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Parkinson's disease-associated human P_{5B}-ATPase ATP13A2 increases spermidine uptake

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P-type ion pumps are membrane transporters that have been classified into five subfamilies termed P_1–P_5. The ion transported by the P_5-ATPases is not known. Five genes, *ATP13A* (ATPase type 13A) *1–ATP13A5*, that belong to the P_5-ATPase group have been identified in humans. Mutations of the human gene *ATP13A2* underlie a form of PD (Parkinson's disease). Previous studies have suggested a relation between polyamines and P_{5B}-ATPases. We have recently shown that the cytotoxicity induced by the polyamine analogue paraquat (1,1′-dimethyl-4,4′-bipyridinium), which is an environmental agent related to PD development, was increased in ATP13A2-expressing CHO (Chinese-hamster ovary) cells. In the present study we showed that ATP13A2-expressing CHO cells exhibit a 2-fold higher accumulation of spermidine. Increasing concentrations of spermidine reduced the viability of CHO cells stably expressing ATP13A2. The higher levels of spermidine attained by the ATP13A2-expressing CHO cells were correlated with an increase in the ATP-dependent spermidine uptake in an isolated subcellular fraction containing lysosomes and late endosomes. The results of the present study support the idea that the human P_{5B}-ATPase ATP13A2 is involved in polyamine uptake.

Key words: Parkinson's disease, P_{5B}-ATPase type 13A2 (P_{5B}-ATP13A2), polyamine, polyamine transport system, P-type ATPase, spermidine uptake.

INTRODUCTION

The P-type superfamily of ion pumps includes membrane transporters energized by hydrolysis of ATP that transport inorganic cations and other substrates across cell membranes. These P-type ATPases are characterized by the formation of a phosphorylated intermediate during their reaction cycle. They are present in prokaryotes and eukaryotes and, on the basis of their conserved core sequences, they have been classified into five subfamilies termed P_1–P_5 or type I–V [1]. The most poorly understood P-type ATPases are those of the P_5 subfamily, which are expressed ubiquitously only in eukaryotes [2,3]. Five genes, *ATP13A* (ATPase type 13A) *1–ATP13A5*, that belong to this group of P_5-ATPases have been identified in humans [3]. The substrate specificity of P_5-ATPases is not known, but almost all publications suggest that these enzymes affect the intracellular level of different cations [4–7]. In fact, several studies proposed that these pumps were Ca^{2+} transporters; nevertheless, by using the microsomal fraction prepared from a stable CHO (Chinese-hamster ovary) cell line expressing the recombinant human ATP13A2, we have recently shown that at least this enzyme does not transport Ca^{2+} under the optimal conditions for the P_2-type Ca^{2+} pumps [8]. The yeast *Saccharomyces cerevisiae* contains two P_5-ATPases named Cod1p [or Spf1p (sensitivity to *Pichia farinosa* killer toxin)] and Ypk9p (yeast ATP13A2). Using DNA sequence alignment it was shown that the mouse gene *Atp13a1* exhibited greater similarity to the yeast gene encoding Cod1p (*Yel031w*) than to the other mammalian isoforms, so they were grouped into the P_{5A} subgroup of type V ATPases; likewise, the DNA sequence of mouse genes *Atp13a2–Atp13a5* were similar to the yeast gene encoding Ypk9p (*Yor291w*) so they were clustered into the P_{5B} group [1]. P_{5A}-ATPases have been identified in the endoplasmic reticulum and seem to have basic functions in protein maturation and secretion. P_{5B}-ATPases localize to vacuolar/lysosomal or apical membranes and in animals play a role in hereditary neuronal diseases [4,7,9,10]. Loss-of-function mutations of the human gene *ATP13A2* were found to underlie an autosomal recessive form of early-onset parkinsonism (Kufor–Rakeb Syndrome) [9]. Furthermore, the interruption by inversion of the long arm of chromosome 3 in the human gene *ATP13A4* was found in patients with ASD (autism spectrum disorder) and SLI (specific language impairment) [10]. Compelling evidence implicates α-syn (α-synuclein) aggregation in the pathogenesis of PD (Parkinson's disease). Accordingly, the α-syn-induced cytotoxicity in yeasts could be suppressed by expression of Ypk9p, one of the P_5-ATPases expressed in yeasts [11]. This genetic interaction between the yeast Ypk9p and α-syn is conserved in neurons because expression of *ATP13A2* is sufficient to rescue α-syn-mediated dopaminergic neuron degeneration in the rat primary midbrain culture [11].

We have recently found that the expression of ATP13A2 made CHO cells more sensitive to paraquat exposure, a toxic polyamine analogue widely used as herbicide [12]. Paraquat is an environmental agent related to PD development [13], which can induce oxidative stress by an increase in the production of cellular ROS (reactive oxygen species) [14,15]. Accordingly, the ROS concentration after paraquat exposure was significantly increased in CHO cells expressing the ATP13A2 pump; and this increment of total ROS level was abolished by spermidine competition [12]. This is interesting because it is known that paraquat utilizes the polyamine transport system to get into the cells [16,17]. The connection between P_5-ATPases, polyamines and PD is suggested

Abbreviations used: ATPγS, adenosine 5′-[γ-thio]triphosphate; ATP13A, ATPase type 13A; CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; HEK, human embryonic kidney; PD, Parkinson's disease; ROS, reactive oxygen species; PSV, polyamine-sequestering vesicle; SAT1, spermidine/spermine N1-acetyltransferase 1; α-syn, α-synuclein; Ypk9p, yeast ATP13A2.

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by the result of a previous study showing that the deletion of the P_{5B}-ATPase CATP-5 (cation-transporting ATPase 5) from *Caenorhabditis elegans*, which is phylogenetically related to the mammalian P_{5B}-ATPases ATP13A2, ATP13A3 and ATP13A4, is responsible for the tolerant phenotype seen in the presence of the toxic spermidine analogue norspermidine [18]. Finally, previous studies have also suggested that polyamines increase α-syn aggregation in *in vitro* systems [19,20]. Altogether, these data make the polyamines good candidates to be the substrates transported by these P_{5B}-ATPases.

In the present study we have characterized spermidine incorporation in CHO cells stably expressing the human ATP13A2 (*ATP13A2*). We found that the expression of ATP13A2 doubled the [^{3}H]spermidine uptake in CHO cells and this increase was associated with a higher ATP-dependent spermidine uptake in the isolated mitochondrial fraction containing lysosomes and late endosomes.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following companies: [^{3}H]spermidine trihydrochloride (32.4 Ci/mmol), PerkinElmer Life and Analytical Sciences; nitrocellulose filters, Millipore; and reagents for cell culture, ATPγS (adenosine 5′-[γ-thio]triphosphate), DTT (dithiothreitol), PMSF, spermidine trihydrochloride and other chemicals, Sigma.

Cell culture and protein expression

The stable CHO cell line expressing the recombinant V5-tagged human ATP13A2 (*ATP13A2*) was described previously [8]. CHO cells were lipofected with the expression vector pcDNA3.1 carrying a mutant of the human *ATP13A2* cDNA, in which the catalytic residue Asp^{508} was substituted by asparagine (ATP13A2-D508N), which was generously donated by Dr Peter Vangheluwe (Faculty of Medicine, Department of Molecular Cell Biology, University of Leuven, Belgium). Stably transfected CHO cells were selected by G418 treatment because of the selectable marker provided by the vector. The detection of the recombinant ATP13A2-D508N was performed by immunoblotting using an antibody against ATP13A2 (Sigma). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 0.1 mM hypoxanthine, 0.01 mM thymidine, 600 μg/ml G418, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % fetal bovine serum. The cells were grown in humidified 5 % CO_2/air on standard plastic culture dishes.

Cell viability assay

CHO cells (2×10^4/well in 100 μl) were plated in 96-well microplates and incubated under the conditions described. At 24–48 h later the medium was removed and the cells were washed twice with Hanks solution and incubated in serum-free medium with increasing concentrations of spermidine trihydrochloride or chloroquine. The viability assay was performed the following day by measuring the activity of the endogenous enzyme hexosaminidase [21]. For this purpose the cells were washed twice with 200 μl of PBS (pH 7.4) and were incubated with 60 μl of substrate solution [7.5 mM *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide (Sigma), 0.1 M sodium citrate (pH 5.0) and 0.25 % Triton X-100] at 37 °C with 100 % humidity for 4 h. The colorimetric product was visualized by adding 90 μl of developer solution [50 mM glycine (pH 10.4) and 5 mM EDTA] and quantified by recording the absorbance at 405 nm in a Microplate Reader Model 550 (Bio-Rad Laboratories).

Radiometric determination of spermidine uptake in intact CHO cells

CHO cells were seeded in 24-well culture plates with an initial density of 5×10^4 cells per well and grown for 72 h as described above. [^{3}H]Spermidine trihydrochloride uptake was performed in serum-free DMEM under the conditions described below and in the Figure legends. At the end of the incubation period, the medium was discarded and the culture dishes were washed three times with PBS. Cells were disrupted by the addition of 0.1 M NaOH (100 μl) and neutralized by an equal volume of 0.1 M HCl. Cell extract (100 μl) was added to 1 ml of OPTI-Phase HiSafe2 liquid scintillation cocktail (PerkinElmer and Wallac Scintillation products) and radioactivity was counted in a scintillation counter. Aliquots (5–20 μl) of this cell extract were transferred to 96-well microplates for protein determination by means of the Bio-Rad Laboratories protein assay, with BSA as a standard. The quantities of transported spermidine are expressed as nmol of spermidine uptake per mg of protein.

Mitochondrial fraction isolation

CHO cells were plated in 145 mm×20 mm cell culture dishes and incubated as described above. When the cells reached confluency the medium was removed, the cells were washed twice with PBS and then resuspended in 1 mM PBS/EDTA by scraping. The cells from three to four dishes were pelleted by centrifugation (10 000 ***g*** for 10 min at 4 °C) and homogenized in 3 ml of hypotonic solution containing 10 mM Tris/HCl (pH 7.4), 1 mM $MgCl_2$, 2 mM DTT and the protease inhibitors 0.1 mM PMSF, 8 μg/ml aprotinin and 1 μg/ml leupeptin. Sucrose (0.5 M, 3 ml), 10 mM Tris/HCl (pH 7.4), 2 mM DTT and 0.3M KCl were added, the mix was re-homogenized and then centrifuged at 10 000 ***g*** for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 25 000 ***g*** for 25 min at 4 °C to precipitate the mitochondrial fraction. The pellet was resuspended in 0.25 M sucrose, 10 mM Tris/HCl (pH 7.4), 0.15 M KCl and 2 mM DTT and homogenized in a Teflon homogenizer. The isolated mitochondrial fraction was frozen in aliquots at − 80 °C. Protein concentration was estimated as described above.

Spermidine uptake assay in the mitochondrial fraction

Spermidine uptake assays were performed as follows. The reaction mixture contained 100 mM KCl, 50 mM Tris/HCl (pH 7.4 at 37 °C), 0.1 μM thapsigargin, 4 μg/ml oligomycin, 20 mM sodium phosphate, 95 μM EGTA, 5 mM $MgCl_2$ and 800 μM spermidine trihydrochloride (labelled with [^{3}H]spermidine trihydrochloride). The reaction was initiated by the addition of the mitochondrial fraction (10 μg of protein), isolated from CHO cells, at 37 °C in the absence or presence of 1.5 mM ATP or ATPγS. The reaction was finished after 1.5 or 12 min by filtering the samples through a 0.45-μm filter. The [^{3}H]spermidine trihydrochloride taken up by the vesicles was determined by counting the radioactivity retained in the filters in a liquid scintillation counter. Uptake activities were expressed per mg of membrane protein. For each data point the activity of the recombinant proteins was estimated by subtracting the radioactivity retained in the filters from the samples incubated without ATP or ATPγS.

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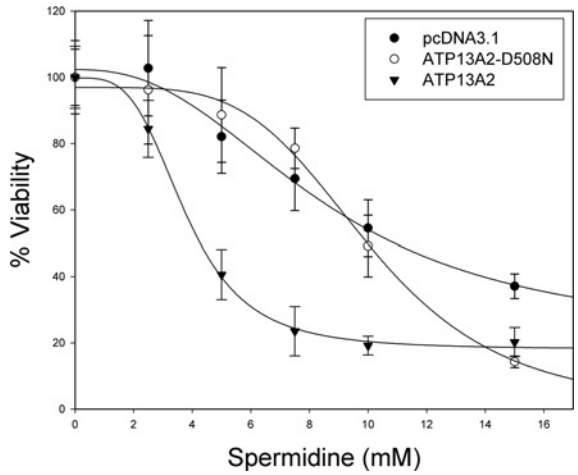

Figure 1 Viability of CHO cells transfected with the empty vector (pcDNA3.1) or expressing the ATP13A2 or ATP13A2-D508N pumps

The cells were incubated for 18 h at 37 °C with increasing concentrations of spermidine trihydrochloride. The viability assay was performed by measuring the activity of the endogenous enzyme hexosaminidase as described in the Materials and methods section. Values are expressed as the percentage of the absorbance at 405 nm measured for untreated cells. Results are means ± S.E.M. for four independent experiments performed six times. The line represents the best fit to the data given by the following equation: viability = min + (max − min)/[1 + (spermidine/EC_{50})$^{-\text{Hillslope}}$] (with min ⩾ 0), for pcDNA3.1: min = 21 ± 19 %, max = 102 ± 3 %, EC_{50} = 8 ± 2 mM and Hillslope = −2.4 ± 0.9; ATP13A2: min = 18 ± 1 %, max = 99 ± 2 %, EC_{50} = 4 ± 1 mM and Hillslope = −3.6 ± 0.3; and ATP13A2-D508N: min 0 ± 20 %, max = 97 ± 2 %, EC_{50} = 10 ± 1 mM and Hillslope = −3.9 ± 1.1.

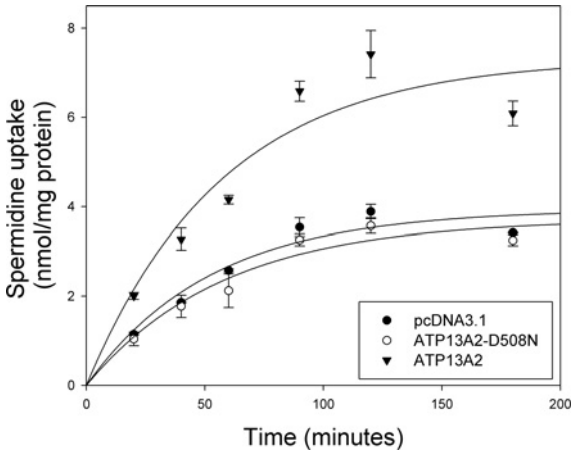

Figure 2 Kinetic behaviour of spermidine uptake in intact CHO cells transfected with the empty vector (*pcDNA3.1*) or expressing the recombinant proteins ATP13A2 or ATP13A2-D508N

[^{3}H]Spermidine (100 μM) was added to CHO cell monolayers in serum-free DMEM at time zero and incubated at 37 °C. Intracellular radioactivity was determined at the indicated incubation periods as described in the Materials and methods section. The activities are expressed as nmol of spermidine uptake per mg of protein. A representative experiment of three independent experiments where each point is the means ± S.D. of duplicate determinations is shown. The line represents the best fit to the data given by the following equation: spermidine uptake = $a \times (1 - e^{-k \times t})$, for pcDNA3.1: a = 3.9 ± 0.3 nmol/mg of protein and k = 0.019 ± 0.005 min^{-1}; ATP13A2: a = 7.3 ± 1.0 nmol/mg of protein and k = 0.017 ± 0.006 min^{-1}; and ATP13A2-D508N: a = 3.7 ± 0.4 nmol/mg of protein and k = 0.017 ± 0.004 min^{-1}.

RESULTS

Cytotoxic effect of spermidine on CHO cells

The effect of spermidine on the viability of stable CHO cells expressing the human ATP13A2 was examined measuring the activity of the endogenous enzyme hexosaminidase by comparison with CHO cells transfected with the empty vector (pcDNA3.1). In addition, cells expressing a mutant of ATP13A2 with the catalytic Asp^{508} replaced by an asparagine residue were used (ATP13A2-D508N). The recombinant proteins were successfully expressed and had the expected migration according to their predicted size of 129 kDa (Supplementary Figure S1 at http://www.biochemj.org/bj/450/bj4500047add.htm). Figure 1 shows the viability of CHO cells incubated for 18 h with increasing concentrations of spermidine; as shown previously [22], the polyamine exerts a dose-dependent cytotoxic effect. It is noteworthy that expression of ATP13A2 increased the toxic effect of the polyamine by making the cells more sensitive to spermidine treatment than CHO cells transfected with the empty vector or those expressing the ATP13A2-D508N mutant pump. A reduction of viability of 50 % was attained at 10, 9.5 and 4.5 mM spermidine in control CHO cells, ATP13A2-D508N- and ATP13A2-expressing CHO cells respectively.

Time course of spermidine uptake in CHO cells

Intact cells were incubated with 100 μM ^{3}H-labelled spermidine and the amount of radioactivity retained in the cells was measured. Figure 2 shows that the spermidine uptake of CHO cells increased with time, reaching a plateau at approximately 100 min. ATP13A2-expressing CHO cells showed approximately a 1.9-fold higher uptake of spermidine than the control cells transfected with the empty vector. This effect was specific for the expression of the ATP13A2 protein since the spermidine uptake of CHO cells expressing the recombinant ATP13A2-D508N mutant pump was similar to that of the control cells transfected with the empty vector.

Temperature dependence of spermidine transport in CHO cells

Because polyamine transport is an energy-requiring process [23], we examined the temperature dependence of the [^{3}H]spermidine uptake in CHO cells. As shown in Figure 3, at 4 °C there was a low level of spermidine associated with the cells which did not show a clear increase with time up to 250 min. When the temperature was raised to 37 °C, the uptake increased with time and reached a level between 5- and 7-fold higher than at 4 °C. At both of the temperatures assayed the CHO cells expressing ATP13A2 retained approximately twice the amount of spermidine than the control cells.

Spermidine concentration dependence of spermidine uptake in CHO cells

As shown in Figure 4, the spermidine incorporation increased gradually at increasing concentrations of spermidine. Both CHO cells transfected with the empty vector and those expressing the ATP13A2 or ATP13A2-D508N proteins reached a maximum at a similar spermidine concentration of approximately 50 μM; showing that no significant change in the apparent affinity of the cells for spermidine was produced by the expression of ATP13A2. The increased spermidine uptake was associated with the expression of a functional ATP13A2, since the spermidine

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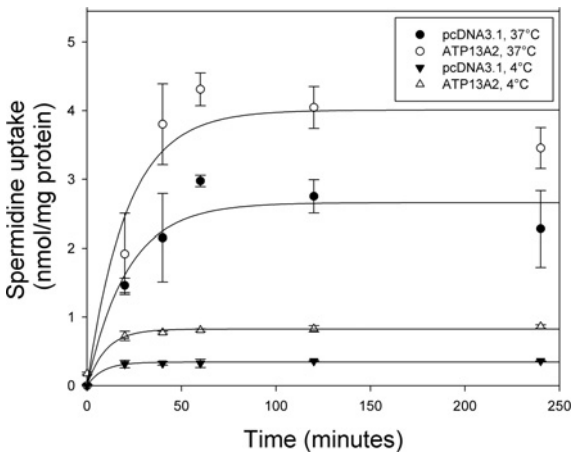

Figure 3 Temperature dependence of spermidine uptake in CHO cells transfected with the empty vector (*pcDNA3.1*) or expressing the recombinant ATP13A2

[^{3}H]Spermidine (2 μM) was added to CHO cell monolayers in serum-free DMEM at time zero, either at 4°C (▲ and △) or 37°C (● and ○). Intracellular radioactivity was determined at the indicated incubation periods as described in the Materials and methods section. The activities are expressed as nmol of spermidine uptake per mg of protein. A representative experiment from two independent experiments is shown where each point is the mean ± S.D. of duplicate determinations. The line represents the best fit to the data given by the following equation: spermidine uptake = $a\,(1 - e^{-k \times t})$, for pcDNA3.1, 37°C: $a = 2.6 \pm 0.2$ nmol/mg of protein and $k = 0.047 \pm 0.015$ min^{-1}; ATP13A2, 37°C: $a = 4.0 \pm 0.3$ nmol/mg of protein and $k = 0.048 \pm 0.016$ min^{-1}; pcDNA3.1, 4°C: $a = 0.343 \pm 0.009$ nmol/mg of protein and $k = 0.119 \pm 0.029$ min^{-1}; and ATP13A2, 4°C: $a = 0.8 \pm 0.1$ nmol/mg of protein and $k = 0.100 \pm 0.047$ min^{-1}.

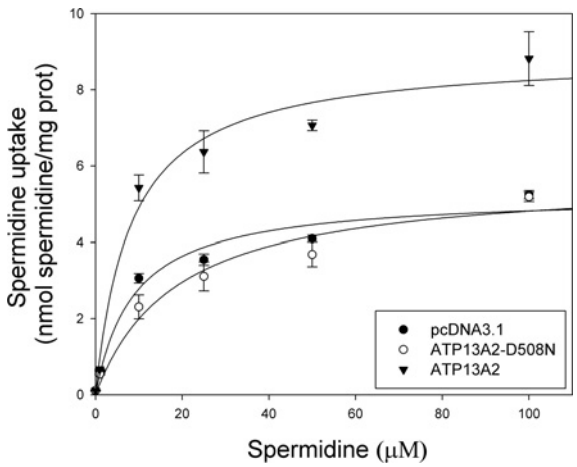

Figure 4 Spermidine dependence of polyamine uptake in CHO cells transfected with the empty vector (pcDNA3.1) or expressing the ATP13A2 or ATP13A2-D508N pumps

Cell monolayers were incubated for 150 min at 37°C with increasing concentrations of [^{3}H]spermidine in serum-free DMEM. Intracellular radioactivity was determined after the incubation period as described in the Materials and methods section. The activities are expressed as nmol of spermidine uptake per mg of protein. A representative experiment from three independent experiments is shown where each point is the mean ± S.D. of duplicate determinations. The line represents the best fit to the data given by the following hyperbolic equation: spermidine uptake = $a \times$Spermidine/(b + Spermidine), for pcDNA3.1: $a = 5.2 \pm 0.4$ nmol/mg of protein and $b = 8.8 \pm 2.9$ μM; ATP13A2: $a = 8.9 \pm 0.6$ nmol/mg of protein and $b = 8.1 \pm 2.4$ μM; and ATP13A2-D508N: $a = 6.8 \pm 0.7$ nmol/mg of protein and $b = 18.5 \pm 6.5$ μM.

incorporation of CHO cells stably expressing the inactive mutant ATP13A2-D508N mutant was significantly lower.

Kinetic displacement of [^{3}H]spermidine by unlabelled spermidine

CHO cells transfected with the empty vector or those expressing the recombinant ATP13A2 were incubated with 10 μM [^{3}H]spermidine at 37°C for 90 min, and the cells were then washed and incubated with 100 mM unlabelled spermidine. Figure 5 shows the residual level of labelled cell-associated spermidine after the addition of unlabelled spermidine. The labelled spermidine decreased with time following similar kinetics in both cell types. CHO cells incubated with DMEM without unlabelled spermidine retained 30% of their initial radioactivity at the end of the incubation (results not shown).

Cytotoxic effect of chloroquine on CHO cells

Because polyamines are accumulated in acidic compartments, the effect of chloroquine, which prevents endosomal acidification, was tested on the viability of CHO cells expressing ATP13A2 or ATP13A2-D508N. Figure 6 shows that chloroquine exerted a dose-dependent cytotoxic effect on the viability of CHO cells. ATP13A2- and ATP13A2-D508N-expressing CHO cells developed the same sensitivity to chloroquine, showing that the active P_5-ATPase was unable to revert the alkalinization of acidic vesicles induced by this compound.

[^{3}H]Spermidine uptake by the mitochondrial fraction of CHO cells

Previous studies have shown that the ATP13A2 protein is present in lysosomes and late endosomes [9]. Accordingly, the mitochondrial fraction containing these vesicles from CHO cells expressing the ATP13A2 or ATP13A2-D508N proteins was isolated. Figure 7 shows that after 1.5 min the ATP-dependent [^{3}H]spermidine uptake of the mitochondrial fraction from ATP13A2-expressing CHO cells was 4-fold higher than that reached by the isolated fraction from CHO cells expressing the ATP13A2-D508N mutant pump. At 12 min, both samples reached the same maximal level of spermidine uptake (27.7 ± 3.2 and 20.7 ± 7.2 μmol spermidine/μg of protein for ATP13A2 and ATP13A2-D508N respectively; results not shown). Spermidine uptake was dependent on ATP hydrolysis, because the non-hydrolysable ATP analogue ATPγS induced only a marginal spermidine uptake.

DISCUSSION

Polyamines are required in various biological processes, in particular for eukaryotic cell cycle progression. There is a precise control of their intracellular pattern by synthesis, catabolism and transport. In the present study we show that the intracellular accumulation of spermidine increases with expression of ATP13A2 suggesting that this P_{5B}-ATPase is involved in the mechanisms responsible for polyamine homoeostasis.

High levels of spermidine seem to be toxic and decrease the cellular viability [22]. ATP13A2-expressing CHO cells exhibited a higher sensitivity to the toxic effect of spermidine. This is in agreement with our previous study showing that the toxicity of paraquat was also increased by ATP13A2 expression in CHO cells [12].

[^{3}H]Spermidine uptake was almost doubled by the stable expression of ATP13A2 in CHO cells. The kinetics of spermidine uptake in ATP13A2-expressing CHO cells was similar to that

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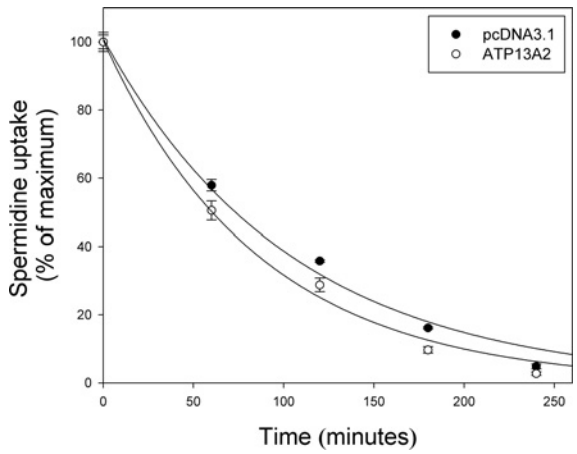

Figure 5 Kinetic displacement of [^{3}H]spermidine by unlabelled spermidine in CHO cells

Cell monolayers were treated for 90 min at 37 °C with 10 μM [^{3}H]spermidine in serum-free DMEM; the cells were then washed and incubated with 100 mM unlabelled spermidine in fresh serum-free DMEM. Intracellular radioactivity was determined at the indicated incubation periods after the addition of unlabelled spermidine as described in the Materials and methods section. The residual spermidine is expressed as the percentage of the maximum uptake reached by ATP13A2-expressing cells or CHO cells transfected with the empty vector (pcDNA3.1). A representative experiment from three independent experiments is shown where each point is the mean $\pm$ S.D. of duplicate determinations. The line represents the best fit to the data given by the following exponential decay equation: residual spermidine $= a \times e^{-k \times t}$. pcDNA3.1: a $= 101 \pm 3$ %, k $= 0.010 \pm 0.001$ min^{-1}; ATP13A2: a $= 100 \pm 3$ %, k $= 0.011 \pm 0.001$ min^{-1}.

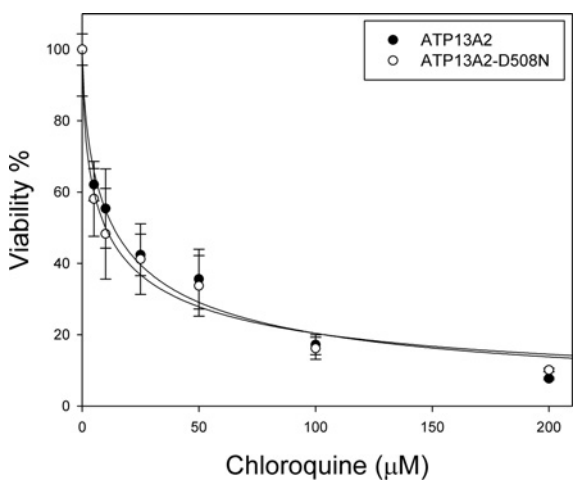

Figure 6 Viability of CHO cells expressing the ATP13A2 or ATP13A2-D508N pumps after chloroquine exposure

The cells were incubated for 18 h at 37 °C with increasing concentrations of chloroquine. The viability assay was performed by measuring the activity of the endogenous enzyme hexosaminidase as described in the Materials and methods section. Values are expressed as the percentage of the absorbance at 405 nm measured for the untreated cells. Results are means $\pm$ S.E.M. for four independent experiments performed in quadruplicate. The line represents the best fit to the data given by the following equation: viability $=$ min $+$ (max $-$ min)/[1 $+$ (chloroquine/EC_{50})$^{-\text{Hillslope}}$] (with min $\geqslant$ 0), for ATP13A2: min $= 0 \pm 1$ %, max $= 99 \pm 6$ %, $EC_{50} = 13 \pm 4$ mM and Hillslope $= -0.68 \pm 0.14$; ATP13A2-D508N: min $= 0 \pm 1$ %, max $= 99 \pm 6$ %, $EC_{50} = 10 \pm 3$ mM and Hillslope $= -0.59 \pm 0.13$.

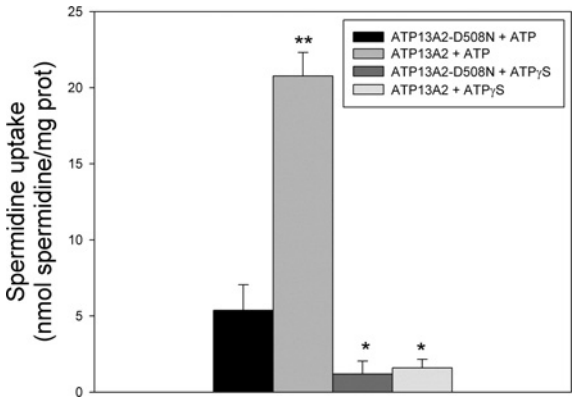

Figure 7 ATP-dependent [^{3}H]spermidine uptake by the mitochondrial fraction of CHO cells

The reaction was initiated by the addition of the mitochondrial fraction (10 μg of protein) isolated from ATP13A2 or ATP13A2-D508N-expressing CHO cells, in a medium containing 800 μM [^{3}H]spermidine in the absence or presence of 1.5 mM ATP or ATPγS at 37 °C. After 1.5 min the reaction was finished by filtering the samples as described in the Materials and methods section. The spermidine uptake is expressed as nmol of spermidine uptake per mg of protein. A representative experiment from three independent experiments is shown where each point is the mean $\pm$ S.D. of duplicate determinations. $^{**}P < 0.01$ compared with the value measured for ATP13A2-D508N-expressing CHO cells (Student's *t* test); $^{*}P < 0.1$ compared with the value measured for ATP13A2-D508N-expressing CHO cells (Student's *t* test).

of control cells and, as described in previous studies [23], was inhibited by decreasing the temperature to 4 °C.

The accepted model of polyamine uptake suggests that polyamine transport is initiated by an unidentified plasma membrane carrier, and is rapidly followed by sequestration into pre-existing PSVs (polyamine-sequestering vesicles) via an undefined energy-dependent mechanism that requires an outwardly directed H^+ gradient [24]. Vesicular sequestration is the rate-limiting step and PSVs co-localize with acidic vesicles of the late endocytic compartment [24]. Because we found that the time dependence of the spermidine uptake in ATP13A2-expressing CHO cells was similar to that of control cells, the higher level of intracellular spermidine would be related to a higher accumulation rather than to an increased transport into the cytosol.

Because the concentration of polyamines into PSVs is higher than in the cytoplasm, the ATP13A2 protein could be directly associated with the active accumulation of polyamines into the vesicles of the late endocytic compartment. This idea is in line with the higher ATP-dependent spermidine uptake of the mitochondrial fraction from ATP13A2-expressing CHO cells. However, it seems appropriate to consider that a higher level of spermidine accumulation in the cell may be brought either by increasing the concentration of spermidine in the vesicles or by increasing the number and/or size of storage vesicles [12]. Favouring the second possibility, the expression of ATP13A2 did not produce a significant change in the apparent affinity of the cells for spermidine and there was not a clear difference in the loss of labelled spermidine between the CHO control and ATP13A2-expressing cells. Further experiments are necessary to establish the exact mechanism by which ATP13A2 increases the accumulation of spermidine.

Although most P-ATPases function as metallic cation transporters, the most closely phylogenetically to the P_5-ATPases are the P_4-ATPases which transport phospholipids [25]. Consequently, polyamines should not be discarded as possible transported substrates of P_5-ATPases. Additional experiments

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examining the details of the ATP-dependent spermidine uptake in the mitochondrial fraction of ATP13A2-expressing CHO cells may help to reach a better understanding of this process.

Because basic drugs, amines and peptides can be accumulated in liposomes simply by imposing a pH gradient across the liposome membrane [26], an active H^+ transporter in the PSV's membrane could also give rise to spermidine uptake without actually transporting this compound. Nevertheless, ATP13A2 expression was unable to revert the increase of lysosomal/endosomal pH induced by chloroquine treatment. This result suggests that this P_5-ATPase does not replace the function of the proton pumps.

In the present study we have shown that expression of the human P_{5B}-ATPase ATP13A2 increases the spermidine accumulation in CHO cells, reinforcing the connection between polyamine metabolism, PD and P_5-ATPases. In agreement with this a recent publication shows a PD-related decrease in the expression of the polyamine catabolic enzyme SAT1 (spermidine/spermine N1-acetyltransferase 1) [27]. SAT1 is the rate-limiting catabolic enzyme in the polyamine metabolic pathway, and a decrease in SAT1 expression results in increased levels of higher-order polyamines, spermine and spermidine. Consequently, if ATP13A2 was an active polyamine transporter located in vesicles of the late endocytic compartment, its mutation would increase the cytoplasmic polyamine content in Kufor–Rakeb Syndrome by diminishing the polyamine uptake by PSVs.

The oxidative stress implicated in the pathogenic pathway of many neurodegenerative diseases, including PD, could be induced by elevated levels of metal ions like cadmium, iron, manganese and selenium. It was recently reported that treatment of HEK (human embryonic kidney)-293 cells expressing ATP13A2 for 12 h with 2 mM $MnCl_2$ reduced the number of dead cells from 28 to 18 % [28]. Because polyamines can chelate metallic cations [29], the manganese tolerance developed by ATP13A2-expressing HEK-293 cells may be a consequence of the higher accumulation of polyamines reported in the present study.

AUTHOR CONTRIBUTION

Diego de la Hera, Gerardo Corradi and Felicitas de Tezanos Pinto performed all experimental work and interpreted results. Hugo Adamo and Felicitas de Tezanos Pinto designed the research and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Parkinson's disease-associated human P_{5B}-ATPase ATP13A2 increases spermidine uptake

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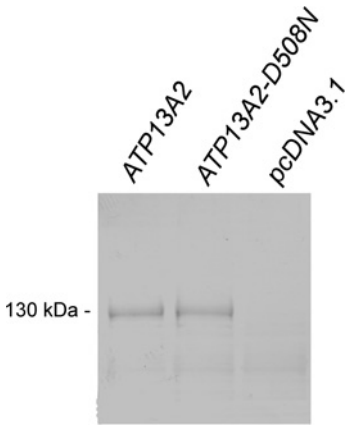

Figure S1 Expression of the recombinant ATP13A2 and ATP13A2-D508N pumps

Immunoblot of the mitochondrial fraction containing lysosomes and late endosomes from CHO cells transfected with cDNAs coding the human ATP13A2, ATP13A2-D508N or the empty vector (pcDNA3.1). Proteins (10 μg) were separated by SDS/PAGE and transferred on to PVDF membranes, and the recombinant proteins ATP13A2 and ATP13A2-D508N were detected with the anti-ATP13A2 antibody (Sigma).

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