Calcium Modulation of Polyamine Transport Is Lost in a Putrescine-Sensitive Mutant of Neurospora crassa

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Received August 3, 1990, and in revised form November 12, 1990

Putrescine transport in Neurospora is saturable and concentrative in dilute buffers, but in the growth medium putrescine simply equilibrates across the cell membrane. We describe a mutant, puu-1, that can concentrate putrescine from the growth medium because the polyamine transport system has lost its normal sensitivity to Ca\(^{2+}\). The wild type closely resembles the mutant if it is washed with citrate and ethylene glycol bis(\(\beta\)-aminoethyl ether)\(N,N'\)-tetraacetic acid. The mutant phenotype also appears in the wild type after treatment with cycloheximide. The results suggest that putrescine uptake is normally regulated by an unstable Ca\(^{2+}\)-binding protein that restricts polyamine uptake. This protein is evidently distinct from the polyamine-binding function for uptake, which is normal in mutant and in cycloheximide-treated wild type cells. The puu-1 mutation, stripping of Ca\(^{2+}\), and cycloheximide treatment all cause an impairment of amino acid transport, indicating that other membrane transport functions rely upon the product of the puu-1 gene. Preliminary evidence suggests that the putrescine carrier is not the Ca\(^{2+}\)-sensitive, low-affinity K\(^+\)-transport system, but K\(^+\) efflux does accompany putrescine uptake.

Most organisms take up polyamines (putrescine, spermidine, and spermine) by an active transport system, despite their ability to make these compounds internally. The system may allow adjustments to the polyamine pools of cells of multicellular organisms; it may serve polyamine catabolic pathways; and it may even allow accumulation of putrescine as an osmoticum.

Neurospora crassa makes polyamines, but does not use them as catabolic substrates. The organism has a polyamine transport system that takes up all three natural polyamines with moderate affinity (\(K_m\)'s: putrescine = 600 \(\mu\)M; spermidine = 240 \(\mu\)M; spermine = 70 \(\mu\)M) from a dilute buffer containing 20 mM Na\(^+\) (1). In the growth medium, however, this system is inhibited by monovalent cations and by Ca\(^{2+}\), and all transport of polyamines takes place by way of a nonsaturable, diffusional system that equilibrates polyamines across the cell membrane (1, 2). The concentrative uptake system appears only in Ca\(^{2+}\)-free buffers having low monovalent cation concentrations. We have in fact questioned whether the natural substrates of the system include the polyamines (1).

We describe here a mutant of \(N.\ crassa\), puu-1, able to concentrate polyamines from low concentrations in the medium, owing to a loss of inhibition of the polyamine uptake system by cell-bound Ca\(^{2+}\). The strain is intoxicated by polyamines in concentrations to which the wild type is indifferent. The results suggest that a component of the cell membrane protects \(N.\ crassa\) from absorbing toxic levels of polyamines—and perhaps other cations—from the environment. The accompanying paper (3) defines the discretionary capacity of \(N.\ crassa\) for polyamines and its intracellular location, using the puu-1 and another putrescine-accumulating strain.

MATERIALS AND METHODS

Strains, media, growth, and genetic techniques. The wild type strain, ORS6a, a strain containing the puu-1 allele, GB1 (IC2296-1a) and (in two experiments) a strain carrying both the puu-1 and the arg-2 (arginase-less, allele UM906) mutations (IC2296-65a) were used in physiological work. GB1 is the only known puu-1 allele. The alcoy stock was used to localize puu-1 genetically in initial studies (4). Other mutant stocks bearing combinations of the following mutations were used in genetic mapping and other work: spe-1 (PE4), arg-2 (CD80), cot-1 (C102), inl (89801), and the permease mutations pmb, pmn, and pmg. All genes are described in the compendium of \(N.\ crassa\) mutants of Perkins et al. (5).
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Standard techniques and media were used for most microbiological and genetic work (6). Vogel's medium N was used for growth studies and stock maintenance. Westergaard's medium was used for genetic crosses. Exponential cultures were grown in boiling flasks, with forced aeration from a glass tube reaching to the bottom (6). Stationary growth was carried out in 10 ml Vogel's medium in 50-ml Erlenmeyer flasks at 32°C. In genetic analysis of the puu-1 mutant, the sensitivity of the puu-1 mutant to 5 mM putrescine in the medium was used to score the marker.

The concentrations of the main salts of Vogel's medium, which we tested individually for their effect on putrescine uptake are as follows: 5 mM Na\textsubscript{2} citrate, 25 mM NH\textsubscript{4}NO\textsubscript{3}, 37 mM K\textsubscript{2}HPO\textsubscript{4}, 0.8 mM MesO\textsubscript{4}, and 0.7 mM CaCl\textsubscript{2}. Trace elements were added at the following concentrations (\mu\textsubscript{M}): Zn, 17; Fe, 2.6; Cu, 1.0; Mn, 0.3; Mo, 0.2.

**Metabolite uptake and determination.** Uptake and excretion of polyamines were measured as described previously (1, 2). Cells were washed gently on Miracloth (Calbiochem), without the compression of severe vacuum filtration when transferring cells from the growth medium to the buffer in which transport was measured. \textsuperscript{14}C-labeled polyamine uptake rates were measured at 25°C in normal or variously modified growth medium (Vogel's medium N), and in Mops-glucose\textsuperscript{b}, buffer, pH 7.2 (10 or 20 mM NaOH neutralized to pH 7.2 with 3-(N-morpholino)propanesulfonic acid, and 0.2% glucose). The buffer will be referred to as Mops buffer, and the Na\textsuperscript{+} concentration will be specified in text, figures, and tables. Substrate saturation curves were performed with different concentrations of polyamine for 5 min (putrescine) or 15 min (spermidine) intervals, and \textit{K}_{\text{m}} and \textit{V}_{\text{max}} determinations were drawn from curves from which the nonsaturable component was subtracted (1). In this work, as in previous work (1), the range of variation of the rate of the nonsaturable transport is about twofold; that of the saturable system of wild type is about 50%, presumably owing to the variation in Ca\textsuperscript{2+} remaining bound to the cells (this work).

For most experiments, we measured transport activity with 1 mM putrescine over a 15-min interval. For the wild type in conditions where uptake was diffusional, uptake was not linear. Instead, internal and external putrescine equilibrated in about 7 min. This component of uptake also prevailed in the puu-1 mutant. In comparison of strains, the diffusional component represented an unacceptably high "blank" (of about 0.2–0.6 nmol per minute per milligram dry weight) in 5-min assays where the saturable system had low activity. The longer interval of uptake rendered the active component more conspicuous and, after subtraction of the diffusional blank, more accurate. Data in the later experiments are reported in terms of nanomoles per milligram per 15 min, without subtracting the diffusional component.

We used doubling time (in minutes) and polyamine pool sizes (see Results) to calculate steady-state parameters of influx and efflux. The rationale was that of Karihu et al. (7), based simply on a growth equation (\textit{A}_{t} = \textit{A}_{0}e^{kt}) where \textit{A}_{t} and \textit{A}_{0} are the values at the end and beginning of an interval (1 min); \textit{t} is time in minutes; and \textit{k} is the growth constant (in 2 divided by doubling time).

Uptake of \textsuperscript{14}C-labeled amino acids of known specific radioactivities was measured by methods similar to those used for polyamine uptake. Activities of three amino acid uptake systems were measured at 25°C for 2 min. The general system substrate was 1 mM \textsuperscript{14}C-arginine in the presence of 0.1 mM L-arginine (9); and the basic system was measured with 0.1 mM \textsuperscript{14}C-arginine in the presence of 0.1 mM leucine (10).

\textit{K}_{\text{m}}, Na\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} were determined by atomic absorption spectroscopy as described previously (11). Polyamine extraction and determination by high performance liquid chromatography were done as described previously (12). K\textsuperscript{+} efflux was measured by loss of cellular K\textsuperscript{+} in various conditions, including unbuffered water, to which certain transport substrates were added. Cells (2–6 mg dry weight equivalent) were collected on 5-um membranes and extracted with trichloroacetic acid, and K\textsuperscript{+} in the extract was determined after appropriate dilution.

Large molar excesses (e.g., 100-fold) of putrescine over spermidine prevailed in putrescine-containing media where small amounts of spermidine excretion might have occurred. Dansylation of spermidine would be compromised under these conditions. We therefore not only isolated polyamines from media on Dowex-50W columns (0.7 × 7.5 cm, 200–400 mesh, 8% crosslinking, H\textsuperscript{+} form), but also separated putrescine and spermidine. Medium (2 ml) from a culture was applied to the column, then the column was washed with 15 ml 1.5 N HCl, and 6 ml of 3.25 N HCl. Putrescine was then eluted in 6 ml 3.5 N HCl; following this, spermidine was eluted in 5 ml 6 N HCl. Spermine (2 mmol) was added as an internal standard for spermidine, and emerged in the 6 N HCl fraction. There was virtually no overlap of putrescine with the other polyamines. Fractions were evaporated to dryness, redissolved in a small amount of HClO\textsubscript{4}-EDTA buffer (12), and dansylated as usual.

G.R.B. and A.D.H. were largely responsible for the isolation and genetics of the puu-1 mutant; R.H.D. and J.L.R. performed the remainder of the work.

**RESULTS**

**Genetics of the puu-1 Mutant**

Strains carrying spe-1 mutations lack ODC and require at least 0.4 mM putrescine for optimal growth (13). A strain carrying a spe-1 mutation was plated (without mutagenesis) on medium containing 0.05 mM putrescine, which normally supports no growth. A single colony appeared and was isolated. It retained an absolute polyamine requirement. Growth of the variant was severely inhibited by 5 mM putrescine, which hardly impaired growth of the parent strain. Genetic work on the variant and its descendants defined a mutation at a new locus, puu-1 (putrescine uptake). The mutation is unrelated to spe-1, which lies on Linkage Group V. Using the putrescine-sensitive phenotype to analyze crosses with alcoy and with cot-1, we localized the mutation to Linkage Group IV. A three-point cross showed that puu-1 lay between cot-1 and arg-2 (Table I).

Additional mutants with the puu-1 growth phenotype were selected after mutagenesis. The severity of the puu-1 mutation varied, with some mutants requiring as much as 10 mM putrescine for optimal growth. A group of mutants was isolated which required at least 10 mM putrescine for growth (Table I).

**TABLE I**

| Progeny of the Cross cot-1 puu-1 arg\textsuperscript{*} × cot\textsuperscript{*} puu\textsuperscript{*} arg-2 |
|---------------------------------|
| Progeny genotype | Number |
|-------------------|--------|
| cot-1 puu-1 arg\textsuperscript{*} | 108 |
| cot\textsuperscript{*} puu\textsuperscript{*} arg-2 | 134 |
| cot\textsuperscript{*} puu-1 arg\textsuperscript{*} | 1 |
| cot-1 puu\textsuperscript{*} arg-2 | 21 |
| cot-1 puu-1 arg-2 | 5 |
| cot\textsuperscript{*} puu\textsuperscript{*} arg\textsuperscript{*} | 8 |
| cot\textsuperscript{*} puu-1 arg-2 | 0 |
| cot-1 puu\textsuperscript{*} arg\textsuperscript{*} | 0 |

Abbreviations used: Mops, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(\beta-aminomethyl ether) N,N'N'-tetrasacetic acid.
trencine uptake or inhibition phenotypes varied widely, and most suffered growth or morphological defects that made them difficult to work with. None was allelic to puu-1. Reversions of the puu-1 mutant were similarly variable. Almost all were due to unlinked suppressor mutations, and none lacked the polyamine transport system. Limited genetic tests showed many reversion events to be nonallelic to one another. Because of the pleiotropy of puu-1 and the diversity of mutations that duplicated or reversed its effect, further genetic studies were deferred in favor of characterizing the original mutant in detail.

A heterokaryon containing arg-2 puu-1 inl+ and arg+ puu+ inl nuclei grew well in minimal medium supplemented with 5 mM putrescine. This indicated that the puu-1 mutation was recessive to its wild type allele and thus represented a deficiency or loss of function.

Altered Transport Characteristics of the puu-1 Mutant

Wild type Neurospora cannot concentrate polyamines from Vogel's medium, because the saturable polyamine transport system is inhibited (1, 2). Putrescine and spermidine equilibrate across the cell membrane by a diffusional mechanism (2). In the same medium, the puu-1 strain displays saturable uptake of putrescine and spermidine (Fig. 1). This accounts for the origin of the puu-1 mutation by the selection method we used. The final slopes of the lines in Fig. 1 revealed that the strains shared the diffusional component of uptake. The apparent Km's of the puu-1 strain were about 3 mM for both substrates under these conditions. After a wash and resuspension in Mops buffer (20 mM Na+), both strains displayed saturable polyamine uptake, with normal Km's for putrescine (0.3–0.7 mM) and spermidine (0.25 mM) (1); the Km's were lower in buffer owing to lack of competitive inhibition by salts in the medium. The Vmax of uptake by puu-1 strains in Mops buffer was 2.5 to 8 times greater than that of the wild type. The putrescine uptake rate and Km of both strains were insensitive to pH variation between 5.2 and 8.2 (data not shown).

The putrescine pool of both Puu+ and Puu− strains grown in minimal medium is approximately 0.8 to 1.0 nmol per milligram dry weight (1, 3). The wild type, grown in the presence of 1 mM putrescine, has a putrescine pool of about 2 to 3 nmol per milligram, or approximately 1 mM in cell water [2.5 μl per milligram dry weight (14)]. This bears out our previous finding that putrescine equilibrates diffusionaly across the cell membrane (2). However, in 1 mM putrescine, the puu-1 strain contains approximately 120 nmol putrescine per milligram, nominally about 48 mM, indicating considerable concentrative transport. The molar value is belied by the fact that most of the putrescine is in the vacuole (3). (If 1 mM spermidine is added to puu-1 cells, the spermidine pool becomes two- or threefold normal. Spermidine at higher concentrations does not seriously inhibit growth.) The difference between strains in their ability to accumulate putrescine is roughly correlated with the Vmax of the strains' saturable uptake systems (see below).

During steady-state growth of either strain in putrescine, the influx rate should be the same as the rate of efflux plus whatever is required to maintain polyamine pools during growth. If the high pool of putrescine in puu-1 was primarily the result of impaired efflux, influx rates at steady-state would be normal or low. If the high pool was owing to a higher rate of influx, the influx rate at steady-state should remain high. A test of these expectations showed that puu-1 uptake at steady-state was much higher than that of the wild type, indicating that influx is altered in the mutant (Fig. 2).

We also calculated the amount of putrescine uptake required to maintain the putrescine and spermidine pools of the two strains during growth in 1 mM putrescine (3), assuming no contribution from biosynthesis. This showed that the measured rate of uptake at steady state (0.11 and 0.5 nmol per minute per milligram for wild type and puu-1, respectively, from Fig. 2) roughly approximated the need (0.14 and 0.6 nmol per minute per milligram, respectively). Only a very small efflux was expected from wild type, and, indeed, little efflux from wild type can be seen upon transfer from 1 mM putrescine to minimal medium. A small, but definite efflux (0.1 nmol per minute per milligram) was actually seen for puu-1 in a parallel experiment, in keeping with its higher intracellular putrescine concentration. Spermidine efflux was not detected.
FIG. 2. Uptake of [14C]putrescine by cultures during steady-state growth in the presence of 1 mM putrescine. Steady-state cultures of wild type (open circles) and puu-1 (closed circles) strains growing in the presence of 1 mM putrescine were washed and introduced to fresh medium containing 1 mM putrescine. A small amount of [14C]putrescine was added to each. Cells (of 2 ml culture) were then harvested at the times indicated and counted.

in either strain, either in the parent culture or in the minimal medium to which it was transferred. These calculations and observations show that the primary effect of the puu-1 mutation is on influx, rather than efflux of putrescine.

The reason that putrescine efflux from puu-1 was not much greater is that excess intracellular putrescine under these conditions is largely vacuolar (3). In the accompanying paper, we show the vacuolar accumulation of putrescine in puu-1 is the result, not the cause, of putrescine accumulation in the cell itself.

Differential Sensitivity of Wild Type and puu-1 to Calcium

Most cations of the medium inhibit putrescine uptake, the effect of Ca2+ being the most severe (1). Unlike omissions of other cations, omission of Ca2+ from the medium led to an increased putrescine uptake rate in the wild type and a slightly decreased uptake rate in puu-1 (Fig. 3). Omission of Ca2+ also allowed the wild type to accumulate a substantial putrescine pool from media containing 1 mM of the diamine (Fig. 4). Varying the Ca2+ concentration of the medium reveals an extreme difference in the sensitivities of wild type and puu-1 cells (Fig. 4). The data suggested that the competitive inhibition of putrescine uptake by Ca2+ previously described for wild type (1) might be less strong in puu-1.

This idea was tested by testing washed cells of the two strains in Mops buffer for the sensitivity of putrescine uptake to Ca2+. Unexpectedly, putrescine uptake was found to be equally sensitive in the two strains (Fig. 5), although they had different $V_{\text{max}}$ for transport. The discrepancy between the strains' sensitivities to Ca2+ in Mops buffer (Fig. 5) and in growth medium (Figs. 3 and 4) was traced to citrate, a metal chelator present at 8 mM in the medium (Table II, Experiment I).

If Ca2+ inhibited putrescine uptake solely by competitive inhibition (Fig. 5), citrate should mitigate the inhibition in both strains to the same degree by reducing the concentration of the free metal. As noted, this was not the case. The differential effect of citrate on the two strains with respect to Ca2+ inhibition therefore suggested (i) that Ca2+ had a second inhibitory action on wild type; (ii) that a high-affinity Ca2+ binding site was involved, such that citrate was only weakly effective against added Ca2+; and (iii) the puu-1 mutant lacked sensitivity to this action of Ca2+, which accounts for its ability to concentrate putrescine from the growth medium. Further experiments supported this scenario.

Cell-Bound Calcium Inhibits Putrescine Uptake Only in Wild Type

Na+ citrate stimulated putrescine uptake by the wild type, even in the absence of Ca2+ (Table II). In the same experiment, Na+ citrate inhibited uptake by puu-1, an effect exerted by the Na+ counterion (NaCl had a similar effect). The stimulation of the wild type system by citrate (actually minimized by the Na+ counterion) suggested that transport in the wild type was controlled by a chelatable factor firmly bound to the cell surface. Ca2+ added back in the presence of citrate inhibited putrescine uptake only in the wild type (Table II). These data, like the effect of
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FIG. 4. Steady-state polyamine pools of mycelia grown in the presence of 1 mM putrescine and varying concentrations of Ca²⁺. Wild type (open circles) and puu-1 (closed circles) were harvested from cultures and putrescine (top) and spermidine (bottom) contents were determined.

Ca²⁺ omission from the medium, suggest that the citrate-chelatable factor originally bound to wild-type cells was Ca²⁺.

Using puu-1 and wild type strains, we compared the effect of a brief rinse of cells in 1 mM EGTA with a 2-min preincubation in 10 mM Na₃ citrate plus 10 mM EGTA, each followed by a wash in Mops buffer (Fig. 6). The brief rinse left the two strains with different uptake rates, and with opposite responses to added citrate (Fig. 6A, 0 Ca²⁺), as seen before. The effect of Ca²⁺ bore out previous experience: Ca²⁺ inhibited both strains similarly, but citrate mitigated this effect only in the mutant (Fig. 6A). The strong chelator wash, however, rendered the wild type phenotype almost identical to that of the mutant (Fig. 6B). At 0 Ca²⁺, the putrescine uptake rates were the same; Na₃ citrate inhibited the strains similarly (via the Na⁺ counterion); and now added Ca²⁺ inhibited neither strain when citrate was present.

This experiment illustrates the membrane-bound nature of the chelatable element and introduces another phenomenon: the lability of the Ca²⁺-binding site. First, briefly rinsed wild type cells retain their low rate of transport, suggesting that the inhibitory Ca²⁺ is still bound to the cells. The further inhibition of transport by added Ca²⁺ is owing to the competitive inhibition common to the two strains (Fig. 5). Briefly rinsed wild type cells, tested in the presence of 10 mM citrate and the absence of Ca²⁺, release cell-bound Ca²⁺, and have an increased rate of uptake (Fig. 6A). However, when more than 0.5 mM Ca²⁺ is also present, it inhibits, owing to the relative ineffectiveness of citrate in protecting the high-affinity binding site from Ca²⁺. The latter site must be missing from puu-1, inasmuch as Ca²⁺ inhibits the mutant only weakly in the parallel test (Fig. 6A).

Turning to cells washed in strong chelator, freed of the wash medium and tested in Mops buffer, we found the wild type relieved of its endogenous inhibitor, and in the absence of Ca²⁺ or citrate, its uptake rate is even higher than that of puu-1. Moreover, it can no longer be inhibited by Ca²⁺ in the presence of citrate. The latter finding suggests that once Ca²⁺ is definitively removed, its site of action is lost rapidly from wild type cells.

The calcium requirement for growth in stationary liquid culture was similar in the wild type and puu-1, and both strains achieved 18 to 24% of their maximal weight in Ca²⁺-free medium containing 10 mM EGTA. Putrescine did not stimulate growth of Ca²⁺-free cultures. The Ca²⁺ content of puu-1 mycelia grown in minimal medium was somewhat higher than in the wild type, but most of the excess could be removed with a brief rinse in 1 mM EGTA. About 10–13% of the intracellular Ca²⁺ of both strains was found in vacuoles. The growth of both strains was inhibited similarly by Verapamil, a Ca²⁺ blocker; trifluoperazine, which inhibits Ca²⁺-binding proteins; and the Ca²⁺ ionophore A23187, indicating no fundamental dif-

FIG. 5. Inhibition of putrescine uptake by Ca²⁺ (as CaCl₂), measured after a brief rinse in Mops buffer (20 mM Na⁺) plus 1 mM EGTA and tested in Mops buffer (20 mM Na⁺). The absolute values for the saturable component of uptake of wild type (open circles) and puu-1 strains (closed circles) at 0 Ca²⁺ were 0.96 and 7.3 nmol putrescine per milligram per minute, respectively. Inset: reciprocal of uptake rate vs inhibitor concentration.
TABLE II

Effects of Medium Ingredients on Putrescine Transport by Wild Type and puu-1 Mycelia

| Wash medium | Addition to Mops uptake medium | Putrescine uptake (nmol/mg/15 min) |
|-------------|--------------------------------|-----------------------------------|
| Experiment I |                                 |                                   |
| Mops + 1 mM Na₃ EGTA | None | Wild type | puu-1 |
|              | 0.7 mM CaCl₂ | 27 | 166 |
|              | 8 mM Na₃ citrate | 78 | 68 |
|              | 0.7 mM CaCl₂ + 8 mM Na₃ citrate | 13 | 75 |
| Experiment II |                                 |                                   |
| Mops + 1 mM Na₃ EGTA | None | 36 | 137 |
|              | 10 mM Na₃ citrate | 64 | 60 |
| Mops + 10 mM Na₃ citrate + 10 mM Na₃ EGTA | None | 105 | 88 |

* Uptake of putrescine from 1 mM putrescine. Cells were prepared by washing them gently and briefly in Mops buffer (20 mM Na⁺) containing 1 mM Na₃ EGTA, or by incubating for 2 min in the Mops buffer (20 mM Na⁺ with 10 mM Na₃ citrate and 10 mM Na₃ EGTA). In all cases, cells were then washed again in Mops buffer (10 mM Na⁺) and tested therein, plus the indicated additions. The figures for Experiment II are also reported in Figs. 6A and 6B (0 Ca²⁺).

* These values represent the basal, diffusional component of uptake.

Other Characteristics of the puu-1 Mutant

The puu-1 strain is more sensitive than the wild type to paraquat, a poison that enters via the polyamine transport system in some mammalian cells (16). The wild type was unaffected by 10 μM paraquat, while puu-1 was inhibited 64%. La³⁺ (10⁻³ M) inhibited growth of the puu-1 mutant about 40%, whereas the wild type was not affected. We do not know whether La³⁺ is transported into cells, nor whether it competes with polyamines in the transport process.

The puu-1 mutant displays only 20 to 50% of the normal activity of the general amino acid uptake system and a

![Graph](image-url)

**FIG. 6.** Response of putrescine uptake by wild type and puu-1 mycelia to citrate and to CaCl₂ after mild and strong chelator treatments. A, mycelia rinsed briefly in 1 mM EGTA before testing in Mops buffer (10 mM Na⁺) with additives. B, mycelia incubated 2 min in 10 mM EGTA and 10 mM citrate, followed by thorough wash in Mops buffer before test of uptake in Mops (10 mM Na⁺). Open symbols, wild type; closed symbols, puu-1. Circles, no citrate; squares, 10 mM Na₃ citrate during uptake test. The figures for 0 Ca²⁺ in (A) and (B) are also reported in Table II, Experiment II.
TABLE III
Effects of Ca\textsuperscript{2+} on K\textsuperscript{+} Efflux from puu-1 Mycelia following Addition of NaCl\textsubscript{2}, Putrescine, and Arginine

| Addition\textsuperscript{b} | No Ca\textsuperscript{2+} | Plus 0.025 mM Ca\textsuperscript{2+} |
|-----------------------------|--------------------------|----------------------------------|
| None                        | 5                        | 13                               |
| 10 mM NaCl\textsubscript{2} | 50                       | 18                               |
| 1 mM putrescine 2HCl         | 69                       | 59                               |
| 1 mM arginine HCl           | 49                       | 46                               |

\textsuperscript{a} The initial pool of K\textsuperscript{+} was 317 nmol per milligram dry weight, nominally 127 mM.

\textsuperscript{b} In all conditions, 0.2% glucose was present.

The effect of cycloheximide on putrescine transport

Cycloheximide, added to wild type cultures in amounts sufficient to stop protein synthesis, induced the ability to concentrate putrescine from the growth medium. By 60 min after cycloheximide addition, the cells resembled puu-1 (Fig. 7). Tests of the concentration-dependence of polyamine transport in cycloheximide-treated cells in Vogel’s medium showed that they had gained activity for the saturable system (data not shown). The same treatment of puu 1 cells, already displaying the saturable transport system in the growth medium, left their polyamine transport rate (Fig. 7) and pools unaltered. The effect of cycloheximide was through its effect on protein synthesis; mutants (cyh-1 and cyh-2) resistant to cycloheximide by virtue of ribosomal alterations were unaffected by the drug (data not shown). After 10 min incubation in cycloheximide, the general amino acid uptake system of the wild type had declined to 54% of normal; the puu-1 mutant was not tested.

FIG. 7. Response of putrescine uptake to cycloheximide. Cells were grown in normal medium, and uptake was tested in the medium after adding 2 mM putrescine. Circles, untreated; triangles, incubated 60 min after addition of 10 \textmu g cycloheximide per milliliter to the cultures. Open symbols, wild type; closed symbols, puu-1 aga.
cellular Ca\(^{2+}\) content and localization, of the effect of Ca\(^{2+}\)-related antimetabolites, and of the growth requirement for Ca\(^{2+}\) reveal no fundamental derangement of Ca\(^{2+}\) metabolism in the mutant. We therefore infer the existence of a cell-surface protein that, when bound to Ca\(^{2+}\), normally blocks polyamine uptake. This protein or function is labile, because sensitivity of polyamine uptake to Ca\(^{2+}\) is lost upon definitive removal of Ca\(^{2+}\), and upon incubating cells with cycloheximide. The observation that cycloheximide treatment and the removal of Ca\(^{2+}\) do not affect polyamine uptake by the puu-1 mutant shows that these treatments target the same system affected by the puu-1 mutation.

Unlike polyamine transport in N. crassa, removal of membrane-bound Ca\(^{2+}\) impairs transport processes in many other organisms. In fact, it impairs amino acid transport even in N. crassa. Calcium chelators partially inactivate the transferrin receptor of rabbit reticulocytes (19) and the uptake of \(\gamma\)-aminobutyrate in synaptosomes (20). Ca\(^{2+}\) depletion slows net uptake of K\(^+\) in corn root (21, 22); it diminishes lysine, arginine, sulfate, glucose, malate, glyceraldehyde-3-P, and uracil transport in tobacco cells (23, 24). Smith (25) used many of the same treatments we have used in his study of Ca\(^{2+}\)-stimulated serine uptake in tobacco cells. He proposed that Ca\(^{2+}\) allows cells to maintain a high membrane potential, upon which serine transport and retention depends. Cells lose transport activity in the presence of K\(^+\) (which depolarizes cells), but do so much more slowly if CaCl\(_2\) is also present (25). Jones and Jennings (26) made the same general finding with respect to the growth of the fungus Dendryphiella salina. The point was made there that Ca\(^{2+}\) reversed Na\(^+\) inhibition of growth by preventing loss of internal K\(^+\).

Why, then, is polyamine transport in N. crassa uniquely stimulated, rather than inhibited, by removal of Ca\(^{2+}\) (and, by analogy, by the puu-1 mutation)? Three component issues allow us to explore this matter: (i) What drives polyamine uptake? (ii) What is the polyamine carrier? (iii) What is the nature of the puu-1’ product?

Inorganic ion transport in N. crassa has been studied in great detail in the laboratories of C. L. Slayman and C. W. Slayman. The membrane potential, established through the activity of the plasma-membrane H\(^+\)-ATPase (27), is the energy source for most secondary transport (28). The membrane potential drives, via K\(^+\)-H\(^+\) symport (29), the establishment of a substantial K\(^+\) gradient (30). (This requires extrusion of 2H\(^+\), mimicking a 1:1 K\(^+\)-H\(^+\) exchange.) If K\(^+\)-replete cells are held in water, they retain K\(^+\), but upon addition of Na\(^+\), they readily exchange internal K\(^+\) for the external cation (14). This has led the Slaymans (15) to analyze a fluent K\(^+\)/Na\(^+\)/H\(^+\) exchange flux characteristic of N. crassa, depending upon the ions present in the cell and in the external medium. Significantly, Ca\(^{2+}\) inhibits the K\(^+\)/K\(^+\) exchange flux, but not the net K\(^+\) accumulation, about 80%, with a concomitant increase of membrane potential (131); summarized in Ref. (29). In other words, Ca\(^{2+}\) makes the membrane less leaky, and energy otherwise dissipated is spared for improved transport of other solutes, as suggested above.

A substantial net efflux of K\(^+\) occurs in our standard Na\(^+\) MOPS buffer, which contains no added Ca\(^{2+}\). Na\(^+\) entry presumably balances K\(^+\) efflux to a large extent under this condition, as the Slayman laboratories have found (15). Addition of putrescine causes an even greater rate of K\(^+\) efflux in the puu-1 strain, concomitant with demonstrable entry of the amine. This behavior resembles previous observations that without Ca\(^{2+}\), addition of imidazole, histidine, Tris, choline, ethanolamine, triethylamine, and NH\(_4\) all caused loss of K\(^+\) and Na\(^+\) (15). Whether these compounds were taken up at the same time was not directly tested, but the membrane potential was not seriously affected (C. L. Slayman, personal communication).

If the effects of Ca\(^{2+}\) on putrescine uptake are opposite to those on amino acids and, in other organisms, most other solutes, we must consider the possibility that putrescine can become part of the exchange flux involving K\(^+\) and Na\(^+\). In this sense putrescine transport as we have measured it here would not be immediately dependent upon membrane potential, but more directly dependent upon the transmembrane gradient of K\(^+\). Energy is ultimately required to establish this gradient, but we have speculated previously that a residual K\(^+\) gradient may account for energy-independent putrescine uptake (1).

We do not know the identity of the putrescine carrier. We feel that to N. crassa, putrescine is a peculiar substrate for transport into the cell. The fungus does not require external polyamines for growth, nor does it use them as C and N sources (1). The ability of N. crassa to take up polyamines is not an obvious advantage, and we have implied that the “polyamine transport system” may have evolved in relation to other needs entirely (1). Thus polyamine transport may be a gratuitous response to unusual circumstances—the absence of Ca\(^{2+}\) and the presence of polyamine.

It is unlikely that the “polyamine transport system” is one of the K\(^+\) carriers, of which there are high- and low-affinity forms in N. crassa and in yeast (32, 33). The data are too preliminary to exclude this possibility, but the puu-1 mutant does retain Ca\(^{2+}\) sensitivity of K\(^+\) efflux upon Na\(^+\) addition (as opposed to addition of putrescine). The fact that K\(^+\), Na\(^+\), and NH\(_4\) all competitively inhibit putrescine transport; that both K\(^+\) and putrescine influx are inhibited by Ca\(^{2+}\), and that many amines, including putrescine, stimulate K\(^+\) efflux may indicate only an indirect coupling of the various transport systems, and independent regulation of these systems by Ca\(^{2+}\).

Cell-bound Ca\(^{2+}\) is a prime influence on the rate of putrescine entry. This leads to consideration of the last question: what is the puu-1 product? Impairment of the puu-1’ product (by mutation, turnover, or removal of Ca\(^{2+}\)) increases putrescine uptake activity. Absence or
impairment of the puu-1" product renders the organism vulnerable to polyamine intoxication (3). Our results suggest that the puu-1" product is a Ca\(^{2+}\)-binding protein that imparts a beneficial impermeability of the plasma membrane to toxic materials (of which putrescine and La\(^{3+}\) are two). The puu-1 product also plays a significant role in the activity of amino acid and perhaps other transport systems. The protein could work by any of a number of means, from improving specificity of an unknown cation carrier by direct contact to changing the character of the bilayer in which this and other carriers are embedded. The pleiotropy of the puu-1 mutation suggests a large scope of involvement with cation transport. Defining the character of the puu-1 gene product will help us to discover its action, and perhaps the identity of the putrescine carrier and other carriers affected by the puu-1 mutation.

ACKNOWLEDGMENTS

We thank Clifford Slayman, Barry Bowman, Frank Harold, John Pitkin, and Laura Williams for extensive discussion in the course of this work. We thank Dr. Timothy Bradley, Department of Developmental and Cell Biology, for use of his atomic absorption spectrophotometer.

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