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Putrescine export in *Xenopus laevis* oocytes occurs against a concentration gradient: evidence for a non-diffusional export process

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Abstract

Putrescine export was found to occur by a non-diffusional highly regulated process using *Xenopus* oocytes as a model system of polyamine transport. Untreated oocytes were observed to possess high endogenous intracellular putrescine and spermidine levels with no detectable polyamine interconversion or biosynthesis over the assay intervals. The putrescine uptake process demonstrated a rapid saturation within a 5 min interval. Spermidine demonstrated a relatively larger uptake capacity with only a minimal ability to export. A kinetic analysis of the concentration-dependence of the putrescine and spermidine uptake processes indicated that the putrescine uptake process may possess two concurrent uptake components while spermidine uptake may possess a two-component process with an allosteric regulation. Elevated intracellular putrescine levels were observed to decrease against a 10-fold higher extracellular concentration gradient in a rapid and specific manner. No noticeable changes in the intracellular levels of other polyamines were observed over the same time interval. The uptake and export rates of putrescine transport also showed a concurrent, rapid and cyclical regulation. These findings support a non-diffusional putrescine export process which is highly regulated.

Keywords: Putrescine; Uptake; Export; Transport; Oocyte; (*Xenopus*)

1. Introduction

Polyamines (putrescine, spermidine and spermine) are small organic polycations that have been proposed to participate in numerous cellular processes due to their characteristic binding to polyanionic sites in DNA, RNA, proteins and cell membranes [1–3]. In particular, cell growth and proliferation have demonstrated a strong dependence on the intracellular polyamine levels [4–6]. Elevated intracellular polyamine levels in cultured cell lines have been observed during rapid cellular growth and mitogen stimulation while the depletion of intracellular polyamines by polyamine biosynthesis inhibitors has conversely been shown to slow or halt cell growth. The increases and maintenance of intracellular polyamine levels have been attributed to polyamine biosynthesis and specific polyamine uptake processes which have been well-characterized in many species [7–11].

By comparison, the putrescine export process remains less well-defined [12–14]. Our previous studies have demonstrated that mammalian cell lines possess a selective putrescine and cadaverine export capacity which can be stimulated by the addition of

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mitogens and ornithine to the cell culture medium [15–19]. This increased putrescine export capacity demonstrated a high degree of regulation and was not correlated to a concomitant increase in the intracellular putrescine level. Though the apparent selectivity of the polyamine export process strongly suggested the presence of a specific high affinity exporter, the possibility remained that the observed putrescine export may have been due to a diffusion-mediated process across the plasma membrane. A diffusion-mediated efflux process would be driven by an outward concentration gradient derived from an increase in intracellular putrescine concentrations from polyamine biosynthesis and interconversion. Estimates of the intracellular putrescine concentration of mammalian cells have been reported as 10–100 μM [20–22] as compared to measurements of extracellular putrescine levels at approximately 1 μM [15–19,23,24]. A diffusion-mediated putrescine efflux process such as facilitated diffusion thus seemed a distinct possibility.

In these series of experiments, we have used *Xenopus laevis* oocytes as a model system to further characterize the putrescine export process. A major advantage of this model system is that mature unfertilized oocytes have little detectable polyamine biosynthesis or interconversion [21,25]. This low level of polyamine metabolism minimizes the possibility that observed changes in the intracellular putrescine levels of oocytes treated with extracellular putrescine are due to processes other than polyamine transport. Additionally, these oocytes have a low endogenous ion conductance compared to mammalian cultured cells [26] thereby reducing the number of non-specific channels by which putrescine could be transported into or out of the oocyte. *Xenopus* oocytes have also been used in the expression of mRNA of cloned transport proteins [27] and provide a potential method of cloning and further characterization of the putative putrescine exporter. From polyamine transport assays conducted on these oocytes, it has been demonstrated that a significant putrescine export process exists in *Xenopus* oocytes and that this process can occur against a high extracellular concentration gradient without changes in the intracellular levels of other polyamines. Periodic or cyclical modulations of the intracellular putrescine levels were also observed against a constitutively high extracellular putrescine gradient. These findings argued against a putrescine export process mediated by diffusion and suggested the existence of a highly regulated energy-dependent or coupled putrescine export process capable of transport up a concentration gradient.

2. Materials and methods

2.1. Materials

[^{3}H]Putrescine was obtained from Amersham Life Sciences with a specific activity of 14.6 Ci/mmol. [^{3}H]Spermidine was purchased from NEN Research Products with a specific activity of 15.3 Ci/mmol. Cytoscint liquid scintillation cocktail was purchased from ICN Biochemicals. All other reagents were obtained from Sigma except for collagenase which was purchased from Boehringer-Mannheim.

2.2. Preparation and selection of Xenopus oocytes

Xenopus laevis oocytes were isolated and maintained as per Quick et al. [28]. Briefly, mature *Xenopus laevis* females were anesthetized in a solution of 3-aminobenzoic acid ethyl ester for 30 min. Oocytes are then removed and defolliculated by enzymatic treatment with 2 mg/ml collagenase over 2.5 h. Defolliculated oocytes were selected for stage VI maturity with a dissection microscope and stored in ND-96 buffer (96 mM NaCl, 5 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing 5% horse serum. Oocytes were kept for 3–5 days at 18°C without a noticeable loss of transport activity.

2.3. Characterization of polyamine transport and intracellular polyamine levels in Xenopus laevis oocytes

Xenopus oocytes and all buffers were equilibrated to room temperature (approx. 22°C) prior to the assays. Intracellular polyamine levels were determined as per Gilbert et al. [23]. Briefly, individual oocytes were incubated in 96-well microtiter plates containing 200 μl/well ND-96 buffer with the specified concentrations of putrescine at room temperature over the indicated time interval. After the desired time interval, oocytes were rapidly washed (less than 1 min) with five 200-μl volumes of ice-cold ND-96

to remove non-specific binding of extracellular putrescine and to minimize decreases of the intracellular polyamine levels due to transport across the plasma membrane. The washed oocytes were placed in 200 μl 0.5 N PCA to promote protein precipitation, sonicated, transferred to 1.5 ml microcentrifuge tubes and placed on ice for 15 min. The samples were then centrifuged at $10\,000 \times g$ for 2 min. The supernatant was incubated with dansyl chloride and quantitated for polyamine content by fluorescence detection HPLC. The possibility of interconversion and degradation of intracellular putrescine was assessed by incubating individual *Xenopus* oocytes in 1 μCi/100 μl [^{3}H]putrescine over 4 h (data not shown). Samples were analyzed by HPLC and the individual peaks were collected and quantitated for radiolabel by liquid scintillation counting. A calculated oocyte volume of 1 μl was used for all concentration determinations.

For both putrescine and spermidine uptake rate determinations, oocytes were incubated at room temperature in ND-96 containing the specified concentration of the tested polyamine. At the indicated time, ND-96 containing the tritiated polyamine analogue of known specific activity was added to initiate the assay. The HPLC protocol was followed as above except that an aliquot of the supernatant was counted by liquid scintillation to determine the uptake rate.

For putrescine export assays, *Xenopus* oocytes were incubated in radiolabelled putrescine of known specific activity for the indicated time intervals. Oocytes were rapidly washed five times with the indicated buffer and placed in a small volume of this buffer. After a 2 min incubation at 22°C, all of the buffer was removed and counted by liquid scintillation. The oocyte was further characterized as described by the uptake method above.

All polyamine uptake and export assays were performed on individual oocytes in triplicate, and most data points are the sum of multiple oocyte preparations. Due to the inherent variability of timed measurements in biological systems and subtle differences in oocyte maturity, however, different oocyte preparations demonstrated varying transport activities for the putrescine uptake and export processes. While the relative transport rates and intracellular polyamine levels were consistent within any given oocyte preparation, comparisons of the absolute uptake and export rates varied by as much as 10-fold between oocytes obtained from different animals. Variations in the intracellular polyamine levels were significantly less with differences no greater than 20% between separate analyses.

3. Results

3.1. Characterization of the intracellular polyamine levels and polyamine transport processes of Xenopus oocytes

From an average calculated oocyte volume of 1 μl, determinations of the intracellular polyamine levels of *Xenopus* oocytes by HPLC analysis demonstrated near millimolar levels of putrescine (approx. 900 μM) and spermidine (approx. 800 μM) in keeping with previously reported observations [25,29]. Spermine was observed at lower intracellular levels of 200–400 μM while cadaverine and acetylputrescine were detected at micromolar levels (data not shown). No other polyamines were identified above the HPLC detection limit of 5 pmol per oocyte. Some variability of the intracellular polyamine levels (approx. $\pm$20%) was observed from different oocyte preparations and different animals.

The potential role of putrescine degradation and interconversion in putrescine transport was assessed by the incubation of oocytes with radiolabelled putrescine over a 1–4 h interval followed by HPLC analysis. An active polyamine metabolic pathway would interchange the intracellular putrescine pool and distribute the tritium radiolabel among the other polyamines. The quantitation of the individual peaks by liquid scintillation counting did not support this possibility, since all counts taken up by the oocytes were recovered and found in the HPLC peak associated with putrescine (data not shown).

The time-dependent uptake of [^{3}H]putrescine and [^{3}H]spermidine were delineated over a 10 min period by monitoring the intracellular accumulation of the tritiated polyamines. The putrescine uptake process rapidly reached a plateau within 5 min at an extracellular putrescine concentration of 0.7 μM (Fig. 1A). At a similar extracellular spermidine concentration, the spermidine uptake process demonstrated a linear increase in radiolabelled spermidine with no decrease

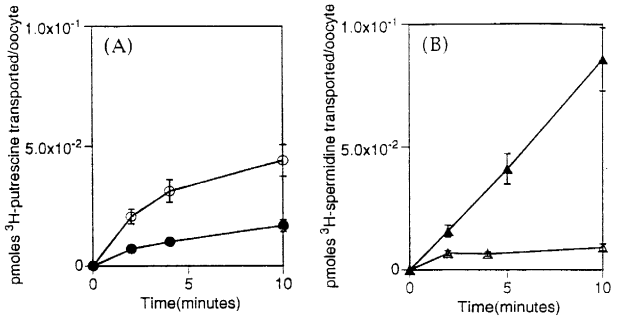

Fig. 1. Time-dependent polyamine transport in *Xenopus laevis* unfertilized oocytes. Measurements of polyamine uptake and export were obtained by incubating oocytes in ND-96 buffer containing either [^{3}H]putrescine or [^{3}H]spermidine at extracellular concentrations of 0.7 μM. Polyamine uptake was assessed as described in Section 2. For the measurements of polyamine export, *Xenopus* oocytes were incubated for 4 h prior to the export assay in ND-96 containing either [^{3}H]putrescine or [^{3}H]spermidine. The oocytes were then washed five times and transferred to fresh ND-96. Aliquots of this buffer solution were taken at the indicated time intervals and counted by liquid scintillation. All points are the mean $\pm$ S.E. of three measurements from three individual oocytes. (A) [^{3}H]Putrescine transport: ●, [^{3}H]putrescine uptake; ○, [^{3}H]putrescine export. (B) [^{3}H]Spermidine transport: ▲, [^{3}H]spermidine uptake; △, [^{3}H]spermidine export.

in rate over the 10 min time interval based upon the time-dependent differences in the linearity of spermidine uptake (Fig. 1B).

A detailed kinetic analysis of the concentration-dependent uptake processes of putrescine and spermidine provided more evidence for distinct uptake processes. By plotting the inverse of the rate constants versus the extracellular polyamine concentrations, characteristic curves were produced which were indicative of specific kinetic models of transport [30]. The curve obtained for the concentration-dependent putrescine uptake process was suggestive of an uptake system with two components (Fig. 2A). The putrescine uptake process may possess a saturable system with a non-saturable component or a saturable system with two highly different affinities. In contrast, the spermidine uptake process demonstrated a cooperative response to increasing extracellular concentrations (Fig. 2B) and may represent a separate process from that described for putrescine uptake. (This finding supports an earlier characterization of spermidine uptake [29].)

Xenopus oocytes also possessed an endogenous putrescine efflux capacity (Fig. 1A). The observed efflux of [^{3}H]putrescine demonstrated a higher level of transport than the [^{3}H]putrescine uptake process in keeping with the higher intracellular putrescine level (approx. 1 mM) as compared to the extracellular putrescine level (approx. 1 μM).

A small but quantifiable spermidine efflux was also observed in *Xenopus* oocytes (Fig. 1B). Even though there was a high intracellular spermidine concentration gradient decreasing to a low extracellular spermidine level, the spermidine efflux process demonstrated significantly less transport than the measured spermidine uptake or putrescine export processes.

3.2. Characterization of intracellular polyamine levels of Xenopus oocytes incubated in 10 mM putrescine

In order to determine whether it was possible for putrescine to be exported against a concentration gradient, the intracellular polyamine levels and putrescine uptake rates were extensively measured in *Xenopus* oocytes incubated in 10 mM putrescine throughout a 6 h period (Figs. 3 and 4). When the oocytes were placed in buffer containing 10 mM putrescine, the intracellular putrescine levels increased rapidly as expected. Within a 15–20 min period, intracellular putrescine levels increased by up

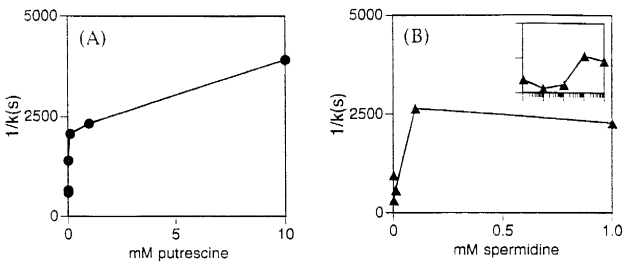

Fig. 2. A kinetic determination of concentration-dependent polyamine uptake in *Xenopus* oocytes. The putrescine and spermidine uptake processes were measured for a 2-min period over a wide range of extracellular polyamine concentrations as described in Section 2. The inverse of the calculated uptake rate constants were plotted against the extracellular polyamine concentration to produce kinetic curves indicative of a two-component putrescine uptake system (A) and a cooperative spermidine uptake system (B). The inset in B is presented in semi-log form for clarity. ●, putrescine; ▲, spermidine.

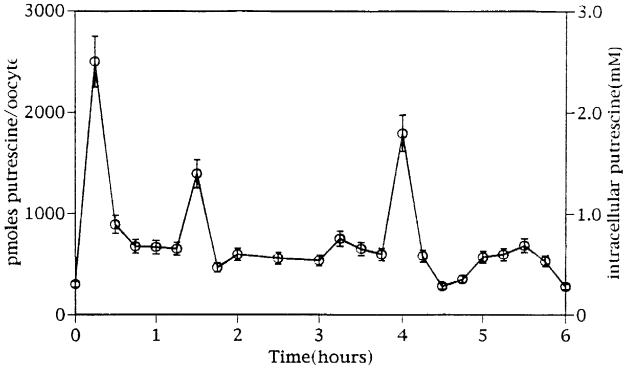

Fig. 3. Time-dependent changes of the intracellular putrescine levels in *Xenopus* oocytes. Oocytes were incubated in ND-96 buffer containing 10 mM putrescine over the indicated time at room temperature. At time *t*, the oocytes were washed five times with fresh ND-96 buffer, placed in 500 μl 0.5 N perchloric acid, and sonicated. Samples were then centrifuged and the supernatant analyzed by HPLC. Intracellular putrescine concentrations were calculated by taking the intracellular oocyte volume to be 1 μl. All points are the mean $\pm$ S.E. of nine oocytes.

to 6-fold. In nearly as rapid a period, however, the intracellular putrescine levels decreased to basal levels. Since there was no apparent metabolic turnover of intracellular putrescine into other polyamines or interconversion of putrescine into other products (see above), the decrease in the intracellular putrescine

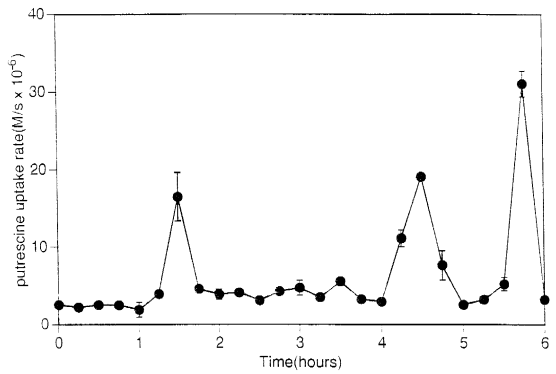

Fig. 4. Time course of the putrescine uptake rate in *Xenopus* oocytes. Oocytes (different from those shown in Fig. 3) were placed in a 96-well titer plate with 10 mM putrescine (pH 7.4) and incubated at room temperature over the indicated time period. At 15-min intervals, radiolabelled [^{3}H]putrescine was added to specified wells, mixed, and incubated for 2 min. The oocytes were then washed five times, placed in 0.5 N PCA and sonicated. Aliquots were taken for liquid scintillation counting and HPLC analysis. All points are the mean $\pm$ S.E. of triplicate determinations from multiple oocyte preparations.

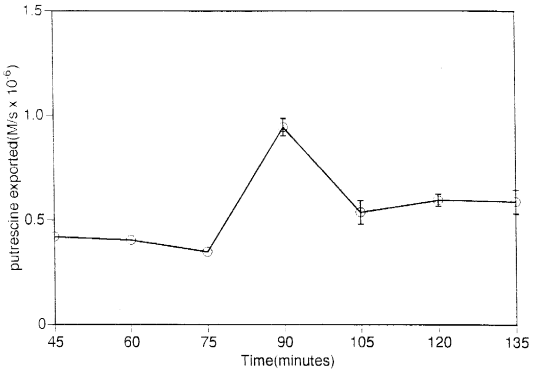

Fig. 5. Time-dependent changes in the putrescine export rate of *Xenopus* oocytes. Oocytes were incubated at room temperature in ND-96 (pH 7.4) containing 10 mM putrescine (ND-96/put). At time *t*, oocytes were placed in an ND-96 buffer containing 10 mM putrescine with a specific activity of 2 μCi/mmol and incubated for 2 min. The oocytes were then washed five times with ND-96/put alone and placed in 200 μl of the buffer for 2 additional minutes. The supernatant from this incubation was counted by liquid scintillation. The export rate was calculated by assuming a limited amount of mixing of the intracellular putrescine pools over the assay interval and an approximately constant specific activity of the [^{3}H]putrescine. All points are the mean $\pm$ S.E. of triplicate assays from multiple oocyte preparations.

level was attributed to transport out of the oocyte against a concentration gradient. Similar rapid cyclical alterations in the intracellular putrescine levels were observed throughout a 6 h period. The periodicity of the intracellular putrescine modulations varied by up to 15 min between different oocytes and oocyte preparations. This time variability led to larger standard errors near the time intervals of the putrescine modulations (Fig. 3, see 1.5 h). Intracellular spermidine and spermine levels did not demonstrate any significant changes over the assay time nor were any increases in acetylated derivatives observed.

3.3. Cyclical changes in putrescine uptake and export rates of Xenopus oocytes incubated in 10 mM extracellular putrescine

To assess the role of putrescine uptake in regard to the intracellular putrescine level, we attempted to measure the unidirectional uptake component of putrescine transport using the short-term labelling protocol as described in Section 2. Rapid and periodic modulations of the putrescine uptake rate were de-

tected (Fig. 4). These increases of putrescine transport activity were seen to occur transiently up to 6 h. A degree of uptake rate variability was seen also for oocytes as per the periodic modulations of the intracellular putrescine levels.

The putrescine export rate was also measured in individual oocytes (Fig. 5). The periodic modulations of the putrescine uptake rate and intracellular putrescine concentration suggested that the putrescine export rate also varied periodically. Due to the large amount of isotope required for the loading of radiolabel into oocytes, a specific time interval of known intracellular putrescine modulation was selected for experimentation. A rapid increase in the putrescine export rate was found to occur over a 15 min interval followed by a subsequent and equally rapid decrease in the putrescine export rate.

4. Discussion

The initial determination of polyamine transport in *Xenopus* oocytes demonstrated an endogenous putrescine export capacity (Fig. 1A) with little detectable ability to export spermidine (Fig. 1B). This export specificity was similar to previous findings reported by this laboratory for mammalian cultured cell lines [15–19] and suggested that oocytes would be an appropriate model system with which to further characterize the putrescine export process. Confirmation of previous reports on the low endogenous polyamine biosynthesis and interconversion found in unfertilized mature *Xenopus* oocytes [21,25] also suggested that this model system would allow for an accurate measurement of putrescine transport independent of metabolic confounders.

It should be noted that measurements of unidirectional polyamine transport in intact cell model systems are exceedingly difficult to obtain. With the exception of electrophysiological techniques, standard quantitative analyses do not clearly distinguish the cumulative contributions to the overall polyamine transport of interacting cellular processes such as unidirectional polyamine uptake and export, biosynthesis, degradation, interconversion and intracellular binding to macromolecules. One or all of these processes can significantly affect the magnitude and direction of the net observed polyamine transport. In the case of *Xenopus* oocytes, however, polyamine biosynthesis, degradation and interconversion did not appear to represent major determinants of net polyamine transport due to the limited amount of observed polyamine metabolism. While intracellular binding of the polyamines certainly contributed to the net observed level of putrescine uptake in oocytes as suggested by the increase of the intracellular putrescine concentration up to 3 mM (Fig. 3), a mechanism of equilibrium binding-mediated putrescine efflux against a 10-fold concentration gradient appeared unlikely. Putrescine uptake and export may thus represent the two major processes in *Xenopus* oocytes by which intracellular polyamine levels can be modulated.

As previously mentioned, a diffusion-mediated putrescine efflux process was considered as a possible transport mechanism due to an outward-directed putrescine concentration gradient across the plasma membrane. It has been observed by this laboratory and others [25,29], however, that oocytes can maintain a naturally high intracellular putrescine concentration of approximately 500–800 μM in vivo and in storage buffer containing no added polyamines. Based upon this large constitutive putrescine concentration gradient from inside to outside of the oocyte and the low endogenous level of polyamine biosynthesis, only a minimal amount of outward diffusion of putrescine was suggested to occur over a given time period.

In this regard, we have determined that putrescine efflux in human erythrocytes can/does occur in part by passive diffusion down a concentration gradient [24]. Using the determined value of the erythrocyte membrane permeability with an intracellular putrescine concentration analogous to that found in *Xenopus* oocytes, a diffusional rate for putrescine efflux was obtained for comparison to the export rates observed for *Xenopus* oocytes. The calculated erythrocyte efflux rate was calculated to be 10^3–10^4-times slower than the observed putrescine export process in oocytes (Fig. 5) at similar intracellular concentrations. The large disparity in the comparative export rates thus made it unlikely that putrescine export in *Xenopus* oocytes occurs solely by passive diffusion across the plasma membrane even when taking into consideration the differences in membrane composition between erythrocytes and oocytes. Spermidine, a related analogue to putrescine with similar

intracellular concentrations in *Xenopus* oocytes, also did not show an appreciable export (Fig. 1) contrary to expectations for a non-specific process like passive diffusion.

In order to assess whether putrescine export occurred by diffusional or active transport processes, oocytes were incubated against a 10-fold extracellular putrescine concentration gradient of 10 mM putrescine. If putrescine export occurred by a diffusion-mediated process (e.g. simple, channel-mediated or facilitated diffusion), a gradual and saturable equilibration of the intracellular putrescine level toward the extracellular putrescine concentration would be expected due to a net putrescine influx. The rapid and specific decrease of the elevated intracellular putrescine levels against a 10-fold concentration gradient (Fig. 3), however, provided strong evidence against a putrescine export process primarily mediated by diffusion. Whereas a gated diffusion process through ion channels or facilitated carriers may have accounted for some of the observed cyclical putrescine uptake for oocytes incubated in high extracellular putrescine concentrations, these channels and carriers could not mediate a net export of putrescine against a concentration gradient. An active export of putrescine thus appeared likely. Transport processes involving nucleotide hydrolysis or coupling to other concentration gradients across the plasma membrane may provide the driving force for putrescine export against the high extracellular concentration gradient.

As observed over a 6 h interval (Fig. 3), the periodic and rapid modulations of the intracellular putrescine level also appeared to support a coordinately regulated transport process. Despite rapid decreases of elevated intracellular putrescine levels, oocytes were never depleted of putrescine below basal levels. A concentration-dependent effect on putrescine export and/or uptake could help to regulate and maintain the intracellular putrescine level and may represent the major determinant of transport regulation. Some support for this possibility is lent by the finding that exogenously applied polyamines have been shown to demonstrate a rapid down-regulation of observed polyamine uptake [31].

While a coordinate regulation of the putrescine uptake and export processes appears likely, it should be noted that the regulation of only one unidirectional transport process is required to demonstrate changes in the intracellular putrescine concentration. A model system with a relatively slow constant putrescine uptake and a regulated putrescine export process can mimic intracellular changes mediated by a coordinately regulated process. This situation applies to a putrescine transport system with constant export and regulated uptake processes as well. Due to the measured increases in putrescine uptake rate (Fig. 4) and export rate (Fig. 5), however, a coordinate regulation of both processes remains a more likely possibility. One possible role of such a regulated transport system may be to regulate intracellular putrescine concentrations over a narrowly defined range in the embryogenic development of the oocyte [32].

In summary, the putrescine export capacity of *Xenopus* oocytes appeared to be quite large. Oocytes were able to maintain a relatively low intracellular putrescine concentration despite exposure to high extracellular putrescine levels over several hours. The pulsatile increases of intracellular putrescine strongly supported the existence of highly regulated putrescine export and uptake processes. This coordination of putrescine export and uptake must demonstrate a strict concentration-dependence to prevent polyamine depletion by uncontrolled putrescine export or osmotic swelling by excessive putrescine uptake. Evidence for an active putrescine export process further suggested that some driving force such as ATP hydrolysis or coupling to electrochemical gradients may be involved in putrescine export against a substantial concentration gradient. These results suggest that future characterizations using electrophysiological methods in *Xenopus* oocytes may be appropriate. Highly sensitive measurements of current–voltage relationships of endogenous polyamine fluxes and electrophysiological characterizations of expressed putative polyamine transporters may allow for a detailed characterization of the putrescine export process.

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