Involvement of proton-coupled SLC49A4-mediated transport in the export of lysosomally trapped pyrilamine

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Abbreviates: SLC, solute carrier; BBB, blood-brain barrier; MES, 2-morpholinoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; CCCP, carbonylcyanide m-chlorophenylhydrazone; FCCP, p-trifluoromethoxyphenylhydrazone; MPP⁺, 1-methyl-4-phenylpyridinium; TEA, tetraethylammonium; BSP, bromosulfophthalein; DIDS, 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid; PAH, p-aminohippuric acid.
Abstract

Our recent study revealed that SLC49A4, known as disrupted in renal carcinoma 2, is a H⁺-coupled lysosomal exporter for pyridoxine (vitamin B6), a cationic compound, and involved in the regulation of its lysosomal and cellular levels. We here examined a possibility that this transporter might also transport cationic amphiphilic drugs (CADs) that are known to undergo lysosomal trapping, using pyrilamine, an H₁-antagonist, as a model CAD and the COS-7 cell line as a model cell system for transient introduction of human SLC49A4 and a recombinant SLC49A4 protein (SLC49A4-AA), in which the N-terminal dileucine motif involved in lysosomal localization was removed by replacing with dialanine for redirected localization to the plasma membrane. The introduction of SLC49A4 into COS-7 cells induced a significant decrease in the accumulation of pyrilamine in the intracellular compartments in the cells treated with digitonin for permeabilization of plasma membranes, suggesting its operation for lysosomal pyrilamine export. Accordingly, functional analysis using the SLC49A4-AA mutant, which operates for cellular uptake at the plasma membrane, in transiently transfected COS-7 cells demonstrated its H⁺-coupled operation for pyrilamine transport, which was saturable with a Michaelis constant of 132 μM at pH 5.5. In addition, many CADs that may potentially undergo lysosomal trapping, which include imipramine, propranolol, verapamil, and some others, were found to inhibit SLC49A4-AA-mediated pyrilamine transport, suggesting their affinity for SLC49A4. These results suggest that SLC49A4 is involved in the lysosomal trapping of pyrilamine, operating for its exit. The CADs that inhibited SLC49A4-AA-mediated pyrilamine transport could also be SLC49A4 substrate candidates.

Significance Statement.

SLC49A4 mediates the transport of pyrilamine in a H⁺-coupled manner at the lysosomal membrane. This could be a newly identified mechanism for lysosomal export involved in its lysosomal trapping.
Introduction

Lysosomes, acidic organelles that play a key role physiologically in metabolic processing of endogenous waste products, are known to sequestrate various cationic amphiphilic drugs (CADs), particularly those with pKa values near or above neutrality, in their luminal interior (de Duve et al., 1974; Andrew et al., 1997; Goldman et al., 2009; Nadanaciva et al., 2011). This sequestration, also known as lysosomal trapping, has been of great interest because such CADs, which typically have ionizable amine groups, include those therapeutically used for the treatments of various neuronal and cardiovascular diseases. Lysosomal trapping could be a factor that leads to high tissue distribution known for those CADs. Accordingly, alterations in their trapping could lead to alterations in their disposition and, consequently, therapeutic effects.

Lysosomal trapping has generally been believed to occur according to the pH partition theory (Kaufmann and Krise, 2007). Whereas the unionized form of a CAD, which is readily permeable across the lipoidal lysosomal membrane by simple diffusion, can be equilibrated at the same concentration inside and outside of the lysosome, the ionized form, which is impermeable, distributes at a higher concentration in the acidic environment inside than in the near neutral environment outside due to a greater extent of ionization in the former, resulting in an accumulation of the CAD inside. However, recent studies (Okura et al., 2008; Okura et al., 2014) have suggested the presence of a H+/organic cation (OC+) antiporter, which is involved in the transport of some CADs, at the plasma membrane facing the vascular lumen in brain capillary endothelial cells constituting the blood-brain barrier (BBB). Therefore, we hypothesized that, likewise, transporters may be involved in the lysosomal membrane transport of CADs. As an attempt to address the issue, we examined a possibility that SLC49A4, known as disrupted in renal carcinoma 2, might be involved in that. SLC49A4 has been identified as a lysosomal exporter for pyridoxine (vitamin B6) in our recent study (Akino et al., 2023).

In analogy to the intestinal pyridoxine/thiamine transporters of SLC19A2 and SLC19A3, which can transport structurally unrelated cationic compounds (Liang et al., 2015; Giacomini et al., 2017), we hypothesized that SLC49A4 might also be involved in the lysosomal export of CADs. Pyrilamine, an H1-
antagonist, was selected as a model CAD known to undergo lysosomal trapping. This drug is also known to be transported into brain capillary endothelial cells by the putative H\(^+/\)OC\(^+\) antiporter (Shimomura et al., 2013). For the functional analyses of SLC49A4, the COS-7 cell line was used as a model cell system for the transient introduction of human SLC49A4 and a recombinant SLC49A4 protein (SLC49A4-AA), in which the N-terminal dileucine motif involved in lysosomal localization was removed by replacing with dialanine by site-directed mutagenesis for redirection to the plasma membrane, exposing intralysosomal segments to the extracellular space (Kalatzis et al., 2001; Sagne et al., 2001; Morin et al., 2004; Savalas et al., 2011). The assessments of cellular pyrilamine uptake using SLC49A4-AA enabled the characterization of SLC49A4-mediated pyrilamine transport in the direction of lysosomal export, as in our previous study on pyridoxine transport (Akino et al., 2023).
Materials and Methods

Cell culture

COS-7 cells were maintained as described previously (Mimura et al., 2017; Akino et al., 2023).

Preparation of plasmids

The complementary DNA (cDNA) of human SLC49A4 (GenBank accession number, NM_032839.3) and the cDNA of SLC49A4-AA were prepared as described previously (Akino et al., 2023) and incorporated into the pCI-neo vector (Promega, Madison, WI) to prepare plasmids for transfection. The plasmids for FLAG-tagged SLC49A4 (FLAG-SLC49A4) and SLC49A4-AA (FLAG-SLC49A4-AA) were generated using the pCI-neo vector modified to fuse FLAG tag to the N-terminus.

Immunofluorescence staining

Immunofluorescence staining was conducted to examine the localization of FLAG-SLC49A4 in transiently transfected COS-7 cells cultured on 35-mm glass bottom dishes as described previously (Akino et al., 2023). The primary antibodies were mouse monoclonal anti-FLAG antibody (FUJIFILM Wako Pure Chemical, Osaka, Japan), rabbit polyclonal anti-ATP1A1 antibody (Proteintech, Rosemont, IL), and rabbit polyclonal anti-lysosomal associated membrane protein 1 (LAMP1) antibody (Proteintech), respectively, for the detection of FLAG-SLC49A4, ATP1A1, a marker for the plasma membrane, and LAMP1, a lysosomal marker. The secondary antibodies were goat polyclonal anti-mouse IgG antibody coupled to Alexa Fluor Plus 488 (Invitrogen, Carlsbad, CA) and goat polyclonal anti-rabbit IgG antibody coupled to Alexa Fluor 594 (Jackson ImmunoResearch, West Grove, PA), respectively. The primary and secondary antibodies were all used at a dilution of 1:500.

Western blot analysis

Western blot analysis was conducted to examine protein expression in transiently transfected COS-7 cells.
cultured on 24-well coated plates as described previously (Furumiya et al., 2015; Akino et al., 2023), using the total cell lysates. The primary antibodies, which were used at a dilution of 1:1,000, were mouse monoclonal anti-FLAG antibody (FUJIFILM Wako Pure Chemical) and mouse monoclonal anti-β-actin antibody (Proteintech), respectively, for the detection of FLAG-SLC49A4 and β-actin as a loading control. The secondary antibody, which was used at a dilution of 1:10,000, was goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch).

**Uptake study**

Uptake assays were conducted as described previously (Mimura et al., 2017; Akino et al., 2023), using transiently transfected COS-7 cells cultured on 24-well coated plates. Briefly, uptake solutions were prepared using Hanks’ solution supplemented with 10 mM MES (pH 6.5 and below) or 10 mM HEPES (pH 7.0 and above) and added with [^3H]pyrilamine (20.1 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO). After termination of [^3H]pyrilamine uptake into the cells, the cells were solubilized for the determination of the associated radioactivity by liquid scintillation counting. Uptake was normalized to cellular protein content determined using BSA as a standard.

In experiments to examine pyrilamine accumulation in transiently transfected COS-7 cells treated with digitonin for permeabilization of plasma membranes, the cells were preincubated in pyrilamine-free uptake solution added with digitonin (50 μM) for 5 min. Uptake assays were conducted in the absence of digitonin using an uptake solution modified by replacing NaCl with K-gluconate and supplemented with 5 mM ATP.

**Data analysis**

Data were handled as described previously (Akino et al., 2023). The saturable uptake of pyrilamine by SLC49A4-AA was analyzed using the Michaelis–Menten model equation as follows: \( v = \frac{V_{\text{max}}}{K_m + s} \). The maximum transport rate \( V_{\text{max}} \) and the Michaelis constant \( K_m \) were estimated by fitting this equation to the experimental profile of the uptake rate \( v \) versus concentration \( s \) of the substrate (pyrilamine).
The data are presented as the mean ± SD with the number of experiments conducted using different preparations of cells. Each experiment was conducted in duplicate as biological replicates. Statistical analysis was performed using a Student’s t-test or, when multiple comparisons were needed, ANOVA followed by Dunnett’s test. The level of $p < 0.05$ was considered statistically significant.
Results

**SLC49A4 decreases pyrilamine accumulation in the intracellular compartments**

To evaluate the accumulation of pyrilamine in the intracellular compartments, which could mostly represent lysosomal accumulation, plasma membranes of COS-7 cells transiently expressing FLAG-SLC49A4 were permeabilized by a treatment with digitonin, a mild detergent. In the immunocytochemical analysis, FLAG-SLC49A4 was shown to be co-localized with LAMP1, indicating its lysosomal localization, in both digitonin-treated and untreated cells (Fig. 1A). The predominant intracellular localization of FLAG-SLC49A4 was also confirmed by quantification of its derived fluorescence intensity in the immunofluorescent images, as assessed in untreated cells (Supp. Fig. 1). In the western blot analysis, the protein of FLAG-SLC49A4 was shown to be present abundantly both in digitonin-treated and untreated cells (Fig. 1B). In addition to the intact FLAG-SLC49A4 protein with the molecular weight of about 70 kDa, a proteolytical product with about 35 kDa was also shown to be present as reported previously (Savalas et al., 2011; Akino et al., 2023). Thus, FLAG-SLC49A4 was confirmed to be almost exclusively localized to lysosomes in digitonin-treated cells as well as in untreated cells. We then used digitonin-treated cells, in which the plasma membrane was permeabilized and, hence, lysosomal accumulation could be evaluated, to examine the function of FLAG-SLC49A4 at lysosomes. As shown in Fig. 1C, the accumulation of pyrilamine (10 nM), which was evaluated at pH 7.4 to mimic the near neutral cytosolic environment, was significantly decreased in FLAG-SLC49A4-expressing cells, compared to mock cells, indicating the operation of FLAG-SLC49A4 for lysosomal pyrilamine export. A similar trend in pyrilamine accumulation was observed in untreated cells, although the difference was not statistically significant (Fig. 1D). A possibility is that the impact of the additional expression of exogenous FLAG-SLC49A4 on the lysosomal accumulation of pyrilamine was not large enough to lead to a change detectable beyond variability in its accumulation at the cellular level. It is notable, however, that the levels of cellular accumulation were comparable to those of lysosomal accumulation, suggesting that the lysosome is the major site of intracellular distribution for pyrilamine.
SLC49A4-AA mediates plasma membrane transport of pyrilamine

To investigate into the transport function of SLC49A4 for pyrilamine, we used the SLC49A4-AA mutant, which is redirected to the plasma membrane in its transiently transfected COS-7 cells, as demonstrated by immunofluorescent staining and western blotting in our recent study (Akino et al., 2023). As shown in Fig. 2A, the uptake of pyrilamine (10 nM) under an acidic extracellular condition (pH 5.5), which mimics the intralysosomal environment, was elevated in cells transfected with plasma membrane-localized FLAG-SLC49A4-AA, compared to mock cells, but not in cells transfected with lysosomal membrane-localized FLAG-SLC49A4. The expression levels of the two transporters were confirmed to be comparable (Fig. 2B). These results indicate the operation of plasma membrane-localized FLAG-SLC49A4-AA for cellular pyrilamine uptake.

Functional characteristics of SLC49A4-AA for pyrilamine transport

We first assessed the time course of pyrilamine uptake in COS-7 cells transiently expressing SLC49A4 (Fig. 3). The uptake of pyrilamine (10 nM) was in proportion to time up to 10 s in SLC49A4-AA-expressing cells; whereas, it remained very low in mock cells. Based on this, the uptake period of 10 s was set for the evaluation of pyrilamine transport in the initial uptake phase.

The pH-sensitive characteristic of pyrilamine transport mediated by SLC49A4-AA was examined for a range of extracellular pHs between 5.0 and 7.5 (Fig. 4A). The specific uptake of pyrilamine (10 nM) by SLC49A4-AA, which was evaluated by subtracting pyrilamine uptake in mock cells from that in SLC49A4-AA-expressing cells, was greatest in the range between pH 5.0 and pH 6.0, and decreased with increasing pH at higher pHs. The condition of pH 5.5, at which regular uptake assays were performed, was in the range where the uptake was greatest. We then examined the effect of the dissipation of the H+ gradient by carbonylcyanide m-chlorophenylhydrazone (CCCP) and p-trifluoromethoxyphenylhydrazone (FCCP), which are protonophores, on the specific uptake of pyrilamine by SLC49A4-AA. These protonophores extensively reduced the specific uptake (Fig. 4B). Nigericin, which dissipates the H+ gradient by a H+/K+-
exchange mechanism, was also effective in reducing the specific uptake (Fig. 4C). These results indicate that an inwardly directed H\(^+\) gradient is required for the pyrilamine transport and, hence, SLC49A4 transports pyrilamine in a H\(^+\)-coupled manner, as found for pyridoxine previously (Akino et al., 2023). It should be noted that pyrilamine can be protonated with pK\(_a\) of 8.9 (Tardioli et al., 2009) and, hence, is mostly cationized in the entire pH range in Fig. 4A. Therefore, it is likely that pyrilamine is transported by SLC49A4 in the cationized form.

We also examined the effect of the other extracellular ions on SLC49A4-AA-mediated pyrilamine transport by replacing NaCl in the uptake solution with an isotonic concentration of KCl, Na-gluconate, K-gluconate, and mannitol (Fig. 5). The specific uptake of pyrilamine (10 nM) was reduced, but only modestly, when Cl\(^-\) was removed by replacement with gluconate. However, the replacement with mannitol exhibited no effect. Thus, Cl\(^-\) may be involved in pyrilamine transport by SLC49A4, but it does not seem to have a major role. This was different from the highly Cl\(^-\)-dependent characteristic of SLC49A4-mediated pyridoxine transport (Akino et al., 2023), although the mechanism behind the difference is unknown.

Kinetic analysis indicated that the SLC49A4-AA-specific uptake of pyrilamine was saturable with a \(V_{\text{max}}\) of 3.41 nmol/min/mg protein and a \(K_m\) of 132 \(\mu\)M (Fig. 6). This \(K_m\) is smaller than that of 522 \(\mu\)M for pyridoxine (Akino et al., 2023), which was determined at a comparable but slightly lower pH of 5.0. Thus, pyrilamine seems to have a higher affinity for SLC49A4 than pyridoxine.

**Effect of various compounds on pyrilamine transport by SLC49A4-AA**

To examine the possibility that SLC49A4 may potentially recognize some other CADs as substrates, we assessed the effect of various CADs (100 \(\mu\)M) on the specific uptake of pyrilamine (10 nM) by SLC49A4-AA transiently expressed in COS-7 cells. As shown in Fig. 7, almost all the tested CADs, with an only exception of chloroquine, inhibited the specific uptake, indicating their affinity for SLC49A4, whereas the other types of cationic drugs did not. Anionic compounds and norfloxacin, a zwitterionic drug, did not, either.
Discussion

The present study has demonstrated the involvement of lysosomal-membrane-localized SLC49A4 in the lysosomal export of pyrilamine. In addition, functional analyses using the plasma membrane-localized SLC49A4-AA mutant indicated its H⁺-coupled operation for pyrilamine transport. Moreover, various CADs were found to inhibit pyrilamine transport by SLC49A4-AA, suggesting that they may be competing substrates. The luminal environment in lysosomes is known to be, similarly to the extracellular environment, electrically more positive than the cytosolic environment (Xu and Ren, 2015) and SLC49A4-AA has been suggested to reside in the configuration exposing intralysosomal segments to the extracellular space in the plasma membrane (Savalas et al., 2011), as generally known for redirected lysosomal membrane proteins (Kalatzis et al., 2001; Sagne et al., 2001; Morin et al., 2004). In addition, the interior side of the lysosomal membrane corresponds to the exterior side of the plasma membrane in that it faces the side opposite to the cytosolic side. Therefore, the operation of SLC49A4-AA for cellular uptake corresponds to that of SLC49A4 for lysosomal export. This transport is feasible electrophysiologically for cationic compounds and could be facilitated by H⁺-coupled cotransport.

Among various CADs, only chloroquine was found not to inhibit pyrilamine transport by SLC49A4-AA. The absence of SLC49A4-mediated export may be in part involved in its well-known high accumulation in lysosomes. High lysosomal accumulation has been suggested to be needed for chloroquine to exert its antimalarial effect (Homewood et al., 1972), whereas overaccumulation may lead to its induced side effects (Frisk-Holmberg et al., 1979; Good and Shader, 1982). Highly hydrophilic cationic drugs, such as TEA, MPP⁺, and cimetidine, were suggested not to have affinity for SLC49A4, as they did not inhibit pyrilamine uptake by SLC49A4-AA (Fig. 5). It is in line with the fact that they do not undergo lysosomal trapping and, hence, do not need to be exported.

Notably, the drugs that were found to inhibit pyrilamine uptake by SLC49A4-AA include several substrates and inhibitors (substrate candidates) of the putative H⁺/OC⁻ antiporters that have been suggested to be present at plasma membranes typically in intestinal epithelial cells and capillary endothelial cells.
constituting the BBB. The reported substrates are apomorphine, clonidine, and diphenhydramine (Shimomura et al., 2013; Okura et al., 2014), and the inhibitors are propranolol, quinidine, and verapamil (Okura et al., 2008; Fischer et al., 2010). The intestinal H⁺/OC⁺ antiporter is known to facilitate the epithelial cellular uptake of CADs (Fischer et al., 2010), but these CADs undergo lysosomal trapping before exiting the cells to the blood side, resulting in delayed absorption (Hayeshi et al., 2008). Since SLC49A4 is expressed in the intestine at an appreciable level and likely to be involved in lysosomal export, as demonstrated for pyridoxine in the Caco-2 cell as an intestinal epithelial model in our previous study (Akino et al., 2023), the enhancement of SLC49A4-mediated export could be a factor to mitigate lysosomal accumulation and accelerate absorption. Because the BBB also has a H⁺/OC⁺ antiporter (Okura et al., 2008; Shimomura et al., 2013; Okura et al., 2014), which is for the endothelial cellular uptake of CADs from the blood, and SLC49A4 at a high expression level, SLC49A4-mediated lysosomal export could also have an impact on the transport of CADs across the BBB. Thus, the lysosomal trapping may be largely regulated by transporters (Fig. 8). There may also be an unidentified transporter, which could be a H⁺/OC⁺ antiporter analogous to the one at the plasma membrane, in the lysosomal membrane for the import of CADs.

Anthracyclines, imidazoacridinones, and tyrosine kinase inhibitors, which are all CADs with anticancer effects, include those known to be trapped in lysosomes and thereby sequestered away from their intracellular target site, cytosol or other organelles (Zhitomirsky and Assaraf, 2016; Zhitomirsky and Assaraf, 2017). In this way, lysosomal trapping may prevent them from exerting cytotoxic effects, resulting in lowered efficacy or chemoresistance (Hrabela et al., 2020). If the SLC49A4-mediated pathway would be available for such CADs to exit lysosomes, it could help deliver them to the site of action. Therefore, designing or finding SLC49A4 substrates could be a strategy to help develop more effective anticancer CADs. The SLC49A4-mediated export could also be beneficial in having antibody-drug conjugates (ADCs) for cancer therapy effective. ADCs enter cancer cells by endocytosis invoked by the binding of the antibody moiety to the specific cell surface antigen and then undergo catabolism to release the drug in the non-conjugated form in lysosomes (Dan et al., 2018). For ADCs using CADs, SLC49A4 may serve as a pathway for the released
drugs to exit lysosomes for exerting cytotoxic effects, similarly to the SLC46A3-mediated pathway which has recently been reported for acidic drugs released from ADCs (Hamblett et al., 2015). Conversely, it would be beneficial for CADs that act in lysosomes to be lysosomotropic without being exported by SLC49A4. Antipsychotic and antidepressant drugs include such CADs that are known to accumulate in lysosomes (Daniel and Wojcikowski, 1997) and act on lysosomal acid sphingomyelinase to exert therapeutic effects (Kornhuber et al., 2008).

In conclusion, our findings in this study indicate that SLC49A4 operates for the lysosomal export of pyrilamine, a CAD known to be trapped in lysosomes. It was also suggested that many other CADs could be SLC49A4 substrate candidates. The functional level of SLC49A4 may have an impact on the disposition and therapeutic effect of lysosomotropic CADs. It should be noted, however, that the conclusions are based on findings in model cell systems expressing SLC49A4-AA or FLAG-SLC49A4, and we cannot exclude the possibility that such recombinant SLC49A4 proteins may be functionally modulated. The function of FLAG-SLC49A4 may also be affected by the digitonin treatment to permeabilize plasma membranes for the assessment of its operation at the lysosomal membrane. Therefore, it is necessary to examine the function of unmodulated SLC49A4 under physiologically relevant conditions in the future.
Footnotes:

Conflict of Interest Statement.

No author has an actual or perceived conflict of interest with the contents of this article.

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Data Availability Statement.

The authors declare that all the data supporting the findings of this study are contained within the paper.
Authorship Contributions.

Participated in research design: Shogo and Tomoya.

Conducted experiments: Shogo and Rei.

Performed data analysis: Shogo and Tomoya.

Wrote or contributed to the writing of the manuscript: Shogo, Tomoya, Takahiro, and Hiroaki.
References


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Figure Legends

Fig. 1
SLC49A4 overexpression decreases pyrilamine accumulation in COS-7 cells treated with digitonin for permeabilization of plasma membranes. (A) Immunofluorescent images showing the localization of FLAG-SLC49A4 (green) with ATP1A1 (red), a marker for the plasma membrane, or LAMP1 (red), a lysosomal marker, in transiently transfected COS-7 cells, which were digitonin-treated or untreated (intact). Scale bar, 10 μm. (B) Western blots showing the protein expression levels of FLAG-SLC49A4 in transiently transfected COS-7 cells, which were digitonin-treated (+) or untreated (-). The blots were obtained using the total cell lysates (1 μg protein aliquots). (C) Lysosomal accumulation of pyrilamine in transiently FLAG-SLC49A4-expressing COS-7 cells, which were treated with digitonin. The accumulation of [3H]pyrilamine (10 nM) in the digitonin-treated cells was determined after 30 min of incubation (pH 7.4 and 37°C) in the absence of digitonin in the uptake solution modified by replacing NaCl with K-gluconate and supplemented with 5 mM ATP. The concentration of digitonin for the treatment was 50 μM. (D) Cellular accumulation of pyrilamine in transiently FLAG-SLC49A4-expressing COS-7 cells. The accumulation of [3H]pyrilamine (10 nM) in untreated cells was determined after 30 min of incubation (pH 7.4 and 37°C) in the regular uptake solution. Data represent the mean ± SD (n = 3). *, p < 0.05 compared with the control.

Fig. 2
SLC49A4-AA mediates plasma membrane transport of pyrilamine. (A) Pyrilamine uptake in COS-7 cells transiently expressing FLAG-SLC49A4 or FLAG-SLC49A4-AA, and in mock cells. The uptake of [3H]pyrilamine (10 nM) was evaluated for 10 s at pH 5.5 and 37°C. Data represent the mean ± SD (n = 3). *, p < 0.05 compared with the control. (B) Western blots showing the protein expression levels of FLAG-SLC49A4 and FLAG-SLC49A4-AA in transiently transfected COS-7 cells. The blots were obtained using the total cell lysates (1 μg protein aliquots).
**Fig. 3**

Time course of pyrilamine uptake in COS-7 cells transiently expressing SLC49A4-AA. The uptake of \[^{3}H\]pyrilamine (10 nM) was evaluated at pH 5.5 and 37°C in SLC49A4-AA-expressing cells (open circle) and mock cells (closed circle). Data represent the mean ± SD (n = 3).

**Fig. 4**

H\(^+\)-dependent pyrilamine uptake by SLC49A4-AA transiently expressed in COS-7 cells. (A) Effect of pH on pyrilamine uptake by SLC49A4-AA. The specific uptake of \[^{3}H\]pyrilamine (10 nM) was evaluated for 10 s at 37°C under various pH conditions. (B) Effect of dissipation of H\(^+\) gradient on pyrilamine uptake by SLC49A4-AA. The specific uptake of \[^{3}H\]pyrilamine (10 nM) was evaluated for 10 s at pH 5.5 and 37°C in the presence of a test agent for H\(^+\)-gradient dissipation, CCCP (50 μM), FCCP (50 μM) or nigericin (10 μM), or in its absence (control) after pretreatment for 5 min with or without the test agent under the same conditions. In experiments using nigericin, the uptake solution was modified by replacing NaCl and KCl with mannitol isotonically. Data represent the mean ± SD (n = 3). *, p < 0.05 compared with the control.

**Fig. 5**

Effect of ionic conditions on pyrilamine uptake by SLC49A4-AA transiently expressed in COS-7 cells. The specific uptake of \[^{3}H\]pyrilamine (10 nM) was evaluated for 10 s at pH 5.5 and 37°C. NaCl in the uptake solution for the control was replaced as indicated. Data represent the mean ± SD (n = 3). *, p < 0.05 compared with the control.

**Fig. 6**

Concentration-dependent uptake of pyrilamine by SLC49A4-AA transiently expressed in COS-7 cells. The specific uptake of \[^{3}H\]pyrilamine was evaluated at various concentrations for 10 s at pH 5.5 and 37°C. The results are presented in the formats of uptake rate versus concentration profile (left panel) and Eadie-Hofstee...
plot (right panel). The $V_{\text{max}}$ was $3.41 \pm 0.10 \text{ nmol/min/mg protein}$ and the $K_{\text{m}}$ was $132 \pm 13 \mu\text{M}$. Data represent the mean ± SD ($n = 3$).

**Fig. 7**

Effect of various compounds on pyrilamine uptake by SLC49A4-AA transiently expressed in COS-7 cells. The specific uptake of $[^3\text{H}]$pyrilamine (10 nM) was evaluated for 10 s at pH 5.5 and 37°C in the presence of a test compound (100 μM) or in its absence (control). MPP⁺, 1-methyl-4-phenylpyridinium; TEA, tetraethylammonium; BSP, bromosulphophthalein; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid; PAH, $p$-aminohippuric acid. The predicted pKa and log P values for each drug that inhibited specific pyrilamine uptake were obtained from DRUGBANK database (https://go.drugbank.com/). Data represent the mean ± SD ($n = 3$). *, $p < 0.05$ compared with the control.

**Fig. 8**

Schematic model showing the suggested role of SLC49A4 in the lysosomal trapping of pyrilamine.
Figure 1

A

Intact cells

FLAG-SLC49A4  ATP1A1  Merge

Digitonin-treated cells

FLAG-SLC49A4  ATP1A1  Merge

B

Digitonin (50 μM)  Mock  FLAG-SLC49A4

Anti-FLAG

Anti-β-actin

C

Accumulation (fmol/mg protein)

Mock (control)  FLAG-SLC49A4

D

Accumulation (fmol/mg protein)

Mock (control)  FLAG-SLC49A4
Figure 2

(A) Uptake (fmol/mg protein) of different treatments: Mock, FLAG-SLC49A4, and FLAG-SLC49A4-AA. The graph shows a significant increase in uptake for FLAG-SLC49A4-AA compared to the other treatments.

(B) Western blot analysis using anti-FLAG and anti-β-actin antibodies. The blot indicates protein bands at 73 kDa, 46 kDa, and 31 kDa for the different treatments.
Figure 3

Uptake (fmol/mg protein)

Time (s)
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Figure 5

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Henderson-Hasselbalch equilibrium

$\text{pK}_a: 8.9$

Lysosome (pH $\approx 5$)

Cytosol (pH $\approx 7.0$)

Extra cellular fluid or plasma (pH $\approx 7.4$)

SLC49A4

H$^+$/OC$^+$ antiporter

Henderson-Hasselbalch equilibrium

Simple diffusion

Pyrilamine

Figure 8