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ORIGINAL ARTICLE

Influence of ornithine decarboxylase antizymes and antizyme inhibitors on agmatine uptake by mammalian cells

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Abstract Agmatine (4-aminobutylguanidine), a dicationic molecule at physiological pH, exerts relevant modulatory actions at many different molecular target sites in mammalian cells, having been suggested that the administration of this compound may have therapeutic interest. Several plasma membrane transporters have been implicated in agmatine uptake by mammalian cells. Here we report that in kidney-derived COS-7cell line, at physiological agmatine levels, the general polyamine transporter participates in the plasma membrane translocation of agmatine, with an apparent Km of 44 ± 7 μM and V_{max} of 17.3 ± 3.3 nmol h^{-1} mg^{-1} protein, but that at elevated concentrations, agmatine can be also taken up by other transport systems. In the first case, the physiological polyamines (putrescine, spermidine and spermine), several diguanidines and bis(2-aminoimidazolines) and the polyamine transport inhibitor AMXT-1501 markedly decreased agmatine uptake. In cells transfected with any of the three ornithine decarboxylase antizymes (AZ1, AZ2 and AZ3), agmatine uptake was dramatically reduced. On the contrary, transfection with antizyme inhibitors (AZIN1 and AZIN2) markedly increased the transport of agmatine. Furthermore, whereas putrescine uptake was significantly decreased in cells transfected with ornithine decarboxylase (ODC), the accumulation of agmatine was stimulated, suggesting a trans-activating effect of intracellular putrescine on agmatine uptake. All these results indicate that ODC and its regulatory proteins (antizymes and antizyme inhibitors) may influence agmatine homeostasis in mammalian tissues.

Keywords Agmatine · Agmatine uptake · Antizyme inhibitor 2 · Antizymes · Ornithine decarboxylase · Polyamine transport system · AMXT-1501 · Diguanidines

Abbreviations

ODC	Ornithine decarboxylase
AZ	Antizyme
AZIN	Antizyme inhibitor
ADC	Arginine decarboxylase
SLC	Solute carrier
OCT	Organic cation transporter
MATE-1	Multidrug and toxin extrusion transport
PTS	Polyamine transport system
DFMO	Alfa-difluoromethylornithine
ORF	Open reading frame

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Introduction

Agmatine (4-aminobutylguanidine), formed by decarboxylation of L-arginine, has been considered as a potential neurotransmitter (Reis and Regunathan 1998, 2000). Previous studies using animal models have shown that this molecule exerts remarkable modulatory actions at many different molecular target sites (see Piletz et al. 2013, for review). Based on these findings, it has been suggested that this compound may have therapeutic potential in the treatment of various complex clinical disorders (Molderings and Haenisch 2012; Piletz et al. 2013). The endogenous basal levels of agmatine in mammalian tissues are very low (nanomolar range) compared to those reported in bacteria and plants (Raasch et al. 1995). Whereas in plants and bacteria it is clear that agmatine synthesis is catalyzed by arginine decarboxylase (ADC), in mammals the percentage of tissue agmatine synthesized endogenously and the fraction absorbed either from the ingested food or from agmatine produced by gut microbiota is still unknown (Molderings and Haenisch 2012). Furthermore, in spite of numerous experiments on the administration of agmatine to different in vitro and in vivo models, data on the agmatine levels reached in cells or tissues are scarce. Experiments dealing with the administration of [^{14}C]-agmatine to rats revealed that agmatine is absorbed from the gastrointestinal tract and distributed in all organs investigated (Molderings et al. 2003a). However, since agmatine can be converted into different metabolites, as clearly shown in studies with rat hepatocytes (Cabella et al. 2001), the potential contribution of exogenous agmatine to the increase of the intracellular agmatine pool is mostly unknown.

Numerous studies have shown that many types of mammalian cells accumulate agmatine, although the main conclusion of many of them is that agmatine enters the cell via the polyamine transport system (PTS) (Babál et al. 2000; Esteban del Valle et al. 2001; Satriano et al. 2001; Molderings et al. 2001, 2003b; Goracke-Postle et al. 2007). However, other studies suggested that agmatine can be transported into cells by transport systems, others than the general polyamine carrier, with less affinity for agmatine than in the case of the general polyamine transporter (Sastre et al. 1997; Cabella et al. 2001; Gründemann et al. 2003; Winter et al. 2011). In this regard, experiments using human embryonic kidney cells transfected with some members of the SLC-family of organic cation transporter (OCT) revealed that OCT-2 and MATE-1 (a multidrug and toxin extrusion transporter) may participate in bidirectional agmatine transport (Gründemann et al. 2003; Winter et al. 2011). Although the mammalian PTS has not been molecularly characterized, it is known that this transport system is inhibited by proteins named antizymes (AZs) (Mitchell et al. 1994; He et al. 1994). These proteins are synthesized in its full-length version only under high polyamine levels, then inhibiting ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, and also preventing the transport of extracellular polyamines (see Hayashi et al. 1996, Coffino 2001, for reviews). Interestingly, it was also shown that agmatine suppresses cell proliferation by induction of antizyme (Satriano et al. 1998).

In a previous paper, we demonstrated that ornithine decarboxylase antizyme inhibitor 2 (AZIN2) stimulated polyamine transport in COS-7 cells derived from monkey kidney tissue (López-Contreras et al. 2008). Azin2 is a paralogous gene of ornithine decarboxylase (Odc), that was early named as Odcp or ODC-like (Pitkanen et al. 2001). Although it was reported later that this gene encodes for the enzyme responsible of arginine decarboxylation and accordingly it was also named Adc (Zhu et al. 2004), this activity could not be confirmed by other groups including ours (Coleman et al. 2004; López-Contreras et al. 2006; Kanerva et al. 2008), which showed that ODCp is indeed an antizyme inhibitor lacking decarboxylase activity (López-Contreras et al. 2006; Kanerva et al. 2008; Snapir et al. 2008). In the present work, we have examined the role of AZIN2 and antizymes in the uptake of agmatine by mammalian cells, as well as the effect of several polyamines and polyamine analogs on this process. The results suggest that AZIN2 may have a regulatory role on agmatine uptake in the cells where this protein is expressed.

Materials and methods

Reagents

L-arginine decarboxylase from *Escherichia coli,* nonradioactive polyamines and arcaine were obtained from Sigma Aldrich (St. Louis, MO). Lipofectamine 2,000 transfection reagent, Dulbecco's Modified Eagle Medium (DMEM), glutamine, fetal bovine serum, penicillin/streptomycin and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA).Bio-Rad protein assay was from Bio-Rad (Hercules, CA). [^{14}C] Putrescine (specific activity 107 mCi/mmol), was from Amersham Biosciences/GE Healthcare (Little Chalfont, UK). Arginine, L-[^{14}C(U)] (specific activity 264 mCi/mmol) and [^{3}H]-agmatine (specific activity 43 Ci/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO).Scintillation solution Ecoscint-H was obtained from National Diagnostics (Atlanta, GA). Quick Change site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Primers were purchased from Sigma Genosys. α-Difluoromethylornithine (DFMO) was kindly supplied by Dr. Patrick M Woster, Department of Pharmaceutical Sciences, Wayne State University, MI. AMXT-1501 was a kind gift from Dr. Mark R. Burns, Aminex Therapeutics Inc., Washington.

Synthesis of diguanidine and imidazoline derivatives

Alkanediguanidines (CD153, CD2, CD3, CD158, CD25), azaalkanediguanidines (CD161, CD7B, CD5), and bis(2-aminoimidazoline) derivatives (CD66C, CD13, CD39, CD27) were synthesized by Dr. Christophe Dardonville following previously reported procedures (Dardonville et al. 2000, 2002, 2003). 5-Aminopentylguanidine (CDIV44) was synthesized by reaction of 5 equivalents of cadaverine with 1 equivalent of S-methylisothiourea sulfate in refluxing water. The precipitated product was recrystallized from ethanol to yield a colorless solid (58 %).^{1}H NMR (300 MHz, D_2O) δ 3.20 (*t*, $J = 6.4$ Hz, 2H), 3.00 (*m*, 2H), 1.65 (*m*, 4H), 1.45 (*m*, 2H) ppm. 13C NMR (75 MHz, D_2O) δ 157.1, 41.2, 39.7, 27.7, 26.8, 23.2 ppm. Elemental analysis calculated for C8H18N4O4S: C, 29.74; H, 7.49; N, 23.12; S, 13.23. Found: C, 29.48; H, 7.51; N, 22.98; S, 12.99.

Synthesis of [^{14}C]-agmatine

160 μM L-[U-^{14}C]-arginine (308 mCi mmol^{-1}) in 0.3 M sodium acetate, pH 5.5 was treated with *Escherichia coli* arginine decarboxylase (1 I.U.) and incubated overnight at room temperature. The enzyme was added again and incubation was continued for another additional hour. The mixture was then purified in a 5,000 NMWL filter unit (Millipore, MA, USA) by centrifugation at 10,000×*g* for 20 min. Radiochemical purity of ^{14}C-agmatine was checked by thin layer chromatography.

Cell culture and transient transfections

The monkey kidney fibroblast-like COS-7 cell line was obtained from the American Type Culture Collection. Cells were cultured in DMEM containing 2 mM glutamine, 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing 5 % CO_2 at 37 °C. Cells were grown to 70–80 % confluence. Transient transfections were carried out with Lipofectamine 2,000 transfection reagent with 0.15 μg of the appropriate plasmid and 2 μl of Lipofectamine per well, in 24-well plates. After 6 h of incubation the transfection medium was removed, and fresh complete medium was added, and cells were cultured for 24 h after transfection. The cells were then used for transport assays. The plasmid pcDNA3 without gene insertion was used as negative control.

Transport assays

For kinetics experiments non-transfected COS-7 cells grown in 24-well plates to about 80 % confluence were used. After removal of the growth medium, cells were washed with PBS and then incubated with 200 μl of fresh DMEM (serum-free) containing different concentrations of agmatine. After incubation at 37 °C for different periods of time, the radioactive medium was removed and the cells were washed three times with cold PBS. Washed cells were lysed by incubation with trypsin or 0.5 % Triton X-100 for 30 min. Finally, a small aliquot of the lysate was used to measure protein by the Bio-Rad protein assay and the remainder was mixed with 3 ml of the scintillation solution and the radioactivity was measured in a Tri-Carb 2,900 TR analyzer (PerkinElmer Life Sciences). Nonspecific accumulation was determined by incubation at 4 °C and subtracted from the total uptake. In the experiments using transfected cells the uptake medium contained 2 μM of [^{14}C]-agmatine or [^{14}C]-putrescine and the incubation period was 30 min.

Polyamine analysis

Cells were homogenized in 0.4 M perchloric acid and after centrifugation at 10,000×*g* for 5 min the polyamines from the supernatant were dansylated according to standard method (Seiler 1983). Dansylated polyamines were separated by HPLC, using a Lichrosorb10-RP-18 column (4.6 × 250 mm) and acetonitrile: water mixtures (running from 70:30 to 96:4 ratio during 25 min of analysis) as mobile phase. 1,6-Hexanediamine was used as internal standard. Detection of the derivatives was achieved using a fluorescence detector, with a 340 nm excitation filter and a 435 nm emission filter. Polyamine content was expressed as nmol/mg protein.

Cloning and plasmids

ODC, AZIN1, AZIN2, AZ1, AZ2, and AZ3 mouse genes were cloned into the expression vector pcDNA3 (Invitrogen) following standard procedures and using the primers described elsewhere (López-Contreras et al. 2006). Antizyme constructs with an appropriate deletion of one nucleotide in the frame-shifting site, for full-length and functional expression, were obtained by mutagenesis (López-Contreras et al. 2006). The ODC construct cloned into the expression vector pcDNA3 was used as template for site-directed mutagenesis using the QuickChange site-directed mutagenesis kit, generating the inactive ODC mutant form C360A using the following primers: forward 5′-GCATCTGGGGACCAACAGTTGATGGCCTTGATCGGATCG-3′ and reverse 5′-CGATCCGATCAAGGCCATCAACTGTTGGTCCCCAGATGC-3′. All constructs were verified by complete sequencing.

Statistics

Unpaired Student's *t* test was used to compare differences between two groups. Kinetics data were analyzed using

GraphPad Prism 5.0 (GraphPad Software, CA, USA). Differences were considered to be statistically significant at $P < 0.05$.

Results

Transport kinetics

Preliminary experiments showed that agmatine uptake by COS-7 cells, using either ^{14}C-agmatine or ^{3}H-agmatine, were linear for at least 30 min (results not shown). Experiments at both 37 and 4 °C were carried out in parallel. Values obtained at 4 °C (non-specific uptake) were always subtracted from the total uptake at 37 °C. Figure 1a shows agmatine uptake in the substrate range from 2.5 µM to 100 µM. Using these values an apparent Michaelis–Menten constant (Km) of 44 ± 7 µM and maximal velocity (V_{max}) of 17.3 ± 3.3 nmol h^{-1} mg^{-1} protein were calculated. However, when agmatine concentrations were extended up to 1 mM, the apparent Km and V_{max} were 649 ± 90 µM and 82,9 ± 6,0 nmol h^{-1} mg^{-1} protein, respectively (Fig. 1b). Linear representation of the data using the Eadie–Hofstee plot (Fig. 1c) suggested the existence of two transport systems for agmatine with different substrate affinity. In the case of the high affinity system, the Km for agmatine was higher than those reported for putrescine, spermidine and spermine (4.5, 1 and 0.8 µM, respectively) using the same type of cells (López-Contreras et al. 2008).

Agmatine uptake by COS-7 cells: influence of polyamines and antizymes

To determine whether agmatine uptake through the high-affinity transport system was mediated by the polyamine transport system (PTS), we performed a series of experiments in which the intra and extracellular polyamine concentrations were modified. Since the physiological levels of agmatine reported in mammalian tissues and blood plasma are in the low micromolar range (Raasch et al. 1995; Lortie et al. 1996, 2000), we used a 2 µM concentration of radioactive agmatine. Treatment of cells for 24 h with 1 mM difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, produced a marked decrease in the cell polyamine content (86, 76 and 51 % of putrescine, spermidine and spermine, respectively). Figure 2a shows that the uptake of agmatine was markedly stimulated (about 12-fold) by the depletion of polyamines caused by DFMO. On the other hand, competition assays clearly demonstrated that the agmatine uptake was significantly inhibited in the presence of exogenous polyamines (Fig. 2b), which suggests that the PTS could mediate the entry of agmatine into the COS-7cells. In addition, the uptake of agmatine

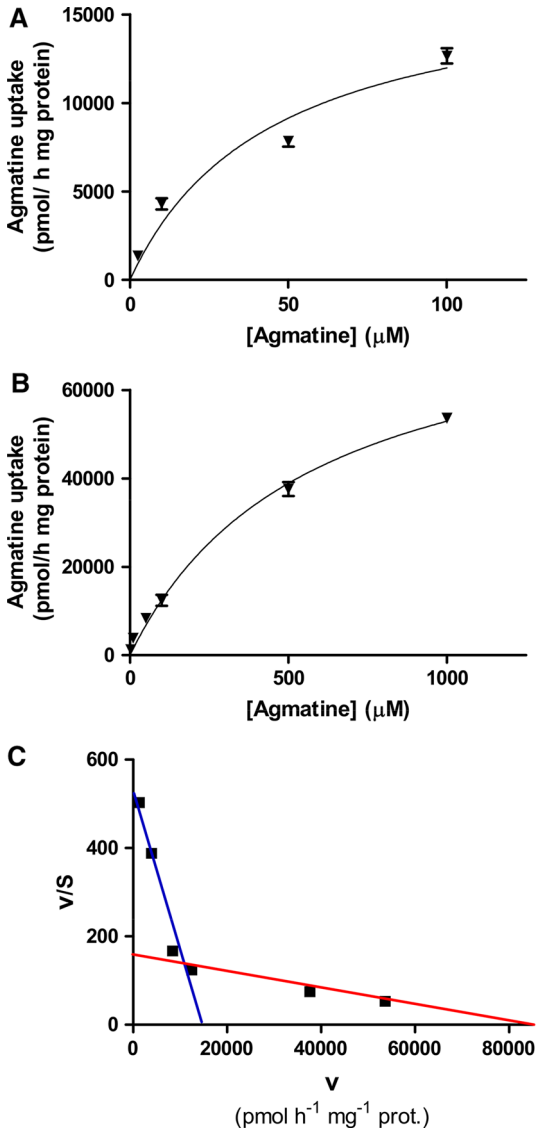

Fig. 1 Kinetics of agmatine uptake by COS7 cells. Specific agmatine uptake was determined as described under "Materials and methods". **a** Direct plot of the mean ± SE values obtained from two independent experiments, using an agmatine range concentration from 0.1 µM to 100 µM. **b** Direct plot of the mean ± SE values obtained from two independent experiments, using an agmatine range concentration from 0.1 µM to 1,000 µM. **c** Eadie–Hofstee plot of the kinetic data obtained as above described. Kinetic parameters were calculated using GraphPad Prism software

was also markedly reduced when cells were preincubated for 3 h with 100 µM polyamines or agmatine, before the uptake assay, especially in the case of spermidine and spermine (Fig. 2c). This effect is likely due to the induction of antizymes in response to the rise in the intracellular levels of polyamines. To corroborate this possibility, COS-7 cells were transiently transfected with different isoforms of AZs. In these assays, as in an early work (López-Contreras et al. 2008), mutated versions of the AZ genes lacking the stop

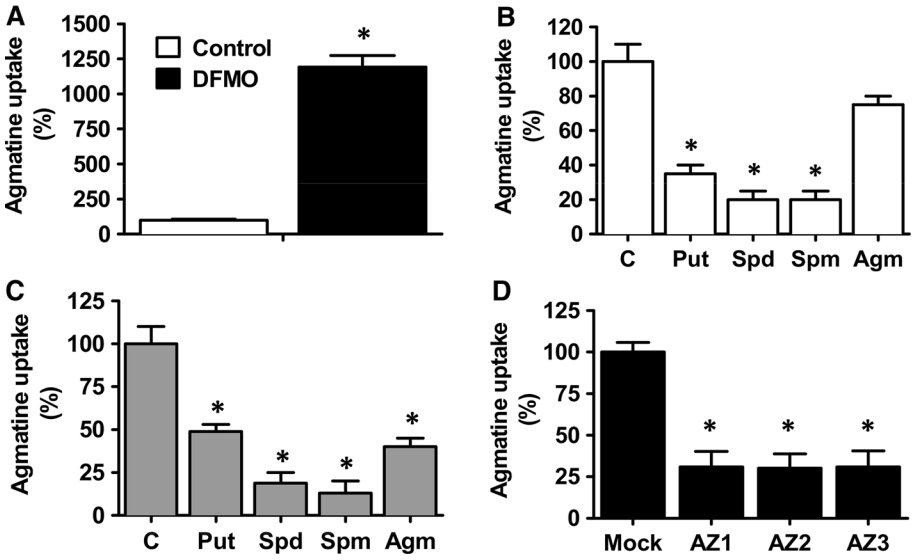

Fig. 2 Influence of different treatments on agmatine uptake by COS7 cells. **a** DFMO (1 mM, 24 h) increased the uptake of 2 μM ^{14}C-agmatine. **b** ^{14}C-agmatine uptake in the absence (control, C) or presence of 20 μM of unlabeled polyamines. **c** Cells were incubated for 3 h with 100 μM of unlabeled polyamines. After washing the cells, agmatine uptake was assayed using 2 μM ^{14}C-agmatine. **d** COS7 cells were transiently transfected with different antizyme constructs (AZ1, AZ2 and AZ3) or the empty pcDNA3 vector (control). After 24 h, the medium was changed and agmatine uptake was assayed using 2 μM ^{14}C-agmatine. Data are percentages of specific transport, taking the control values as 100 %. Results are given as mean ± SE of three experiments. *$P < 0.01$ vs. control according to Student's *t* test

codon of the ORF1, which synthesize antizymes without frame-shifting requirement, were used. Figure 2d shows that all AZs tested (AZ1, AZ2 and AZ3) negatively affected agmatine uptake. Taken into consideration that there is a percentage of the COS-7 cultured cells that have not been transfected, the actual suppressing effect should be higher than that shown in Fig. 2d. Taken together, these results suggest that in COS-7 cells, agmatine uptake might be mediated by the general PTS.

Influence of antizyme inhibitor 2 on agmatine uptake

To test the possible influence of AZIN2 on agmatine transport, COS-7 cells were transfected with the expression vector containing the mouse Azin2 gene, and agmatine uptake was measured and compared with the one corresponding to mock-transfected cells. As shown in Fig. 3a, agmatine uptake was stimulated about sixfold in the cells expressing high levels of AZIN2. Azin1, a paralogous of Azin2, as expected also stimulated agmatine uptake. Likewise, both antizyme inhibitors also produced very similar stimulatory effects on putrescine uptake (Fig. 2b). Interestingly, whereas in cells transfected with ODC, agmatine uptake was also markedly enhanced, reaching values similar to those found in Azin2- and Azin1-transfected cells (Fig. 3a), the uptake of putrescine was not increased by ODC but, on the contrary, it was decreased to 32 % of control values (Fig. 3b). No effect of ODC on arginine uptake was observed (data not shown). To analyze whether this opposite effect of ODC on agmatine and putrescine uptake could be related with the expected increase in the intracellular levels of putrescine caused by ODC overexpression, cells transfected with ODC were simultaneously treated with DFMO, and agmatine uptake measured and compared with that found in non-treated cells. Figure 3c clearly shows that the inhibition of ODC by DFMO abrogated the increase in agmatine uptake. In addition, in cells transfected with the mutant ODC C360A, an inactive form of ODC (Coleman et al. 1993), agmatine uptake was also not stimulated (Fig. 3c).

Effect of guanidine derivatives on agmatine uptake

Since agmatine is an aminoguanidine compound that has dicationic nature at physiological pH, we decided to test the effect of several aminoalkylguanidine, diguanidine and bis(2-aminoimidazoline) derivatives (Dardonville and Brun 2004), with different structure and electrical charge, on the uptake of agmatine by COS-7 cells. Figure 4 shows the structure of aminoalkylguanidines (group A), diguanidines and azaalkanediguanidines (group B) and bis(2-aminoimidazolines) (group C). The effect of these compounds on agmatine uptake is shown in Fig. 5. CDIV44 (5-aminopentylguanidine) produced an inhibitory effect close to 50 %. The effect of the bisguanidines was dependent on the length of the aliphatic chain, with arcaine

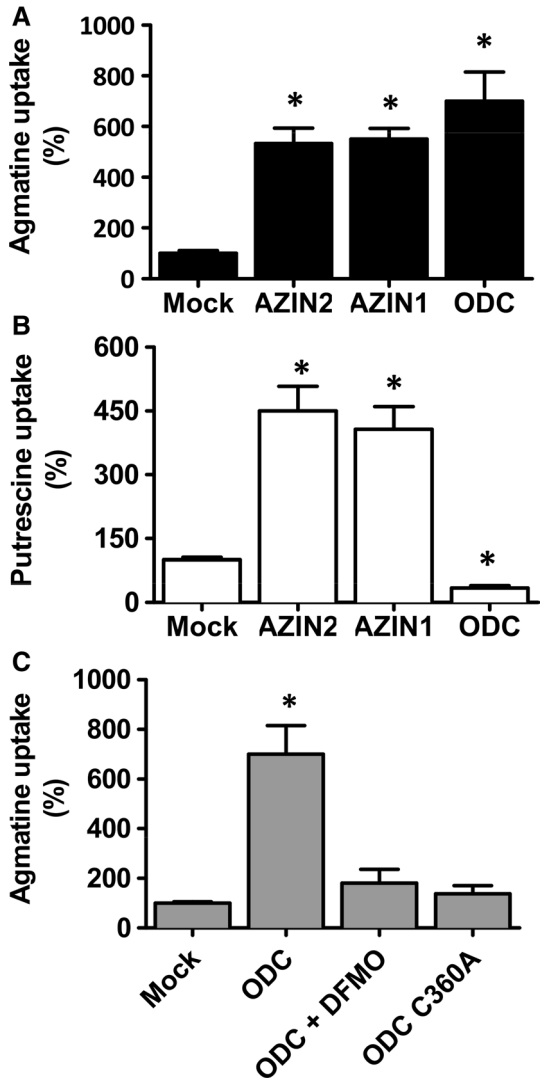

Fig. 3 Agmatine and putrescine uptake in COS7 cells transfected with ODC or with the antizyme inhibitors AZIN1 and AZIN2. **a** COS7 cells were transiently transfected with ODC, AZIN1, AZIN2 or the empty pcDNA3 vector (control). After 24 h, the medium was changed and agmatine uptake was assayed using 2 μM ^{14}C-agmatine. **b** COS7 cells were transiently transfected with ODC, AZIN1, AZIN2 or the empty pcDNA3 vector (control). After 24 h, the medium was changed and putrescine uptake was assayed using 2 μM ^{14}C-putrescine. **c** Cells were transfected with ODC or mutant ODC C360A. In the ODC + DFMO-treated cells, 1 mM DFMO was added 5 h before the uptake assay using 2 μM ^{14}C-agmatine. Data are percentages of specific transport, taking the control values as 100 %. Results are given as mean ± SE of three experiments. *$P < 0.01$ vs. control

(1,4-diguanidinobutane) having an inhibitory effect similar to CDIV44, and no effect when the aliphatic moiety contained more than 6 carbon atoms (CD2, CD3, CD158) or aromatic rings (CD25). Azaalkanediguanidines with propylene arms showed higher inhibitory effects (75 % CD5 and 58 % CD7B). The bisaminoimidazolines CD13 and CD66C also acted as inhibitors of agmatine uptake (58 and 42 %, respectively). Of note is that the most potent inhibitors among the different compounds assayed here were trications, with a spatial charge distribution close to that of spermidine. Since these competition assays suggest again that the PTS may be responsible for agmatine uptake, the effect of AMXT-1501, a potent polyamine transport inhibitor (Burns et al. 2009; Samal et al. 2013), on agmatine uptake was assayed. This lipophilic lysine-spermine conjugate produced a dramatic decrease on agmatine transport in mock transfected COS-7 cells as well as in Azin2- and Odc- transfected cells (Fig. 6). The inhibitory effect of AMXT-1501 was higher than that produced for the most potent guanidine compound.

Discussion

The results presented here indicate that agmatine uptake by COS-7 cells can be accomplished by at least two different transport systems with different affinity for agmatine. The high affinity system, with an apparent Km for agmatine of 44 ± 7 μM, could correspond with the mammalian general polyamine transport system. This assumption is based on several facts: (1) at 2 μM agmatine in the uptake medium, a concentration very close to that reported existing in rat plasma (Lortie et al.1996; Lortie et al. 2000), agmatine accumulation was inhibited by the presence of any of the major physiological polyamines putrescine, spermidine and spermine; (2) the preincubation of COS-7 cells with polyamines before the uptake assay markedly inhibited agmatine uptake, very likely due to the induction of antizymes; (3) the transfection of COS-7 cells with any of the three antizyme isoforms, dramatically decreased agmatine uptake, similarly to that found earlier for polyamines (López-Contreras et al. 2008); (4) in cells transfected with either Azin1 or Azin2, agmatine uptake was markedly stimulated, presumably by the blockade of the endogenous antizymes; (5) the treatment of cells with DFMO highly increased the uptake of agmatine, in accordance with studies showing that the depletion of polyamines by the ODC inhibitor activates polyamine transport (Pegg 1987); (6) the marked inhibitory action of the potent polyamine transporter inhibitor AMXT-1501 (Samal et al. 2013) on agmatine uptake. Therefore, these results are in agreement with studies using other types of cells that suggested that agmatine and polyamines share the same transporter (Babál et al. 2000; Esteban del Valle et al. 2001; Satriano et al. 2001; Molderings et al. 2001; Goracke-Postle et al. 2007). However, it is unclear the relevance that this transporter may have on agmatine distribution in the body under physiological conditions, since the reported levels of agmatine in blood plasma and tissues of rats and humans are below the Km values (Raasch et al. 1995; Lortie et al. 1996, 2000;

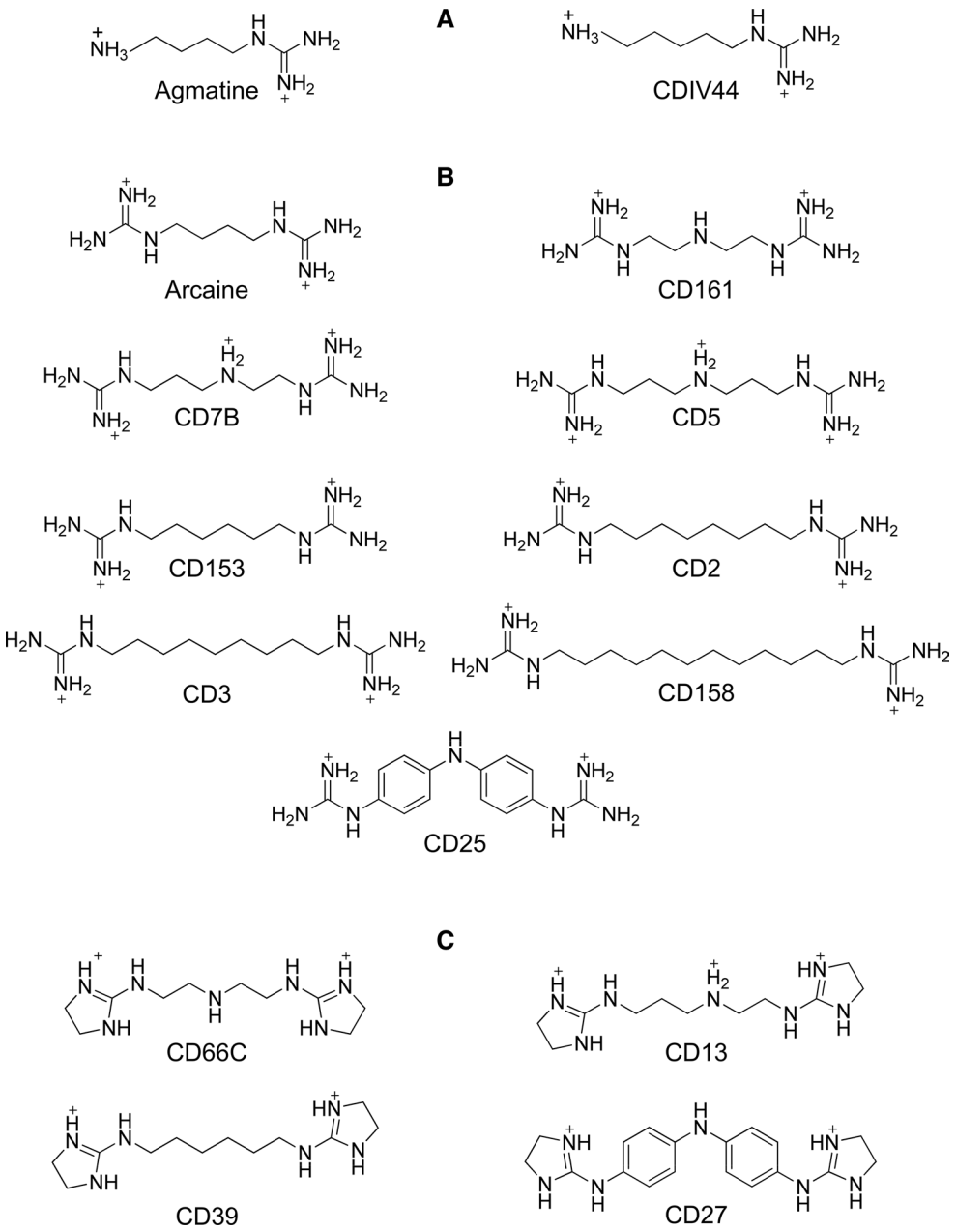

Fig. 4 Chemical structure of the compounds tested as inhibitors of agmatine uptake. **a** Aminoalkylguanidines. **b** Diguanidines and azaalkanediguanidines. **c** Bis(2-aminoimidazolines)

Halaris et al. 1999; Zhang and Kaye 2004). Nevertheless, it is likely that the general polyamine transporter may be relevant for treatments based in the exogenous administration of agmatine or for the uptake of luminal agmatine in the digestive tract, where agmatine concentration may be higher than in plasma or tissues.

Although there is evidence supporting the existence of two subtypes of PTS in the plasma membrane: diamine- and polyamine-preferential transporters (Seiler and Dezeure 1990; Poulin et al. 2011), so far, the mammalian PTS has not been molecularly characterized. The results presented here on the inhibitory effect of diguanidines and bisaminoimidazolines on agmatine uptake, in which the similarity of electric charge distribution with polyamines appears to be more important than the nature of the groups carrying the charge, suggest again that polyamines share their transporter with agmatine and other guanidines, although the affinity of agmatine for this transporter is lower than that of polyamines (López-Contreras et al. 2008).

Our results clearly show that antizymes and antizyme inhibitors affect agmatine uptake. This is in agreement with previous results showing the respective negative and positive effects of these proteins on polyamine transport (Mitchell et al. 1994; He et al. 1994; López-Contreras et al. 2008; Snapir et al. 2008). Interestingly, the parallelism

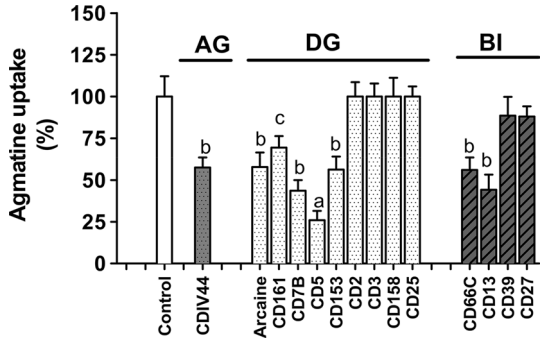

Fig. 5 Effects of competitors on agmatine uptake. Transport experiments were carried out using 2 μM ^{14}C-agmatine in the absence (control, C) or presence of 20 μM of the different competitors. *AG* aminoalkylguanidines, *DG* diguanidines, *BI* bis(2-aminoimidazolines). Data are percentages of specific transport, taking the control values as 100 %. Results are given as mean ± SE of three experiments. Statistical significance: *a* $P < 0.001$ vs. control; *b* $P < 0.01$ vs. control; *c* $P < 0.05$ vs. control

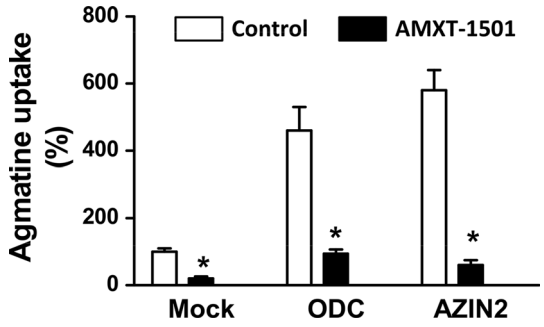

Fig. 6 Inhibitory effect of AMXT-1501 on agmatine uptake by COS7 cells. Twenty hours after transfection, cells were incubated for 30 min with 2 μM ^{14}C-agmatine (control) or with radiolabeled agmatine containing 20 μM of the polyamine transport inhibitor AMXT-1501. Data are percentages of specific transport, taking the control values of cells transfected with the empty vector as 100 %. Results are given as mean ± SE of three experiments. *$P < 0.01$ vs. control

between agmatine and putrescine uptake observed in the cells transfected with antizyme inhibitors was not detected in the case of ODC-transfected cells, making unlikely that this differential effect of ODC on agmatine and putrescine uptake could be mediated by interaction with antizyme. The fact that the stimulatory effect of ODC on agmatine uptake was dependent on the decarboxylating activity, rather than on the amount of ODC protein, suggests that high levels of intracellular putrescine may stimulate agmatine uptake. The underlying mechanism for this finding is presently unknown, but one possibility is that the binding of endogenous putrescine to the putative polyamine carrier could increase its affinity for agmatine. Another possibility is an exchange between putrescine and agmatine through a putative diamine exchanger, as it has been described for putrescine and arginine in colon epithelial cells (Uemura et al. 2008).

In addition to the PTS, different studies have reported that agmatine can be also transported by other specific carriers. Thus, members of the SLC-organic cation transporter (OCT), especially OCT2 (Winter et al. 2011; Gründemann et al. 2003; Higashi et al. 2014), the extraneuronal monoamine transporter (Gründemann et al. 2003) and the multidrug and toxin extrusion transporter-1 (Winter et al. 2011) can participate in the uptake of agmatine by mammalian cells. For all these transporters the Km values for agmatine are markedly higher than Km for the PTS. The low affinity transport system with the apparent Km of 649 μM found here for the COS-7 cells could correspond to any of the above mentioned transporters. Although under physiological conditions the PTS may have preference for agmatine uptake, the other systems may also play a role for cell accumulation of the aminoguanidine when pharmacological doses of agmatine are administered.

Agmatine has been considered as a potential neurotransmitter (Reis and Regunathan 1998, 2000; Molderings and Haenisch 2012; Piletz et al. 2013). However, little is known about the subcellular localization of agmatine in mammalian cells. As shown by immunocytochemical analysis, agmatine-like immunoreactivity appears to be mainly localized in tubular and small synaptic vesicles in the axon and axon terminals of rat hippocampus (Reis et al. 1998), as well as in large dense-core vesicles and in the endoplasmic reticulum of rat neurons (Otake et al. 1998). In addition, polyamines are present in mast cell secretory granules and spermidine is released after degranulation (García-Faroldi et al. 2010). For neurotransmitters, vesicle storage is a key process. Several vesicular neurotransmitter transporters have been characterized that are responsible for loading the signaling molecules (Omote and Moriyama 2013).To our knowledge, not a single putative agmatine vesicular transporter has been identified. However, very recently, the gene SLC18B1 has been recognized as coding for a mammalian vesicular polyamine transporter in the brain (Hiasa et al. 2014). It cannot be excluded that this transporter might also participate in the accumulation of agmatine in secretory vesicles.

Recent studies have demonstrated that AZIN2 is expressed not only in certain brain regions but also in mast cells (Kanerva et al. 2009), in gonadal secretory cells (Makitie et al. 2009), and in the adrenal medulla and pancreatic Langerhans islets (López-García et al. 2013). Furthermore, at cellular level AZIN2 has been mainly localized associated to membranous structures. In COS-7- and HEK 293-transfected cells the protein was localized in both the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and the cis-Golgi network (López-Contreras et al. 2009), whereas in neural cells, it was found in post-Golgi

vesicles of the secretory pathway (Kanerva et al. 2010). In addition, AZIN2 has been also found in close association with serotonin-containing granules of mast cells (Kanerva et al. 2009). All these data have suggested that AZIN2 may be implicated in vesicular trafficking and secretory processes. Although our results showing the stimulatory effect of AZIN2 on cellular agmatine uptake, suggest that exogenous agmatine may accumulate in tissues where AZIN2 is expressed, no data are available on its possible effect on vesicular polyamine or agmatine levels. The analysis of polyamines and agmatine in vesicular structures on available Azin2 knockout mice (López-García et al. 2013) may shed light on this possibility.

In conclusion, our results indicate that ornithine decarboxylase antizymes and antizyme inhibitors affect agmatine uptake by COS-7 cells, very likely by their effects on the general polyamine transporter. The possibility that AZIN2 may affect agmatine homeostasis in secretory cells where this antizyme inhibitor is mainly expressed awaits further investigation.

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Conflict of interest The authors declare that they have no conflict of interest.

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