Biochemical and Biophysical Research Communications **280,** 307–311 (2001)
doi:10.1006/bbrc.2000.4101, available online at http://www.idealibrary.com on IDEAL®

Agmatine Uptake by Cultured Hamster Kidney Cells

Alicia Esteban del Valle, José Carlos Paz, Francisca Sánchez-Jiménez, and Miguel Ángel Medina
Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain

Received November 20, 2000

Agmatine, the product of arginine decarboxylation, has been recently found in a wide variety of animal tissues. In spite of the emergent interest on agmatine in animals, the mechanism of agmatine uptake in mammalian cells has been scarcely studied. An analysis of radiolabeled agmatine uptake was carried out by using a classical, kinetic approach with BHK-21 hamster kidney cells in culture. A high affinity, temperature- and energy-dependent agmatine transport system in BHK-21 kidney cells is here kinetically characterized which seems to be a "general" transporter shared by di- and triamines and different to a highly specific carrier for the tetraamine spermine. © 2001 Academic Press

Key Words: **agmatine; uptake; kidney; polyamines; DFMO.**

Arginine is an amino acid critical to normal growth and multiple physiological processes, being the precursor of urea, proline, glutamate, creatine, and other important bioactive compounds, such as nitric oxide (1). An enormous interest has been focused upon the key roles of nitric oxide in animal physiology (2). Many years ago, it has been established that arginine is a substrate for arginine decarboxylase (E.C. 4.1.1.19) in bacteria and plants (3), yielding the diamine agmatine, but it was believed that this enzyme was not expressed in mammals. In 1994, agmatine was identified as an endogenous clonidine-displacing substance in the brain (4). Afterwards, agmatine has been detected in many different tissues and organs, including aorta, spleen, adrenals, small intestine, skeletal muscle, stomach, brain, liver and kidney (5–7). Although agmatine is also present in food and intestinal flora, the distribution of tissue agmatine does not correlate to tissue blood flow, suggesting local synthesis by arginine decarboxylase. In fact, arginine decarboxylase has been partially cloned in rat kidney (8), and its activity has been demonstrated in a number of cells, tissues and organs (9–13).

In addition, agmatine is another bioactive metabolite of arginine in mammals. In plants and bacteria, agmatine is a metabolic intermediate in a pathway for polyamine biosynthesis; in fact, hydrolysis of agmatine by agmatinase (E.C. 3.5.3.11) yields putrescine, a precursor for spermidine and spermine biosynthesis. Recently, agmatinase activity has been found in mammalian brain (1, 14). Thus, agmatine may also be a precursor for polyamine biosynthesis in mammals.

Several effects are induced by agmatine, including promotion of catecholamine release from adrenal chromaffin cells (4), stimulation of insulin release (15), and inhibition of human coronary artery vascular smooth muscle cell growth (16). The multiple modulator effects of agmatine on arginine and polyamine metabolism seem especially relevant: agmatine has been shown to be a competitive inhibitor of nitric oxide synthases (17, 18), an activator of *S*-adenosylmethionine decarboxylase (19), an inducer of translational frameshift of antizyme mRNA (20), an inhibitor of ornithine decarboxylase and polyamine uptake (19, 20), and a potent inducer of spermidine/spermine acetyltransferase activity (19).

In kidney, agmatine is degraded to guanidinobutylaldehyde by diamine oxidase. The production and degradation of agmatine has been described as a novel endogenous regulatory system in the kidney (10). In fact, agmatine seems to contro natriuresis by functioning as a physiological agonist of I_1 imidazoline receptors (21), and it can increase absolute proximal reabsorption and single nephron glomerular filtration rate through I_2 imidazoline receptors (10).

In spite of the findings suggesting an interference of agmatine on polyamine uptake, scarce information is currently available on the mechanism of agmatine uptake. A low affinity, Na^+-independent uptake system for agmatine has been described in rat brain synaptosomes (22), and it has been hypothesized that agmatine can suppress proliferation in transformed cells after targeting them via selective transporters (23).

In the present work, we describe for the first time the kinetic characterization of a high affinity uptake system for agmatine in kidney, by performing transport experiments in cultures of BHK-21 hamster kidney cells.

0006-291X/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

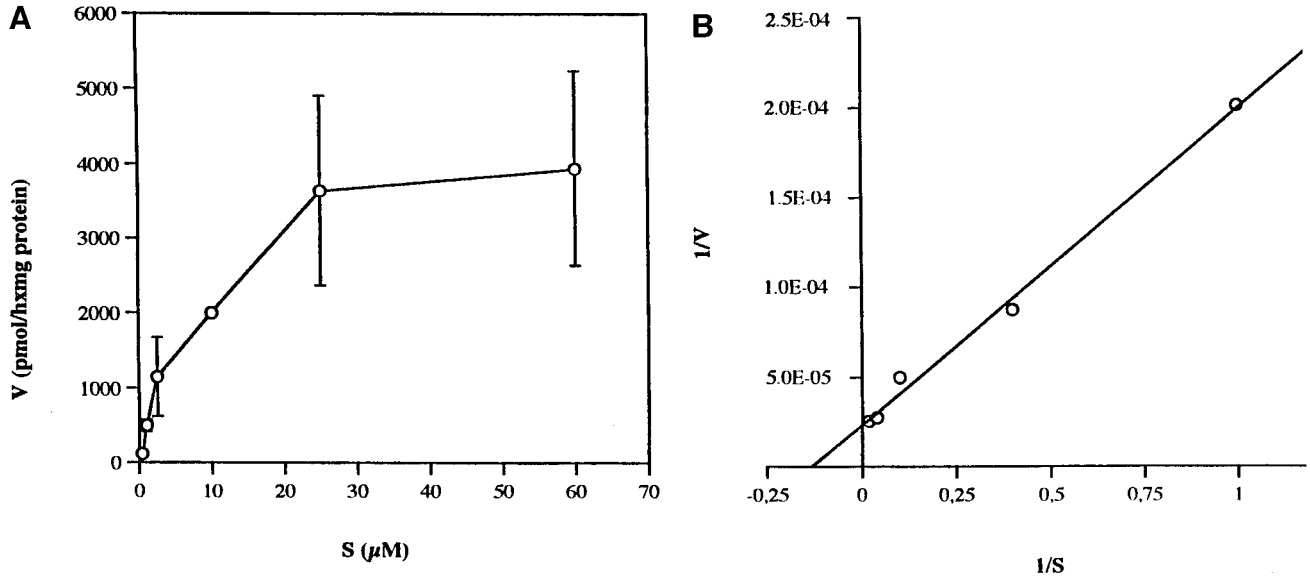

FIG. 1. Kinetics of agmatine uptake by BHK-21 cells. Specific agmatine uptake by BHK-21 cells was determined as described under Materials and Methods. (A) Direct plot of the means ± SD values obtained from two independent experiments carried out by triplicate. (B) Double reciprocal plot of mean values.

MATERIALS AND METHODS

Materials. Sterile plasticware was from Nunc. DMEM medium, fetal calf serum, trypsin-versane and antibiotics were from Bio-Whittaker. [^{3}H]-Agmatine (50 Ci/mmol) was supplied by American Radiolabeled Chemicals. Ecoscint H liquid scintillation counting solution was from National Diagnostics. All other reagents were from Sigma.

Cell culture. BHK cells were obtained from the American Type Culture Collection. They were grown in DMEM medium supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin, and amphotericin B) at 37°C in 90 mm diameter tissue culture dishes under 5% CO_2 atmosphere. When cells were confluent, they were detached with trypsin-versane and subcultured at a split ratio of 1:4.

Transport assays. The cells used for transport experiments were seeded into 24-well cluster dishes and were used when reached 50–75% of subconfluency. The buffered media used in transport experiments were phosphate buffered salines containing 0.15 M sodium (PBS-Na), potassium (PBS-K), or choline (PBS-choline) chlorides, or 0.3 M mannitol (PB-mannitol). Serum-containing DMEM was retired from wells, cells were washed twice with PBS and transport was initiated by adding 0.5 mL of the buffered medium containing different concentrations of agmatine. Experiments at both 37°C and 0°C were always carried out in parallel. After 30 min of incubation, the uptake was terminated by removing the transport medium and washing with 2 × 0.5 mL aliquots of ice-cold PBS. Plates were drained and 0.4 mL of 0.5% Triton X-100 were added. Cells were then removed with a cell scrapper and suspended in this medium, 250 μL samples were dissolved in 4 mL of scintillation solution for counting and protein was determined using the method described by Bradford (24). The nonsaturable, linear component of uptake due to simple diffusion measured in the experiments carried out at 0°C was always substrated from the total amount of agmatine transported at 37°C.

RESULTS AND DISCUSSION

We carried out kinetic experiments making the usual assumption that polyamine uptake has two components: one saturable, corresponding to the carrier-mediated uptake, and the other linear, corresponding to nonspecific uptake, that is, passive diffusion following Fick's law. Since polyamine transport is energy dependent, diffusional uptake at 0°C is a measurement of nonspecific uptake, including binding to cell surface (25). For this reason, we always carried out parallel experiments at both 37°C and 0°C.

Figure 1 shows that the specific uptake of agmatine (uptake at 37°C minus uptake at 0°C) by BHK-21 hamster kidney cells incubated in PBS-Na is a saturable process. In the conditions used, nonspecific uptake accounted for less than 10% of total uptake at agmatine concentrations lower than 60 μM (results not shown). From the linear representation of data using either Lineweaver-Burk (not shown) or Hanes-Woolf (Fig. 1b) plots, the following kinetic parameters were calculated: a K_m of 9.9 ± 1.4 μM and a V_{max} of 4867 ± 802 pmol/h/mg protein. The affinity of the transport system for agmatine is in the range of those published for other polyamines in whole cells (26) and three orders of magnitude higher than that shown for agmatine in rat brain synaptosomes (22).

The potential Na^+-dependence of agmatine transport by BHK-21 cells was examined by isosmotically substituting NaCl with either KCl, choline chloride or mannitol in the assay medium. Figure 2 shows that substitution of sodium ions with choline or potassium ions inhibited, at least partially, agmatine uptake. On the contrary, agmatine uptake slightly increased by replacing NaCl with an osmotically equivalent concentration of mannitol.

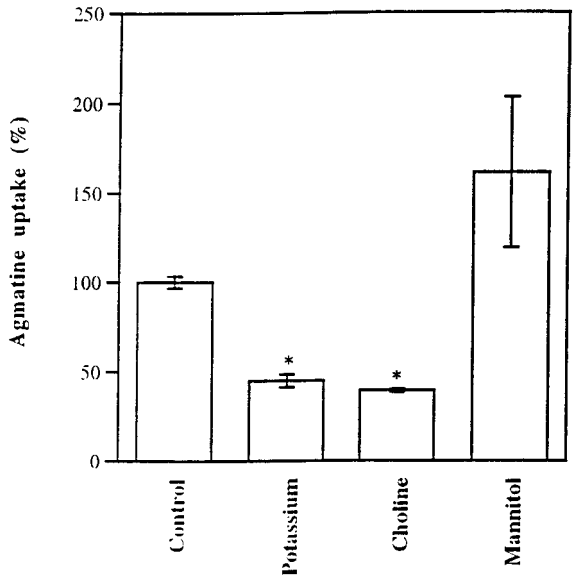

FIG. 2. Ionic effects on the transport of agmatine by BHKJ-21 cells. Transport experiments were carried out in parallel in different phosphate buffered media containing isosmotic concentrations of either sodium, potassium or choline chloride, or mannitol. In all the cases, agmatine concentration was 10 μM. Data are percentages of specific transport, taking the values in PBS-Na as 100%, and they are given as means ± SD of the means values of three determinations in three independent experiments. *Significant ($P < 0.01$) versus PBS-Na values according to a Student's t test.

Some authors consider the fraction of total uptake in the presence of sodium ions which is inhibited by isoosmotical concentrations of choline chloride as a "Na^+-dependent" uptake (27,28). In this sense, part of the agmatine uptake by BHK-21 cells could be considered as a "Na^+-dependent" transport. However, a Na^+-dependent transport system is defined as that secondary active transport in which the substrate is taken up in symport with sodium ions. Since NaCl can be substituted by isosmotical concentrations of mannitol in our system, we cannot talk of a true Na^+-dependent uptake. In proliferant cells, plasma membrane potential is primarily determined by a potassium-ion diffusion potential (29). Therefore, increasing the concentration of extracellular potassium-ions should depolarize the plasma membrane, in accordance with the Nernst equation. As shown in Fig. 2, uptake of 10 μM agmatine by BHK-21 cells incubated in PBS-Na is decreased by almost 60% in PBS-K. Thus, the "Na^+-dependent" uptake could be described here more precisely as a plasma membrane potential-dependent uptake (29–32).

To study the substrate specificity of agmatine uptake by BHK-21 cells in PBS-Na, experiments were carried out in which radiolabelled agmatine competed with other unlabelled polyamines added 50-fold in excess. Figure 3 summarizes the results obtained and shows that most of the polyamines tested inhibited agmatine uptake very significantly, especially putrescine, spermidine and cadaverine. Only the tetraamine spermine was a poor inhibitor of agmatine uptake. Thus, in high contrast with published data for agmatine uptake into rat brain synaptosomes (22), our results suggest that agmatine shares a transport system common to other polyamines and that spermine is the only polyamine which seems to have a specific carrier different to the "general" carrier for polyamines.

Figure 4 shows that agmatine uptake is highly increased (more than 3-fold) in cells pretreated for 48 h with 3 mM DFMO, a suicide inhibitor of ornithine decarboxylase, the first and rate-limiting enzyme of the pathway for ornithine-derived poyamines (putrescine, spermidine, and spermine). These data seem to reinforce our suggestion on the presence of a transport system shared by agmatine and other polyamines in BHK-21 kidney cells.

Increased accumulation of evidence points to calcium-ion as a pivotal element in the regulation of polyamine transport (25). In human breast cancer cells, depletion of the extracellular calcium ions significantly inhibits putrescine uptake, and the addition of 420 μM $CaCl_2$ in the extracellular medium induces a 2-fold increase of putrescine uptake (29). In rabbit intestinal brush border membrane vesicles, addition of calcium-ions also increases putrescine uptake (33). We

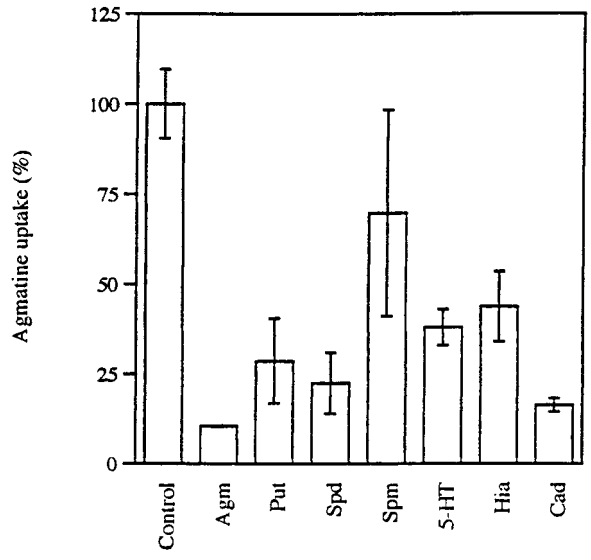

FIG. 3. Substrate specificity of agmatine uptake by BHK-21 cells. Uptake experiments were carried out in the presence of 2 μM agmatine as the substrate and 100 μM unlabelled polyamines and analogs as inhibitors. The effects of unlabelled agmatine (Agm), putrescine (Put), spermidine (Spd), spermine (Spm), 5-hydroxytryptamine or serotonin (5-HT), histamine (Hia) and cadaverine (Cad) were tested. Data are percentages of specific transport, taking the control values as 100%, and they are given as means ± SD of the means values of three determinations in three independent experiments. All the differences versus control values were significant ($P < 0.01$) according to a Student's t test.

compared 10 μM agmatine transport in the absence or presence of 1 mM $CaCl_2$ in the assay medium and we could not find any significant differences in the respective rates of uptake (Fig. 4). On the other hand, the presence of the calcium-ion chelator EGTA (1 mM) significantly decreased agmatine uptake (Fig. 4). Once again, these results are in contrast with those published for agmatine uptake into rat brain synaptosomes (22).

Figure 5 shows that neither neutral amino acids (glutamine), nor cationic amino acids (arginine or ornithine) inhibited agmatine uptake by BHK-21 kidney cells. These data are in contrast with our previous results on the activating effect of glutamine on ornithine transport by Ehrlich cell plasma membrane vesicles (34). Interestingly, a slight but significant increase in agmatine uptake can be observed in the presence of ornithine, the substrate for ornithine decarboxylase and entrance point to the biosynthetic pathway of polyamines.

In conclusion, we have kinetically characterized a high affinity, temperature and energy dependent agmatine transport system in BHK-21 kidney cells which seems to be a "general" transporter shared by di- and triamines and different to a highly specific carrier for the tetraamine spermine.

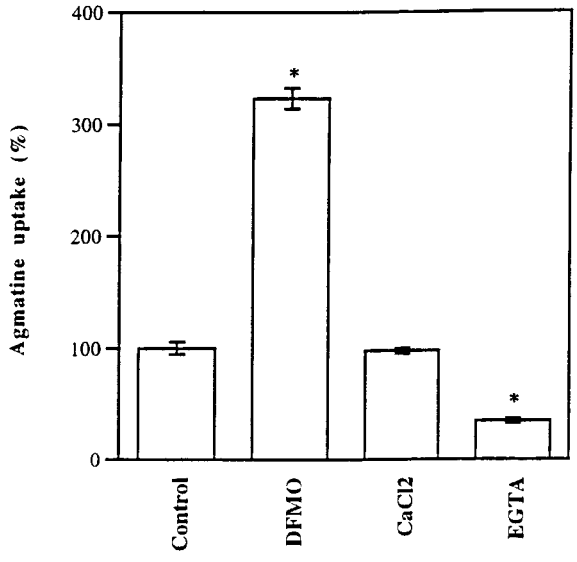

FIG. 4. Effects of DFMO pretreatment and calcium ion presence or absence on agmatine uptake by BHK-21 cells. Uptake experiments were carried out as described under Material and Methods. In experiments with DFMO, cells were pretreated for 48 h with 3 mM DFMO. In experiments on the effect of calcium ions, 1 mM calcium chloride or 1 mM EGTA was added to the assay medium. Data are percentages of specific transport, taking the control values as 100%, and they are given as means ± SD of the means values of two determinations in two independent experiments. *Significant ($P < 0.01$) versus control values according to a Student's t test.

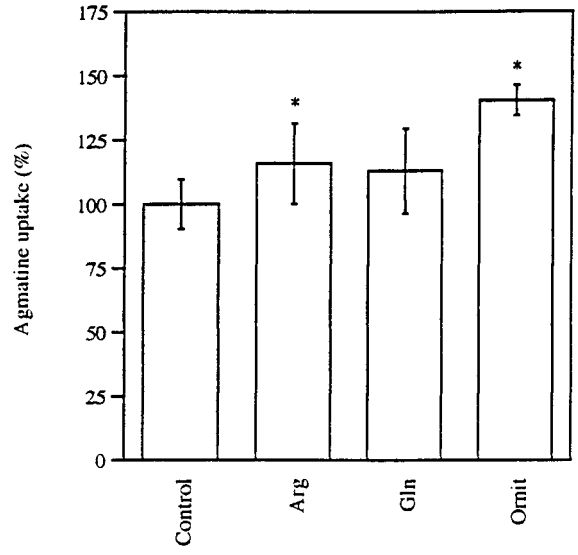

FIG. 5. Effects of some amino acids on agmatine uptake by BHK-21 cells. Uptake experiments were carried out in the presence of 2 μM agmatine as the substrate and 100 μM unlabelled arginine (Arg), glutamine (Gln), or ornithine (Ornit). Data are percentages of specific transport, taking the control values as 100%, and they are given as means ± SD of the means values of two determinations in two independent experiments. *Significant ($P < 0.01$) versus control values according to a Student's t test.

ACKNOWLEDGMENTS

Supported by Grant SAF 98-0150 from the CICYT. A.E.V. had a predoctoral fellowship from the Spanish Government.

REFERENCES

1. Wu, G., and Morris, S. M., Jr. (1998) Arginine metabolism: Nitric oxide and beyond. *Biochem. J.* **336,** 1–17.
2. Nathan, C., and Xie, Q. W. (1994) Nitric oxide synthases: Roles, tolls and controls. *Cell* **78,** 915–918.
3. Wu, W. H., and Morris, D. R. (1973) Biosynthetic arginine decarboxylase from *Escherichia coli*. *J. Biol. Chem.* **248,** 1687–1695.
4. Li, S., Regunathan, S., Barrow, C. J., Eshraghi, J., Cooper, R., and Reis, D. J. (1994) Agmatine: An endogenous clonidine-displacing substance in the brain. *Science* **263,** 966–969.
5. Stickle, D., Bohrer, A., Berger, R., Morrisey, J., Klahr, S., and Turk, J. (1996) Quantitation of the putative neurotransmitter agmatine as the hexafluoroacetylacetonate derivative by stable isotope dilution gas chromatography and negative-ion chemical ionization mass spectrometry. *Anal. Biochem.* **238,** 129–136.
6. Molderings, G. J., Burian, M., Menzel, S., Donecker, K., Homann, J., Nihius, M., and Gothert, M. (1999) Imidazoline recognition sites and stomach function. *Ann. N.Y. Acad. Sci.* **881,** 332–343.
7. Raasch, W., Regunathan, S., Li, G., and Reis, D. J. (1995) Agmatine, the bacterial amine, is widely distributed in mammalian tissues. *Life Sci.* **56,** 2319–2330.
8. Morrissey, J., McCracken, R., Ishidoya, S., and Klahr, S. (1996) Partial cloning and characterization of an arginine decarboxylase in the kidney. *Kidney Int.* **47,** 1458–1461.
9. Li, G., Regunathan, S., and Reis, D. J. (1995) Agmatine is syn-

thesized by a mitochondrial arginine decarboxylase in rat brain. *Ann. N.Y. Acad. Sci.* **763,** 325–329.

10. Regunathan, S., Feinstein, D. L., Raasch, W., and Reis, D. J. (1995) Agmatine, the decarboxylated arginine, is localized and synthesized in glial cells. *Neuroreport* **6,** 1897–1900.
11. Regunathan, S., Youngson, C., Raasch, W., Wang, H., and Reis, D. J. (1996) Imidazoline receptors and agmatine in blood vessels: a novel system inhibiting vascular smooth muscle proliferation. *J. Pharmacol. Exp. Ther.* **276,** 1272–1282.
12. Lortie, M. J., Novotny, W. F., Peterson, O. W., Vallon, V., Malvey, K., Mendonca, M., Satriano, J., Insel, P., Thompson, S. C., and Blantz, R. C. (1996) Agmatine, a bioactive metabolite of arginine. Production, degradation and functional effects in the kidney of the rat. *J. Clin. Invest.* **97,** 413–420.
13. Sastre, M., Galea, E., Reis, D. J., and Regunathan, S. (1998) Metabolism of agmatine in macrophages: Modulation by lipopolysacharides and inhibitory cytokines. *Biochem. J.* **330,** 1405–1409.
14. Sastre, M., Regunathan, S., Galea, E., and Reis, D. J. (1996) Agmatinase activity in rat brain: A metabolic pathway for the degradation of agmatine. *J. Neurochem.* **67,** 1761–1765.
15. Sener, A., Lebrun, F., Blaicher, F., and Malaisse, W. J. (1989) Stimulus-secretion coupling of arginine-induced insulin release. Insulinitropic action of agmatine. *Biochem. Pharmacol.* **38,** 327–330.
16. Regunathan, S., and Reis, D. J. (1997) Stimulation of imidazoline receptors inhibits proliferation of human coronary artery vascular smooth muscle cells. *Hypertension* **30,** 295–300.
17. Galea, E., Regunathan, S., Eliopoulos, V., Feinstein, D. L., and Reis, D. J. (1996) Inhibition of mammalian nitric oxide synthases by agmatine, an endogenous polyamine formed by decarboxylation of arginine. *Biochem. J.* **316,** 247–249.
18. Regunathan, S., Feinstein, D. L., and Reis, D. J. (1999) Antiproliferative and anti-inflammatory actions of imidazoline agents: Are imidazoline receptors involved? *Ann. N. Y. Acad. Sci.* **881,** 410–419.
19. Vargiu, C., Cabella, C., Belliardo, S., Cravanzola, C., Grillo, M. A., and Colombatto, S. (1999) Agmatine modulates polyamine content in hepatocytes by inducing spermidine/spermine acetyltransferase. *Eur. J. Biochem.* **259,** 933–938.
20. Satriano, J., Matsufuji, S., Murakami, Y., Lortie, M. J., Schwartz, D., Kelly, C. J., Hayashi, S., and Blantz, R. (1998) Agmatine suppresses proliferation by frameshift induction of antizyme and attenuation of cellular polyamine levels. *J. Biol. Chem.* **273,** 15313–15316.
21. Penner, S. B., and Smyth, D. D. (1996) Natriuresis following central and peripheral administration of agmatine in the rat. *Pharmacology* **53,** 160–169.
22. Sastre, M., Regunathan, S., and Reis, D. J. (1997) Uptake of agmatine into rat brain synaptosomes: Possible role of cation channels. *J. Neurochem.* **69,** 2421–2426.
23. Satriano, J., Kelly, C. J., and Blantz, R. C. (1999) An emerging role for agmatine. *Kidney Int.* **56,** 1252–1253.
24. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
25. Seiler, N., Delcros, J. G., and Moulinoux, J. P. (1996) Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell. Biol.* **28,** 843–861.
26. Seiler, N., and Dezeure, F. (1990) Polyamine transport in mammalian cells. *Int. J. Biochem.* **22,** 211–218.
27. Rinehart, C. A., Jr., and Chen, K. Y. (1984) Characterization of the polyamine transport system in mouse neuroblastoma cells. Effects of sodium and system A amino acids. *J. Biol. Chem.* **259,** 4750–4756.
28. Rannels, D. E., Kameji, R., Pegg, A. E., and Rannels, S. R. (1989) Spermidine uptake by type II pneuomocytes: Interactions of amine uptake pathways. *Am. J. Physiol.* **257,** L346–L353.
29. Poulin, R., Lessard, M., and Zhao, C. (1995) Inorganic cation dependence of putrescine and spermidine transport in human breast cancer cells. *J. Biol. Chem.* **270,** 1695–1704.
30. Nicolet, T. G., Scemama, J. L., Pradayrol, L., Seva, C., and Vaysse, N. (1990) Characterization of putrescine and spermidine transport systems of a rat pancreatic acinar tumoral cell line (AR4-2J). *Biochem. J.* **269,** 629–632.
31. Morgan, D. M. (1992) Uptake of polyamines by human endothelial cells. Characterization and lack of effect of agonists of endothelial function. *Biochem. J.* **286,** 413–417.
32. Poulin, R., Zhao, C., Verma, S., Charest-Gaudreault, R., and Audette, M. (1998) Dependence of membrane putrescine and spermidine transport on plasma-membrane potential: Identification of an amiloride binding site on the putrescine carrier. *Biochem. J.* **330,** 1283–1291.
33. Brachet, P., and Tomé, D. (1992) Putrescine uptake by rabbit intestinal brush-border membrane vesicles. *Biochem. Int.* **27,** 465–475.
34. Medina, M. A., Urdiales, J. L., Núñez de Castro, I., and Sánchez-Jiménez, F. (1991) Diamines interfere with the transport of L-ornithine in Ehrlich-cell plasma-membrane vesicles. *Biochem. J.* **280,** 825–827.