Polyamine transport system mediates agmatine transport in mammalian cells

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Satiriano, Joseph, Masato Isome, Robert A. Casero, Jr., Scott C. Thomson, and Roland C. Blantz. Polyamine transport system mediates agmatine transport in mammalian cells. Am J Physiol Cell Physiol 281: C329–C334, 2001.—Agmatine is a biogenic amine with the capacity to regulate a number of nonreceptor-mediated functions in mammalian cells, including intracellular polyamine content and nitric oxide generation. We observed avid incorporation of agmatine into several mammalian cell lines and herein characterize agmatine transport in mammalian cells. In transformed NIH/3T3 cells, agmatine uptake is energy dependent with a saturable component indicative of carrier-mediated transport. Transport displays an apparent Michaelis-Menten constant of 2.5 μM and a maximal velocity of 280 pmol·min⁻¹·mg⁻¹ protein and requires a membrane potential across the plasma membrane for uptake. Competition with polyamines, but not cationic molecules that utilize the y⁻ system transporter, suppresses agmatine uptake. Altering polyamine transporter activity results in parallel changes in polyamine and agmatine uptake. Furthermore, agmatine uptake is abrogated in a polyamine transport-deficient human carcinoma cell line. These lines of evidence demonstrate that agmatine utilizes, and is dependent on, the polyamine transporter for cellular uptake. The fact that this transport system is associated with proliferation could be of consequence to the antiproliferative effects of agmatine.

THE DECARBOXYLATION OF ARGININE by arginine decarboxylase (ADC) produces agmatine. In mammals, agmatine has the capacity to regulate intracellular polyamine levels (31, 36) and nitric oxide synthase activity (1, 2, 6) and has been ascribed roles in association with neurotransmitter receptors (13, 14, 21, 27) and modulation of opioid analgesia (12) and as an ADP-ribose acceptor (19). Distribution of agmatine in organs can vary widely with high levels in organs that display low-to-undetectable ADC activity (15–17, 23). For example, in blood vessels, agmatine is found in the endothelium and vascular smooth muscle cells, yet only the endothelial cells express ADC (25). The disparity between cellular agmatine content and synthesis implies cellular uptake from extracellular sources. Furthermore, nonreceptor-mediated regulation of intracellular polyamine content and nitric oxide generation would require intracellular agmatine acquisition by most cells. Agmatine uptake by mammalian cells has yet to be characterized.

Polyamines (putrescine, spermidine, and spermine) are cationic molecules essential for entry into and progression through the cell cycle, and thus proliferation. Polyamine uptake directly correlates with the rate of cellular proliferation (4, 8, 9, 18, 20). In bacteria and plants, agmatine is a precursor of polyamines (35). An agmatine-putrescine antiporter mediates transport of these molecules in Enterococcus faecalis (5), whereas a unidirectional energy-dependent system is utilized to transport agmatine and polyamines in Escherichia coli (11). In the present study, we examined mechanisms of agmatine uptake in a cultured fibroblast-derived cell line, a renal proximal tubule cell line, and a lung carcinoma cell line. We present evidence that the polyamine transport system mediates agmatine uptake in these mammalian cells. Agmatine exhibits antiproliferative effects that are associated with its capacity to deplete intracellular polyamine levels (31, 36). Competition of agmatine with polyamines for cellular uptake offers a mechanism by which agmatine can effectively contribute to the attenuation of intracellular polyamine levels and thus proliferation.

MATERIALS AND METHODS

Chemicals and supplies. α-Difluoromethylornithine (DFMO) was kindly supplied by Dr. C. Macias of ILEX Oncology (San Antonio, TX). Agmatine was purchased from Aldrich (Milwaukee, WI). 3H radioisotopes of putrescine, spermidine, and arginine were purchased from NEN (Boston, MA), and 3H-agmatine was from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise noted.

Cell preparations. Ras/3T3 (Ha-ras-transformed NIH/3T3) (37) or MCT (kidney proximal tubule) (7) cell lines were used.

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for all experiments, with the exception of the polyamine transport mutant studies. Ras/3T3 and MCT cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA). Serum-starved cells were grown in DMEM supplemented with 0.5% calf serum for 3 days before and including uptake. For polyamine transport mutant studies, NCI-H157 non-small cell lung carcinoma (non-SCLC) cells with a neomycin resistance vector (H157v) and the transport-deficient mutant cells (H157mt) were grown in RPMI 1640 supplemented with 10% FBS.

Transport experiments. Transport experiments were performed as previously described (29). In brief, cells were grown in six-well culture plates for 3 days until 70–90% confluent. Wells were washed with HEPES buffer (25 mM Na+-free HEPES, 5 mM KCl, 0.9 mM CaCl2, 1 mM MgSO4, 5.6 mM d-glucose, and 137 mM NaCl). The addition of HEPES buffer containing 10 μM [3H]agmatine, [3H]putrescine, or [3H]spermidine at ~200,000 counts per minute well started a 5-min incubation (uptake) period, unless otherwise stated. The amount of isotope and incubation time was increased in evaluation of transport at high, nonradioactive agmatine and spermidine concentrations (Fig. 1B). Three rapid washes with ice-cold phosphate-buffered saline (PBS) and lysis in 3 N NaOH terminated the reactions. The Bio-Rad protein assay (Bio-Rad, Hercules, CA) used a small aliquot of each lysate for protein determination, and the remainder of the sample was counted in a beta-scintillation counter to evaluate uptake. Nonspecific binding (blank) was determined as stated above, except the labeled addition was immediately terminated (time 0) by PBS washes and lysis. When applicable, experimental agents were administered for a 30-min pretreatment period in HEPES buffer and continued throughout the uptake period. Choline chloride (137 mM) replaced sodium chloride in Na+-free HEPES buffer.

Statistical evaluations. Variations between samples within groups were analyzed by ANOVA, with significance determined by Fisher’s protected least significant differences post hoc test. StatView software (version 4.5, Abacus Concepts) was used for these analyses. Systat software (SPSS) was used to estimate a rate constant and maximum uptake.

RESULTS

Transport kinetics. Rapid agmatine uptake was observed in several mammalian cell types, including Harras-transformed NIH/3T3 fibroblast (Ras/3T3) and MCT cell lines. Here we show the results of the fibroblast-derived Ras/3T3 cell line. Uptake in Ras/3T3 cells was linear for at least 60 min (not shown). Agmatine import demonstrated a saturable component, with an apparent Michaelis-Menten constant (Km) and maximal velocity (Vmax) of 2.5 μM and 280 pmol·min⁻¹·mg⁻¹ protein, respectively (Fig. 1, A and inset). Extending agmatine concentrations to 1,000 μM (Fig. 1B) revealed a nonsaturable uptake component in these transformed cells. Spermidine uptake also demonstrated saturable kinetics at low concentrations with a nonsaturable component apparent at high concentrations (Fig. 1).

Energy and Na⁺ dependence. To determine energy dependence, Ras/3T3 cells were pretreated for 30 min with 0.1 mM dinitrophenol (DNP) and 1 mM iodoacetate (IA). This procedure inhibits ATP synthesis by uncoupling oxidative phosphorylation. Uptake was also evaluated at 4°C. Both DNP plus IA and transport at 4°C markedly suppressed agmatine uptake (Table 1). Na⁺ dependence was evaluated by substituting choline chloride for sodium chloride in the HEPES incubation buffer. Choline chloride buffer diminished agmatine transport (Table 1). Disruption of the Na⁺ gradient by pretreating cells with the Na⁺-K⁺-pump inhibitor ouabain (0.1 mM) or the ionophore monensin (5 μM) tended to decrease transport, but the results were not significantly different from those for untreated cells. Pretreatment with the ionophore gramicidin D (4 μg/ml) inhibited transport (Table 1). Similar
results were obtained for uptake of the polyamines putrescine and spermidine (Table 1).

**Competition experiments.** Agmatine is a cationic molecule that is unlikely to effectively traverse the cell membrane by simple diffusion. Arginine, the precursor molecule of agmatine, utilizes the inducible \( y^+ \) system transporter, among others. Polyamines utilize polyamine transporters (Fig. 2). We evaluated agmatine uptake in competition experiments with polyamines, paraquat (a polyamine transport inhibitor), arginine, and other cationic molecules that utilize the arginine \( y^+ \) system transporter. Labeled agmatine (Fig. 3A) or putrescine (Fig. 3B) uptake was evaluated in the absence (control) or presence of unlabeled competitors. Unlabeled competitors were in 100-fold molar excess (1 mM), except for agmatine doses at 1-, 10-, and 100-fold molar excess (10, 100, and 1,000 \( \mu M \), respectively; Fig. 3B) as indicated.

Unlabeled agmatine, as a positive control, effectively competed with labeled agmatine for uptake (Fig. 3A). Polyamines and paraquat were also effective competitors. Ornithine, lysine, \( N^G \)-monomethyl-L-arginine, and arginine, all of which utilize the \( y^+ \) system transporter, were ineffective competitors of agmatine uptake.

Uptake of \([^{3}H]\)putrescine, used as a marker for polyamine transport, was also evaluated in the absence and presence of unlabeled competitors. Agmatine, shown at 1-, 10-, and 100-fold molar excess, was an effective competitor for putrescine transport (Fig. 3B). Molar excess (100-fold) unlabeled polyamines, or the polyamine transport inhibitor paraquat, were used as controls. Unlabeled ornithine, lysine, and arginine were negative controls and were ineffective competitors of polyamine transport. Inhibition of putrescine uptake by spermidine implies a single polyamine transporter, as anticipated for a fibroblast-derived cell line (33). MCT cells displayed an identical competition profile to that of the Ras/3T3 cells (not shown).

**Alteration of polyamine transport activity.** DFMO is a potent inhibitor of the first and rate-limiting enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC). DFMO inhibition of ODC activity resulted in a compensatory increase in polyamine transport (4, 29). Administration of 5 mM DFMO into the culture media of Ras/3T3 cells for 3 days before the uptake reaction increased putrescine transport (Fig. 4).

### Table 1. Ion and energy dependence for agmatine and polyamine uptake in Ras/3T3 cells

<table>
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<tr>
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<th>Agmatine</th>
<th>Putrescine</th>
<th>Spermidine</th>
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<tbody>
<tr>
<td>Control</td>
<td>211 ± 17</td>
<td>213 ± 40</td>
<td>248 ± 16</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>91 ± 9*</td>
<td>108 ± 31*</td>
<td>165 ± 17*</td>
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<tr>
<td>Ouabain (0.1 mM)</td>
<td>177 ± 13</td>
<td>165 ± 17</td>
<td>237 ± 19</td>
</tr>
<tr>
<td>Mesnénin (5 ( \mu M ))</td>
<td>186 ± 18</td>
<td>187 ± 46</td>
<td>230 ± 23</td>
</tr>
<tr>
<td>Gramicidin D (4 ( \mu g/m))</td>
<td>99 ± 14*</td>
<td>127 ± 25*</td>
<td>148 ± 10*</td>
</tr>
<tr>
<td>DNP + IA</td>
<td>51 ± 8*</td>
<td>102 ± 14*</td>
<td>61 ± 9*</td>
</tr>
<tr>
<td>4°C</td>
<td>32 ± 7*</td>
<td>65 ± 11*</td>
<td>34 ± 7*</td>
</tr>
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Values represent uptake in pmol·min\(^{-1}\)·mg\(^{-1}\) protein ± SD of 3 experimental observations. *\( P < 0.01 \) compared with control group.

DISCUSSION
Here we characterize agmatine uptake in mammalian cell lines and demonstrate that it is via the polyamine transport system. Mammalian polyamine transport is energy dependent and saturable, suggesting that it is carrier mediated. Agmatine uptake exhibits a saturable component, with an apparent \( K_m \) of 2.5 \( \mu M \) and a \( V_{max} \) of 280 pmol·min\(^{-1}\)·mg\(^{-1}\) protein in Ras/3T3 cells, similar to that observed for spermidine at 2.6 \( \mu M \) and 347 pmol·min\(^{-1}\)·mg\(^{-1}\) protein, respectively.

Quiescent cells, as a rule, display little, if any, polyamine uptake. To make the Ras/3T3 cells more quiescent, they were serum starved for 3 days in 0.5% FBS before uptake. Serum starvation decreased uptake of putrescine relative to the untreated Ras/3T3 cells, suggesting a downregulation of polyamine transporters associated with quiescence (Fig. 4). Changes in agmatine uptake closely paralleled those of putrescine in response to either DFMO or serum starvation (Fig. 4).

**Agmatine uptake in polyamine-deficient cells.** A polyamine transport-deficient cell line from human non-SCLC cells (NCI-H157) has been described (34). We compared uptake of agmatine, putrescine, and arginine in the parental H157 cell line, H157v, and H157mt. Uptake in the parental H157 cell line was not different from that in the neomycin vector-containing line, H157v (Fig. 5). The polyamine transport-deficient mutant cells, H157mt, displayed markedly diminished uptake of agmatine and putrescine relative to control H157 and H157v cells. Suppressing active transport at 4°C did not further decrease uptake in H157mt cells. This suggests that the uptake noted in the polyamine transport-deficient cells is not carrier mediated. Agmatine uptake, evaluated as a negative control, was not affected in the mutant cell line (H157, 2,675 ± 271; H157v, 2,619 ± 265; H157mt, 2,807 ± 415 pmol·min\(^{-1}\)·mg\(^{-1}\) protein).

Fig. 2. Structures of arginine, agmatine, and polyamines. These molecules contain a common N-C\(_4\)-N sequence. Paraquat does not contain a N-C\(_4\)-N sequence, but the distance between the positively charged nitrogens are thought to mimic the 4-carbon spacing of polyamines.
The $K_m$ of agmatine uptake is similar to its plasma concentration (14). We detected a concentration-dependent linear increase in agmatine and spermidine uptake above the saturation levels predicted from Fig. 1A (Fig. 1B). Uptake at 4°C also increases with concentration. However, subtracting uptake at 4°C from total uptake is insufficient to negate the nonsaturable component observed for agmatine and spermidine. These data suggest that the nonsaturable uptake cannot be accounted for by simple diffusion and raises the possibility of either nonspecific transport at these high substrate concentrations or an additional agmatine/polyamine transporter in these cells. The existence and characterization of this potential transport system and whether it is the outcome of Ha-ras transformation requires further study.

Agmatine uptake is energy dependent and largely, but not completely, Na$^+$ dependent (Table 1). Ouabain tended to suppress agmatine transport, but the effect was not significantly different from control untreated cells. Aziz et al. (3) also did not observe changes in putrescine uptake in response to ouabain in bovine pulmonary artery smooth muscle cells. Gramicidin D, but not monensin, significantly suppresses agmatine uptake (Table 1). A similar response to these agents was reported for putrescine uptake in bovine lymphocytes (10). Gramicidin D is an ionophore selective for monovalent cations, whereas monensin is an electroneutral exchanger of Na$^+$ for protons. Together, these results imply that a membrane potential, though not necessarily a Na$^+$ electrochemical gradient, is required for agmatine transport into these cells. Poulin et al. (22) first observed the requirement for a plasma membrane potential in mammalian cell polyamine transport in a human breast cancer cell line. The polyamines putrescine and spermidine demonstrate a profile of

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**Fig. 3.** Effects of competitors on agmatine uptake. Transport experiments monitoring 10 μM [3H]agmatine (A) or [3H]putrescine (B) uptake in Ras/3T3 cells for a 3-min incubation period in the absence, control, or presence of unlabeled competitors. Competitors are in 100-fold molar excess of labeled control, except agmatine, as noted in micromolars in B. Control, solid bars; agmatine, open bars; polyamines, crosshatched bars; paraquat, hatched bars; arginine and other $y^+$ system transporter substrates, horizontally lined bars. 1-NMMA, $\dot{N}^\circ$-monomethyl-$\dot{L}$-arginine; cpm, counts per minute; prot, protein.

**Fig. 4.** Effect of altering polyamine transport on agmatine uptake. Evaluation of 10 μM [3H]agmatine or [3H]putrescine uptake into untreated Ras/3T3 cells, control, or cells pretreated for 3 days with α-difluoromethylornithine (DFMO; 5 mM) or serum starved for 3 days in media containing 0.5% fetal bovine serum. Values are means ± SD of 3 observations. DFMO and serum-starved groups were significantly different from control groups for both agmatine and putrescine ($P < 0.05$).

**Fig. 5.** Agmatine uptake in polyamine transport-deficient cells. Uptake of 10 μM [3H]agmatine or [3H]putrescine into the human nonsmall cell lung carcinoma cell line H157, with a neomycin-resistant vector, H157v, or the polyamine transport-deficient mutant, H157mt. Uptake in H157mt cells was also evaluated at 4°C (last group). Values are means ± SD of 3 observations. Uptake of agmatine or putrescine into H157mt was significantly different from either H157 or H157v ($P < 0.0001$).
Na\(^+\) and energy dependence similar to that of agmatine in Ras/3T3 cells (Table 1).

Polyamines and paraquat, but not cationic molecules that utilize the y\(^+\) system transporter, inhibit agmatine uptake (Fig. 3A). Although suggestive, this does not establish that agmatine utilizes the polyamine transporter. Polyamines could, for example, merely hinder agmatine access of agmatine transporters. The converse experiment, agmatine inhibition of polyamine uptake (Fig. 3B), argues against this possibility. That cellular uptake of agmatine is via the polyamine transport system is further supported by the parallel changes observed in agmatine and polyamine uptake upon altering polyamine transport by DFMO administration or serum starvation (Fig. 4).

We used a polyamine transport-deficient large-cell lung carcinoma cell line to further examine the relationship between agmatine uptake and polyamine transporters. Active transport of both agmatine and polyamine are abrogated in the polyamine transport-deficient cell line (Fig. 5). The results at 4°C suggest that the remaining transport observed in the H157mt is not due to active transport. The polyamine transport-deficient cells clearly indicate the dependence of agmatine on polyamine transporters, at least in H157 carcinoma cells, for cellular uptake. Unlike the uptake in Ras/3T3 cells, uptake of agmatine in H157 and H157v cells was not equivalent to that of putrescine. Selective polyamine transporters have been described in some mammalian cell types; however, most cells, including fibroblasts, have a single transporter (for review, see Ref. 33). It is unknown whether lung cells express multiple transporters with overlapping selectivities or a single nonselective transporter, as expressed in fibroblasts. Another possible explanation for this discrepancy could be the unique capacity of the lungs to take up polyamines by diffusion (24). Uptake at 4°C was observed and was higher for putrescine than for agmatine in H157mt (Fig. 5) and Ras/3T3 (Table 1) cells. Mammalian polyamine transporters are not yet cloned; therefore, further evaluation of our model systems at the molecular level could not be assessed.

Agmatine is an important mammalian neurotransmitter molecule (for review, see Ref. 26). It is synthesized in the brain and taken up by synaptosomes from where it is released by depolarization. Incorporation of agmatine into brain synaptosomes occurs by a yet unknown energy-dependent, but Na\(^+\)-independent, mechanism that is independent of polyamine transport (28). Expression of polyamine transporters in synaptosome vesicles has not been established. However, the report by Galea et al. (6) describes active agmatine transport by a mechanism that is different from that described here by the polyamine transport system. The presence of discrete transporters in the plasma membrane and intracellular vesicles could allow discrimination of agmatine and polyamines to intracellular sites and an additional degree of regulation of intracellular agmatine and polyamine distribution.

This study provides evidence that cellular agmatine uptake is via the polyamine transport system. Rapid polyamine uptake has been noted in rapidly proliferating cells (9) and many tumor cell lines (4, 8, 18, 20), compared with the differentiated or nontumorigenic cells of the same lineage. Thus agmatine transport via polyamine transporters allows agmatine to compete with polyamines for cellular uptake and could target agmatine uptake to rapidly proliferating cells. The targeting of agmatine uptake to, and the effects on, rapidly proliferating cells is currently under investigation (30). The utilization of the polyamine transport system by agmatine could effectively contribute to the depletion of intracellular polyamine levels and, thus, the antiproliferative potential of agmatine.

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