Characterization of a Diamine Exporter in Chinese Hamster Ovary Cells and Identification of Specific Polyamine Substrates*

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Export of the diamine putrescine was studied using inside-out plasma membrane vesicles prepared from Chinese hamster cells. Putrescine uptake into vesicles was a saturable and an ATP- and antizyme-independent process. Excess amounts of a series of diamines or monoacetyl spermidine, but not monoacetyl putrescine, spermidine, or spermine, inhibited putrescine transport. Putrescine uptake into vesicles prepared at pH 7.4 was suppressed at pH 5, compared with pH 7.4; and was not inhibited by valinomycin in the presence of potassium ions. Reserpine and verapamil blocked [3H]putrescine uptake into inverted vesicles. Verapamil treatment caused an increase in intracellular contents of putrescine, cadaverine, and N2-acetylputrescine, in unstressed proliferating cells, or of N4-acetylputresme, in cells subjected to heat shock to induce acetylation of spermidine at N4. These data indicate that putrescine export in Chinese hamster cells is mediated by a non-electrogenic antiporter capable of using protons as the counter ion. Physiological substrates for this exporter include putrescine, cadaverine, and monoacetyl spermidine and have the general structure NH$_3$\((CH_2)_{n}\)-NH$_2$ + R at acidic or neutral pH.

The polyamines spermidine and spermine, and their diamine precursor putrescine, are essential for eukaryotic cell proliferation (1, 2). Intracellular content of these amines is modulated by changes in the activity of the key enzymes in the synthetic and catabolic pathways (1, 2). However, increasing evidence indicates that polyamine transport, including uptake and export, can also play an important role in the control of intracellular polyamine pools (3). Polyamine export, or efflux from the cells, has been observed in a variety of mammalian cell types (3). Polyamine export, or efflux from the cells, has been observed in a variety of mammalian cell types (3). Polyamine export, or efflux from the cells, has been observed in a variety of mammalian cell types (3).

MATERIALS AND METHODS

Chemicals—[1,4-3H]Putrescine dihydrochloride (27.0 Ci/mmol) was obtained from Amersham Life Science Inc. α-Difluoromethylornithine (DFMO) and the FAD-dependent polyamine oxidase (PAO) inhibitor, MDL 72.521, were generous gifts from Marion Merrell Dow Inc. (Cincinnati, OH). Polyamines, reserpine, verapamil, and other chemicals were purchased from Sigma. Cell culture medium, fetal bovine serum, and supplementary antibiotics were from Life Technologies, Inc.

Cell Culture and Treatments—CHO-K1 cells, obtained from American Type Culture Collection (Rockville, MD), were grown in F-12 nutrient mixture supplemented with 10% fetal bovine serum plus 100 IU/ml penicillin, 100 μg/ml streptomycin, C55.7 (M-ODC) and C55.7 (Tb-ODC) were ornithine decarboxylase (ODC)-deficient CHO cells (C55.7) transfected with either mouse (M) or trypanosome (Tb) ODC, as described elsewhere (17). These cells grew in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, antibiotics, and 300 μg/ml G418.

Preparation of Inside-out Vesicles—The procedure for preparation of inverted membrane vesicles was modified from the methods described by Schaub et al. (18) and by Saxena and Henderson (19), and began with the isolation of plasma membranes to minimize contamination from other cellular membranes. CHO-K1 cells (0.5–1 × 10^7) were harvested from the cell culture and washed with 50 ml of hypotonic buffer (0.5 mM sodium phosphate, pH 7.4). The cell pellet was suspended in 50 ml of hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride) and gently stirred on ice for 3 h. The cell lysate was then centrifuged at 100,000 × g for 45 min at 4 °C. White fluffy material around the hypotonic buffer, and homogenized with 20 strokes in a Potter-Elvehjem homogenizer. The homogenate was then layered over a 38% sucrose solution buffer.

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1 The abbreviations used are: DAX, diamine exporter; CHO, Chinese hamster ovary; DFMO, α-difluoromethylornithine; PAO, polyamine oxidase; ODC, ornithine decarboxylase; M-ODC, murine ODC; Tb-ODC, trypanosome ODC; MES, 4-morpholineethanesulfonic acid; SSAT, spermidine/spermine N4-acetyltransferase.

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Putrescine Export in CHO Cells

and centrifuged at 100,000 × g for 30 min at 4 °C. The turbid layer at the interface was collected into 25 ml of TS buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose and 50 mM NaCl) and pelleted by centrifugation at 100,000 × g for 30 min at 4 °C. After suspending the pellet in 5 ml of TS buffer, vesicles were formed by passing the suspension through a 27-gauge needle with a syringe. Inside-out vesicles were enriched by applying to a column of wheat germ agglutinin linked to CNBr-activated Sepharose 4B (equilibrated with TS buffer). Unbound inside-out vesicles were collected and further concentrated by centrifugation at 100,000 × g for 30 min at 4 °C. The final inside-out vesicles were resuspended in TS buffer and stored at −70 °C until use. The purity of inside-out vesicles was estimated by sialic acid accessibility (20, 21). Protein was determined using the Pierce BCA (bicinchoninic acid) protein assay reagent with bovine serum albumin as a standard.

Measurement of [3H]Putrescine Uptake by Inside-Out Vesicles—Assay mixtures were prepared on ice and contained membrane vesicles (100 μg of protein), 1.0 mM ATP, 10 mM MgCl₂, 0.2 mM CaCl₂, 10 mM dithiothreitol, an ATP generating system (10 mM creatine phosphate, and 100 μg/ml creatine kinase), and 1 μM [3H]putrescine in assay buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4) at a final volume of 100 μl. The incubation was carried out at 37 °C, and uptake was stopped by placing on ice and diluting with 1 ml of ice-cold stop buffer (250 mM sucrose, 150 mM NaCl, 10 mM MES, pH 5.5). The vesicles were collected by rapid filtration onto premoistened Millipore HAWP 0.45-μm filters, washed with four 1-ml portions of ice-cold stop buffer. Radioactivity on the filters was measured in a scintillation counter. The determination of nonspecific binding (blank) was performed by adding ice-cold stop buffer to vesicles prior to adding label and was subtracted from transport measurements.

Polyamine Analysis—For the determination of intracellular polyamine contents, cells were harvested and sonicated in 0.1 N HCl. The cell lysate was then adjusted to 0.2 N HClO₄, and incubated at 4 °C for 2 h to separate acid-soluble polyamines from macromolecules. After centrifugation at 15,000 × g for 10 min, the acid-soluble fraction was subjected for polyamine content measurement by using the reverse-phase high performance liquid chromatography procedure described by Seiler and Knodgen (22), while the acid insoluble pellet was assayed for protein content.

RESULTS

Characteristics of Intravesicular Accumulation of [3H]Putrescine—Inside-out plasma membrane vesicles were prepared from CHO cells and purified from right-side-out vesicles by passage through a column of wheat germ agglutinin linked to CNBr-activated Sepharose 4B, as described by others (18, 19). The purity of the inside-out vesicles was determined by the accessibility of the membrane-associated sialic acid to neuraminidase. The amount of sialic acid liberated by the neuraminidase treatment in the absence and presence of 0.1% (v/v) Triton X-100 were 38 ± 5 nmol/mg of protein and 117 ± 16 nmol/mg of protein, respectively. From these values, we estimate that a minimum of 68% of the total population of the vesicles were inside out.

The results in Fig. 1A represent a typical time course experiment measuring intravesicular putrescine accumulation when vesicles were incubated in the presence of 1.0 μM [3H]putrescine. The process was linear for 30 min at 37 °C, and then remained at a maximal value through 120 min of incubation. Uptake measurements at varying concentrations of [3H]putrescine are shown in Fig. 1B. These data show that the transport of [3H]putrescine into the vesicles is a saturable process and suggest that this diamine is transported and not simply binding to the membrane preparation.

The data shown in Fig. 2 demonstrate that the association of [3H]putrescine with the vesicles is osmotically sensitive. The amount of [3H]putrescine taken up by vesicles in 1 h was inversely related to the osmolarity in the extravesicular medium. Since the vesicles shrink in hypertonic medium, these results also suggest that putrescine is transported into the vesicles, rather than simply binding to the vesicle surface.

Putrescine import into cells is ATP-dependent (23) and negatively regulated by the ODC antizyme (24). To assess the potential impact of residual right-side-out vesicles on our measure of putrescine export, we determined the dependence of ATP and antizyme on this system. Incubation of inverted vesicles with 1 μM [3H]putrescine at 37 °C, in the presence or absence of ATP and/or an ATP regenerating system (creatine phosphate and creatine kinase) had no effect on [3H]putrescine uptake into the vesicles (data not shown). A rat antizyme cDNA clone (25) was expressed as a fusion protein with glutathione S-transferase in a bacterial expression system and purified using GSH-agarose, as described by others (26). Concentrations of the antizyme fusion protein that inactivated greater than 90% of ODC enzyme activity had no effect on [3H]putrescine uptake into the inverted vesicles (data not shown). Thus, right-side-out vesicles appear to be inactive in this system, as neither ATP nor antizyme affect [3H]putrescine uptake measurements.

Competitive Inhibition of [3H]Putrescine Transport into the Vesicles by Other Amines—To determine the specificity of the

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**Fig. 1.** Transport of [3H]putrescine into the inside-out vesicles. **A**, time course. Membrane vesicles (100 μg of protein) were assayed for putrescine uptake by the addition of 1 μM [3H]putrescine into the assay mixture. The vesicle-associated radioactivity was measured at the indicated time points. Data are representatives of a single experiment which has been repeated in triplicate. **B**, substrate concentration dependence. Various concentrations of [3H]putrescine were added into the assay mixture containing the vesicles, and the transport was assayed after incubation for 10 min at 37 °C.

**Fig. 2.** Osmotic sensitivity of [3H]putrescine accumulation into the inside-out vesicles. Membrane vesicles were preincubated in buffer containing various concentrations of sucrose for 1 h at 4 °C. Uptake was started after this preincubation by adding 1 μM [3H]putrescine into the assay mixture. Each value is the mean of triplicates. Error bars are not shown when smaller than the symbol.
transport system mediating the uptake of [3H]putrescine into the vesicles, inhibition of intravesicular accumulation of [3H]putrescine by other polyamines was examined (Fig. 3A). As expected, addition of excess, unlabeled putrescine inhibits 90% of [3H]putrescine uptake into the vesicles. There was no inhibitory effect of the triamine spermidine, the tetraamine spermine, and monoacetylspermidine on [3H]putrescine uptake in this system. These data suggest that certain diamines and polyamines carrying +2 charges, in addition to putrescine, are substrates for DAX.

**Effect of pH on [3H]Putrescine Transport in Inverted Vesicles**—Uptake of [3H]putrescine into the inverted vesicles is pH-dependent. As seen in Table I, uptake is suppressed dramatically in vesicles prepared at pH 7.4 when the reaction solution is adjusted to pH 5.0, compared with pH 7.4. However, when vesicles are first equilibrated to a lower pH to reduce the pH inside the vesicles and then placed in a reaction mixture at pH 7.4, uptake is significantly stimulated, compared with parallel vesicles incubated at pH 7.4 prior to addition of radiolabeled putrescine.

**Effect of Valinomycin on [3H]Putrescine Transport**—To determine if putrescine is transported electrogenically, we added potassium ions and/or the potassium ionophore valinomycin to the reaction mixture and measured uptake into inverted vesicles. Valinomycin in the presence of potassium ions will establish an inward-directed positive voltage, and thus should suppress transport of radiolabeled putrescine if this amine is electrogenically exported. Control reactions containing additional sucrose (100 mM) with and without valinomycin were carried out to control for possible osmotic effects of increased potassium ions. As shown in Table II, the presence of valinomycin actually stimulates uptake of labeled putrescine into the inverted vesicles. The presence or absence of potassium ions has no significant effect on DAX activity. Thus, the DAX appears to function via a non-electrogenic mechanism.

**Inhibition of [3H]Putrescine Transport by Reserpine or Verapamil**—Reserpine, an antihypertensive plant alkaloid, is a potent inhibitor of vesicular monoamine transporter in nervous system (28). This drug has also been shown to decrease P-glycoprotein-mediated drug efflux by binding to P-glycoprotein and inhibiting its efflux function. The inhibitory effects of reserpine on structurally and functionally divergent efflux transporters suggest that this drug might also affect putrescine efflux. Fig. 4A shows that when reserpine was added into the transport assay mixtures, [3H]putrescine uptake decreased in a reserpine dose-dependent manner, with an IC50 of approximately 40 μM.

Verapamil, a calcium channel antagonist, also inhibits export processes mediated by P-glycoprotein without inhibition of calcium influx (29). The inhibitory effect of verapamil on putrescine efflux has been demonstrated in macrophage-like RAW 264 cells and Reuber H35 hepatoma cells (13–15). As shown in Fig. 4B, verapamil was a potent inhibitor of DAX in the inverted membrane vesicle system. Approximately 90% of [3H]putrescine uptake was inhibited in the presence of 10 μM verapamil.
2–3-fold increases in intracellular putrescine contents in the two transfected cell lines, and that C55.7 (Tb-ODC) accumulated higher putrescine contents than C55.7 (M-ODC) because of the more stable ODC protein. Spermidine and spermine levels do not significantly change in cells treated with or without verapamil (data not shown). In addition to the increased intracellular putrescine contents, cells also accumulate cadaverine and N\textsuperscript{8}-acetyl spermidine (Table III). These results suggest that DAX mediates the eflux of the diamines putrescine and cadaverine and N\textsuperscript{8}-acetyl spermidine in log phase cells. These amines carry a +2 charge under physiological conditions. The verapamil-dependent accumulation of all of these amines is inhibited by treatment with an ODC inhibitor, DFMO, indicating that the accumulation of these amines, including cadaverine, is ODC-dependent. Reserpine treatment of these cells causes a similar increase in intracellular levels of putrescine, cadaverine, and monoacetylspermidines (data not shown).

N\textsuperscript{1}-Acetylspermidine is another physiologically relevant polyamine carrying a +2 charge. Induction of the spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT), which catalyzes the synthesis of N\textsuperscript{1}-acetyl spermidine and N\textsuperscript{3}-acetyl spermidine, is a prominent response of cells to certain physical and chemical stresses (30, 31). To determine whether the putrescine export system also mediates N\textsuperscript{1}-acetyl spermidine efflux, we examined the effects of verapamil on intracellular N\textsuperscript{1}-acetyl spermidine accumulation after heat shock. An inhibitor of the FAD-dependent PAO, MDL 72,521, was used to block the catabolism of monoacetylspermidine to shorter chain amines (32).

Intracellular N\textsuperscript{1}-acetyl spermidine levels are undetectable in the absence of the PAO inhibitor under all conditions tested (Table IV). Verapamil alone or verapamil plus heat shock cause a significant increase in intracellular putrescine, but not N\textsuperscript{1}-acetyl spermidine, presumably because this monoacetylspermidine is catabolized to putrescine by the action of PAO. The PAO inhibitor by itself is sufficient to cause detectable intracellular accumulation of N\textsuperscript{1}-acetyl spermidine without a change in intracellular putrescine content. Addition of heat shock, verapamil, or both caused further increases in both putrescine and N\textsuperscript{1}-acetyl spermidine. Stimulation of SSAT by heat shock, under conditions of inhibition of putrescine and N\textsuperscript{1}-acetyl spermidine export (verapamil) and N\textsuperscript{1}-acetyl spermidine catabolism (MDL72,521), results in an increase in the putrescine pool size from 12.4 (control, no treatments) to 32.4 nmol/mg of protein and the N\textsuperscript{1}-acetyl spermidine pool size from non-detectable levels (limit of detection 0.05 nmol/mg of protein, control group) to 15.7 nmol/mg of protein. N\textsuperscript{1}-Acetylspermine levels were not

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**TABLE III**

Accumulation of putrescine, cadaverine, and N\textsuperscript{8}-acetyl spermidine in ODC-transfected C55.7 cells treated with verapamil in log phase

| Cell line | Treatment | Putrescine | Cadaverine | N\textsuperscript{8}-AcSpd |
|-----------|-----------|------------|------------|--------------------------|
| C55.7 (M-ODC) | None | 2.83 ± 0.65 | ND\textsuperscript{a} | ND |
| | Vpm\textsuperscript{a} | 7.70 ± 1.32 | 0.67 ± 0.25 | 0.39 ± 0.16 |
| | Vpm + DFMO | ND | ND | ND |
| C55.7 (Tb-ODC) | None | 10.97 ± 3.78 | ND | ND |
| | Vpm | 24.67 ± 2.36 | 1.64 ± 0.70 | 0.94 ± 0.36 |
| | Vpm + DFMO | ND | ND | ND |

\textsuperscript{a} Vpm, verapamil.
\textsuperscript{b} ND, not detectable; limit of detection is 0.05 nmol/mg protein.

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**TABLE IV**

Accumulation of polyamines in C55.7 (Tb-ODC) cells after heat shock.

| Treatment | Inhibitor | Polyamine content (nmol/mg protein) |
|-----------|-----------|-----------------------------------|
|           | Putrescine | N\textsuperscript{1}-Acetylspermidine |
| None      | None      | 12.4                                | ND\textsuperscript{a} |
|           | Vpm\textsuperscript{b} | 18.9                                | ND\textsuperscript{a} |
|           | MDL\textsuperscript{c} | 14.2                                | 2.2 |
|           | Vpm + MDL | 25.4                                | 6.6 |
| HS\textsuperscript{a} | None | 10.6                                | ND\textsuperscript{a} |
|           | Vpm | 22.1                                | ND\textsuperscript{a} |
|           | MDL | 19.2                                | 3.5 |
|           | Vpm + MDL | 32.4                                | 15.7 |

\textsuperscript{a} HS, heat shock.
\textsuperscript{b} Vpm, verapamil.
\textsuperscript{c} MDL, MDL72,521.
\textsuperscript{d} ND, not detectable; limit of detection is 0.05 nmol/mg protein.
detectable, presumably because this amine can be further acetylated by SSAT to \(N^1,N^{12}\)-diacetylputrescine (33). This species is not detectable by our high performance liquid chromatography method, which requires at least one free primary amine (22).}

**DISCUSSION**

In this study, we demonstrated transport of \(\text{[}^3\text{H}\text{]}\)putrescine into inverted plasma membrane vesicles prepared from Chinese hamster cells. This process was saturable, indicating that putrescine export in these animal cells is not a simple diffusion process. Fukumoto and Byus (34, 35) have also found that putrescine export is not a simple diffusion process in two other vertebrate cell models. However, putrescine is exported in *Neurospora crassa* by a non-saturable, diffusional mechanism (36, 37).

In this cell-free system, \(\text{[}^3\text{H}\text{]}\)putrescine uptake into the inside-out vesicles was not dependent on the availability of ATP. This result is corroborated by the findings shown in Fig. 1, which indicate that uptake into the vesicles is a non-concentrative phenomenon, as intravesicular \(\text{[}^3\text{H}\text{]}\)putrescine concentration after reaching equilibrium was less than the extracellular concentration of this diamine. Our estimate of intravesicular putrescine concentration is based on the following measurements. We were able to derive membrane vesicles containing \(~1~\text{mg of vesicular protein from} \times 10^8~\text{cells}$. Using a particle sizing method (38), we measured the vesicle and cell size distributions and determined that the vesicles were \(~0.2–1~\mu\text{m in radius, while the cellular radius was} \approx 10~\mu\text{m.}~\text{If one cell becomes} \times V \text{vesicles, the spherical surface area of the vesicles remains constant (4\pi r^2 = N\times4\pi r^2, where} R \text{is the cell radius and} r \text{is the vesicle radius), and the vesicular volume is equal to the cell volume ([4/3]\pi R^3) times (r/R). Thus, the highest estimate of intravesicular putrescine concentration is given by the smallest vesicle diameter and is: (\text{~1 pmol/mg of protein/1 mg of vesicular protein/1.5} \times 10^8~\text{cells/4187 pmol/cell})^{1/2} (10~\mu\text{m/0.2~\mu m}), which \text{is} \approx 0.1~\mu\text{m or less, when the extravesicular concentration is} 1~\mu\text{m.}~\text{For vesicle diameters of} \approx 1~\mu\text{m, the intravesicular concentration is} \approx 0.01~\mu\text{m putrescine when extravesicular putrescine concentrations are} 1~\mu\text{m.}~\text{An ATP-independent putrescine efflux system has been demonstrated in bacteria (11), where excretion of putrescine is catalyzed by the antiporter protein, which exchanges putrescine and ornithine. The mechanism of putrescine export is different in mammalian cells, however, since levels of extracellular ornithine do not correlate well with the rates of putrescine export (13).}

Putrescine transport in the CHO cell inverted membrane system is pH-dependent. Lowering the extravesicular pH relative to the intravesicular pH, thus increasing the proton concentration outside relative to that inside the vesicles, suppresses uptake into the vesicles. Conversely, lowering the intravesicular pH relative to the extravesicular pH, thus increasing the proton concentration inside relative to that outside the vesicles, stimulates putrescine uptake into the vesicles. These results could be due to one of two mechanisms. In one scenario, DAX could be transporting only deprotonated putrescine. Lower pH inside the inverted vesicles then would result in an increase in the total amount of labeled putrescine transported, but the rate of transport would be unaffected. Alternatively, if protonated putrescine is transported by a proton antiport mechanism, then lower pH inside the vesicles would cause an increase in both the rate and amount of putrescine transport. From Fig. 1A, it can be seen that a 10-min incubation is a measure of reaction rate. Thus, the results in Table I, showing an increase in reaction rate caused by a decrease in intravesicular pH, suggest that putrescine is being transported in this system by an antiport mechanism capable of using protons as the counterion. These data do not allow us to establish the stoichiometry of the antiport mechanism. DAX does not appear to be an electroneutral transporter, since valinomycin in the presence of KCl did not inhibit putrescine uptake in the inverted vesicles. Valinomycin, in the presence of potassium ion, will cause an inward-directed (+) membrane potential (\(\Delta\Psi\)) (39). If the mechanism of putrescine antiport is an electroneutral process, uptake of \(\text{[}^3\text{H}\text{]}\)putrescine would be suppressed by a (+) \(\Delta\Psi\). Interestingly, putrescine uptake was stimulated by valinomycin in the presence or absence of KCl. However, in the presence of valinomycin, the effects of potassium ion were not significantly different than those in absence of this ion.

Verapamil and reserpine are reagents that have been found to be able to interfere with some efflux transporters, such as P-glycoprotein and a vesicular monoamine transporter. The inhibitory effect of verapamil on putrescine export has been reported in the macrophage-like RAW 264 cells and H35 hepatozoma cells (13–15). Here we have shown that putrescine transport into the inside-out vesicles was inhibited by both drugs, although reserpine was not as potent as verapamil which may be related to its limited solubility. This inhibitory effect was confirmed in intact cells. Treatment of the cells with either reserpine or verapamil caused increases in intracellular putrescine contents. These results support the conclusion that putrescine efflux is not a simple diffusion process in intact CHO cells.

The specificity of transport was demonstrated in the inverted membrane system by competition with unlabeled putrescine. The finding that putrescine transport was also inhibited by acetylspermidine (+2 charge), a number of diamines (+2), but...
not by monoacetylputrescine (+1), spermidine (+3), or spermine (+4), indicate that the transport system might mediate export of certain other diamines and acetyl-polymamines with a (+2) charge as well. This conclusion is supported by our whole cell studies, using verapamil to block the putrescine exporter. We observed that putrescine, cadaverine, and both N⁸- and N⁴-monoacetyl spermidine, all carrying +2 charges, accumulated in the presence of this agent. The levels of polyamines carrying charges other than +2 were not affected by verapamil. Fig. 5 summarizes these results, suggesting that DAX exports a variety of amines with the general structure R-NH²-(CH₂)₃-NH₃⁺.

Havel et al. (40) reported the presence of cadaverine (1,5-diaminopentane), the product of lysine decarboxylation, and putrescine in extracellular medium of RAW 264 and H35 cultures. Their studies indicated that cadaverine, which was not detectable inside cells, was formed through the decarboxylation of lysine by ODC, and then removed from the cells with a high degree of efficiency. When we treated cells with verapamil, we also found accumulation of cadaverine, which was suppressed by the ODC inhibitor DFMO, indicating that lysine is a physiological substrate of ODC in animal cells. In the presence of verapamil, the intracellular cadaverine pool is nearly 10% the size of the intracellular putrescine pool in Chinese hamster cells.

The accumulation of N⁸- and N⁴-acetyl spermidine in the presence of verapamil and the conclusion that DAX exports these molecules are consistent with the expression of acetyltransferases responsible for the formation of these acetylpolymamines. The N⁸-acetyltransferase is expressed in log phase growth of animal cells (41), while SSAT activity is induced by certain stresses, including heat shock (30, 31). We observed the accumulation of N⁸-, but not N⁴-, acetyl spermidine in log phase cells treated with verapamil. N⁴-Acetyl spermidine was only observed in verapamil-treated cells in response to inducing stresses.

Further elucidation of the mechanism of export of amines by DAX will require isolation of the purified exporter. The inverted membrane system described here provides a system for accomplishing this goal and, subsequently, cloning of the gene(s) encoding DAX. Recently, we have been able to identify a gene responsible for spermidine export in Bacillus subtilis. Ahmed et al. (42) described a multidrug transporter, named Blt, in these bacteria and cloned another downstream gene in the same cistron, which they called BltD. BltD is a spermidine/spermine acetyltransferase, while Blt facilitates spermidine export (43). Blt is highly homologous to another bacterial multidrug exporter gene, NorA, which has been shown to be proton antiporter (44). Consequently, we conclude that the mechanism of spermidine export in B. subtilis involves a proton antiporter mechanism and is different than the electrodiffusion mechanism identified in Xenopus laevis (12). Thus, our conclusion, that the mechanism of export of the diamine putrescine by DAX in animal cells involves a mechanism that is different from that found in bacteria (11), is similar to the observation that the mechanism of spermidine export is different in pro- and eu-karyotic systems.

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