.75 ± 0.16	63, 1
+L-Arg	1	1.17 ± 0.14	97, 7
	10	0.84 ± 0.17	70, 7

Physiological Significance

Glycine mainly occurs during photorespiration where it is shuttled effectively between mitochondria and peroxisomes. The vacuolar glycine carrier as well as the Arg transport system (19) might present a "valve" for accumulating amino acids at high light intensities, corresponding to high rates of photorespiration and amino acid synthesis supported by a high assimilatory force (see Heber *et al.* [12]). Thus, the

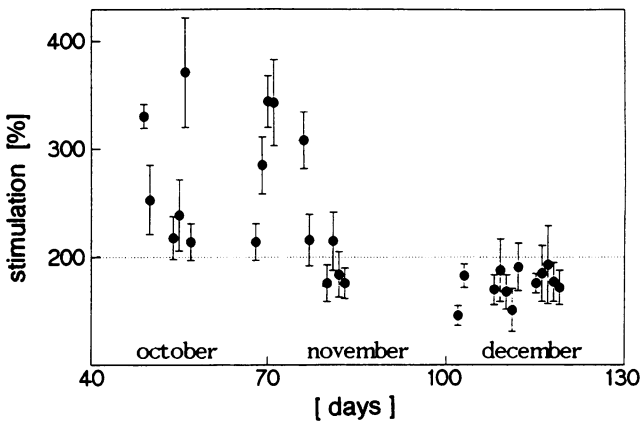

Figure 8. Change of ATP stimulation of glycine uptake with time of the year. Shown is percentage of stimulation of glycine uptake with 5 mM NaATP with respect to an ATP free standard. One hundred percent corresponds to the ATP free standard in each individual experiment. All data are based on two parallel experiments with at least five time points each.

synthesized end products could be removed effectively from the cytosol into the vacuole and feedback down-regulation of the synthesizing systems would be prevented. Preliminary data (Fig. 8) point to a variation of ATP-stimulated transport with respect to time of the year, a decrease of stimulation with the coming of winter. Because winter is a time of little assimilatory activities, a system as described above, a fast overflow mechanism in times of high metabolic activity, would be unnecessary. A possible explanation for the smaller stimulation in winter could be lower presence of carrier proteins. For further investigations of amino acid and other transport systems in plants, this seasonal variation should not be neglected.

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