

Identification of a vesicular nucleotide transporter

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ATP is a major chemical transmitter in purinergic signal transmission. Before secretion, ATP is stored in secretory vesicles found in purinergic cells. Although the presence of active transport mechanisms for ATP has been postulated for a long time, the proteins responsible for its vesicular accumulation remains unknown. The transporter encoded by the human and mouse SLC17A9 gene, a novel member of an anion transporter family, was predominantly expressed in the brain and adrenal gland. The mouse and bovine counterparts were associated with adrenal chromaffin granules. Proteoliposomes containing purified transporter actively took up ATP, ADP, and GTP by using membrane potential as the driving force. The uptake properties of the reconstituted transporter were similar to that of the ATP uptake by synaptic vesicles and chromaffin granules. Suppression of endogenous SLC17A9 expression in PC12 cells decreased exocytosis of ATP. These findings strongly suggest that SLC17A9 protein is a vesicular nucleotide transporter and should lead to the elucidation of the molecular mechanism of ATP secretion in purinergic signal transmission.

ATP | chromaffin granule | purinergic signaling | storage and exocytosis | synaptic vesicle

Vesicular storage and subsequent exocytosis of neurotransmitters is essential for chemical transmission in neurons and endocrine cells. Thus far four distinct classes of transporters are known to participate in the uptake of neurotransmitters into neuronal synaptic vesicles and secretory granules in endocrine cells. These are vesicular monoamine transporters, vesicular acetylcholine transporters, vesicular inhibitory amino acid transporters, and vesicular glutamate transporters (VGLUTs) (1–7). These vesicular transporters mediate the active accumulation of their respective neurotransmitters through an electrochemical gradient of protons across the membrane generated by vacuolar proton-ATPase. ATP is stored in secretory vesicles and subsequently exocytosed, which leads to the various purinergic responses, such as central control of autonomic functions, pain and mechanosensory transduction, neural-glia interactions, control of vessel tone and angiogenesis, and platelet aggregation through purinoceptors, thus establishing its role as a chemical transmitter (8–12). Because the concentration of nucleotides in the vesicles was maintained at ≈ 0.1 – 1 M, an active transport mechanisms to accumulate nucleotides has been postulated (8–18). Although evidence increasingly supports the presence of a vesicular ATP transporters in secretory vesicles such as synaptic vesicles and adrenal chromaffin granules (13–19), the protein responsible for the ATP accumulation has not yet been identified.

Here, we report the expression and function of human and mouse SLC17A9, an isoform of the SLC17 phosphate transporter family. We present evidence that the SLC17A9 protein acts as a vesicular nucleotide transporter (VNUT) and that it plays an essential role in vesicular storage of ATP in the ATP-secreting cells.

Results and Discussion

Gene Organization and Expression of SLC17A9. SLC17 is a type I phosphate transporter family consisting of eight genes that are classified into three distinct subfamilies: (i) SLC17A1–4 (NPT1,

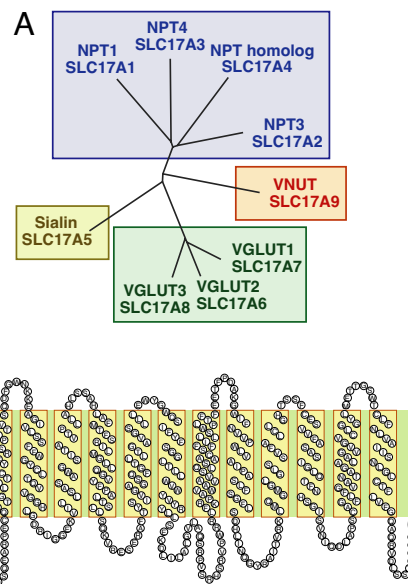


Fig. 1. Phylogenetic tree of SLC17 family (A) and putative secondary structure of SLC17A9 protein (B).

NPT3, NPT4, and $\text{Na}^+/\text{PO}_4^{2-}$ -cotransporter homologue), Na^+ and inorganic phosphate cotransporters, (ii) SLC17A5 (sialin), a lysosomal $\text{H}^+/\text{sialic acid}$ cotransporter, and (iii) SLC17A6–8, VGLUT (5). We found that a fourth subfamily, tentatively designated SLC17A9, was identified in a genome data bank screening (Fig. 1A). SLC17A9 was located on chromosome 20 and contained 14 exons and 13 introns [supporting information (SI) Fig. S1]. The SLC17A9 protein was 430 aa residues long with 12 putative transmembrane helices with ≈ 23 – 29% identity and 41–48% similarity to that of other SLC17 members (Fig. 1B and Fig. S2). Orthologues of other mammalian species have been identified. Although amino acid sequences of the amino-terminal region in mammals exhibited species-specific variation, the remaining regions showed the relatively well conserved features (83% identity) (Fig. S3).

We cloned cDNA-encoded SLC17A9 (Fig. S2). Northern blot analysis indicated that the SLC17A9 and mouse counterpart (mSLC17A9) were widely expressed in various organs, but predominantly in the adrenal gland, brain, and thyroid gland

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The authors declare no conflict of interest.

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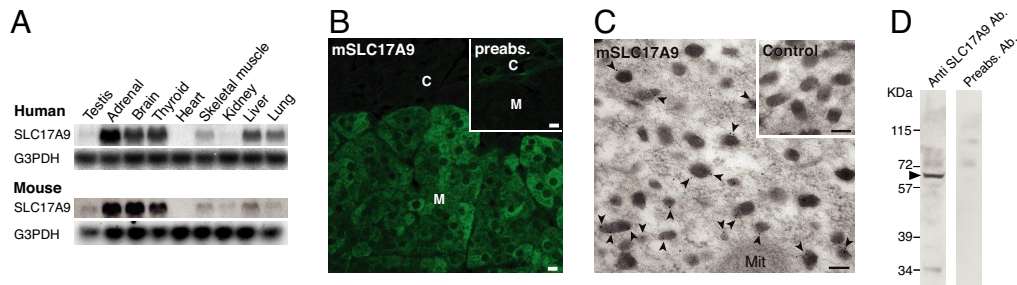


Fig. 2. Expression of SLC17A9 and its association with chromaffin granules. (A) Northern blotting showing expression of SLC17A9 in human and mouse brain and adrenal gland. Expression of G3PDH is also shown as a control. (B) Immunohistochemical localization of SLC17A9 protein in mouse adrenal gland. (Inset) Shown is control staining with preabsorbed antibodies. C, cortex; M, medulla. (C) Immunoelectronmicroscopy showing that SLC17A9 proteins (gold particles) are associated with chromaffin granules. (Inset) Shown is background labeling with control serum. Mit, mitochondrion. (D) Western blot indicates the presence of a SLC17A9 counterpart in the membrane of bovine adrenal chromaffin granule with a relative mobility of 61 kDa. The preabsorbed antibodies did not bind to the protein. The positions of marker proteins are indicated on the left. (Scale bars: B, 10 μ m; C, 100 nm.)

(Fig. 2A). In mouse adrenal gland, the mSLC17A9 protein was specifically expressed in the medulla and was associated with chromaffin granules (Fig. 2 B and C). The presence of a SLC17A9 counterpart in bovine adrenal chromaffin granule membrane has been confirmed (Fig. 2D).

SLC17A9 Protein Is an ATP Transporter. We hypothesized that SLC17A9 is responsible for the storage of nucleotides in chromaffin granules, in which high concentrations of ATP and other nucleotides (≈ 0.2 M) are accumulated in a membrane potential ($\Delta\psi$)-dependent manner (8, 13, 16). To test this working hy-

pothesis, the SLC17A9 protein was expressed in High Five cells, solubilized from the membranes, and purified by Ni-NTA column chromatography (Fig. 3A). The purified fraction showed two major protein bands (68 and 63 kDa) on SDS polyacrylamide gel, which corresponded to SLC17A9 protein because of their

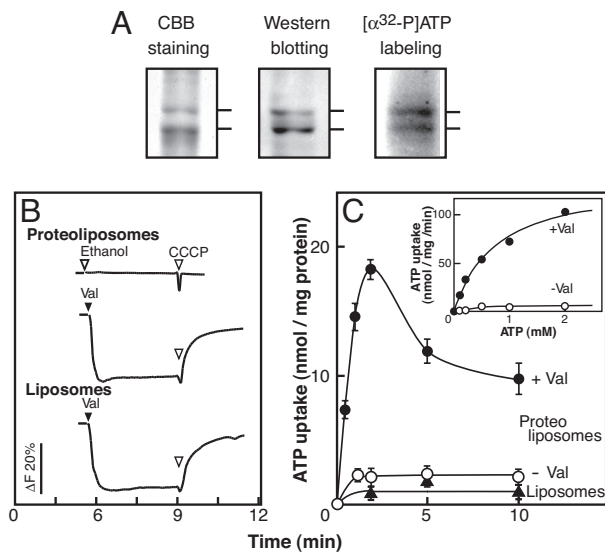


Fig. 3. Purification and reconstitution of SLC17A9 protein. (A) Purification. (Left) Purified fraction (7 μ g protein) was analyzed in an 11% PAGE in the presence of SDS and visualized by Coomassie brilliant blue staining. (Center) A duplicate gel was analyzed by Western blotting with anti-SLC17A9 antibodies. (Right) Labeling of SLC17A9 protein upon UV light illumination with [α - 32 P]ATP. (B) Formation of $\Delta\psi$ (positive inside) was measured by oxonol-V fluorescence quenching. Proteoliposomes (0.5 μ g protein) or liposomes (20 μ g lipid) containing trapped Na^+ were suspended in buffer containing 0.15 M K-acetate plus 4 mM KCl and 1 μ M oxonol-V, and fluorescence quenching was measured. Final concentration of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were added at 1 μ M. (C) Time course of [α - 32 P]ATP uptake. Na^+ -trapped proteoliposomes or liposomes were suspended as above in the presence or absence of valinomycin. Upon the addition of [α - 32 P]ATP, samples were taken at the indicated times, and radioactivity taken up by the proteoliposomes was counted. (Inset) Dose dependence of ATP uptake.

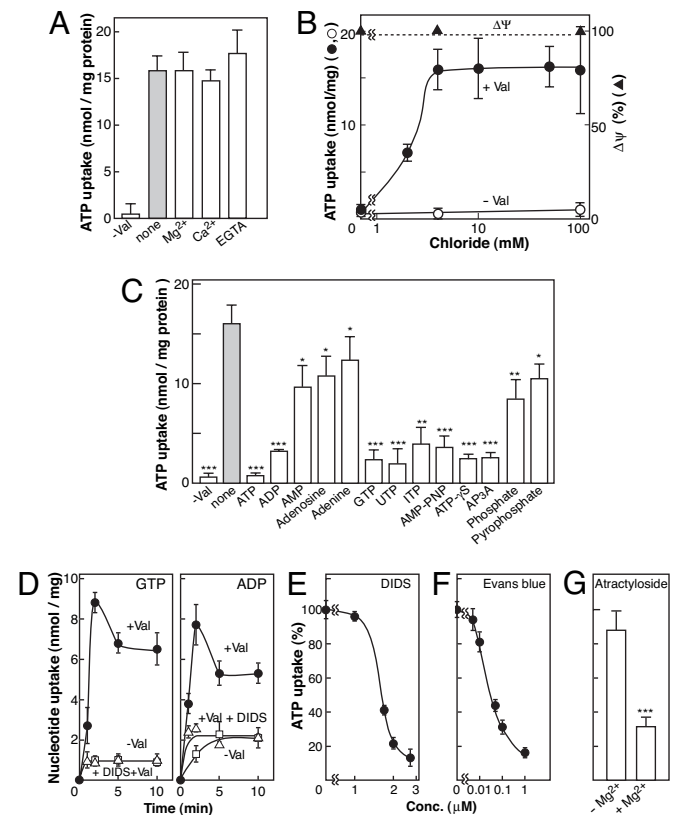


Fig. 4. Characterization of nucleotides transport by SLC17A9 protein. (A) The effect of divalent cations on the [α - 32 P]ATP uptake in the absence or presence of 2 mM Mg^{2+} or Ca^{2+} or 0.5 mM EGTA. (B) Dependence of [α - 32 P]ATP uptake on $[\text{Cl}^-]$. The uptake was measured after 2 min. Part of the potassium acetate in the reaction mixture was replaced with the indicated concentration of KCl. The magnitude of $\Delta\psi$ (oxonol V-fluorescence quenching) was little affected under the assay conditions used. (C) [α - 32 P]ATP uptake in the presence of various nucleotides at 1 mM. (D) Uptake of radiolabeled GTP or ADP transport in the presence or absence of valinomycin. When indicated, DIDS at 2 μ M was included. (E–G) The effect of DIDS, Evans blue, and atractyloside on [α - 32 P]ATP uptake in the presence or absence of Mg -acetate. Atractyloside was included at 200 μ M. *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

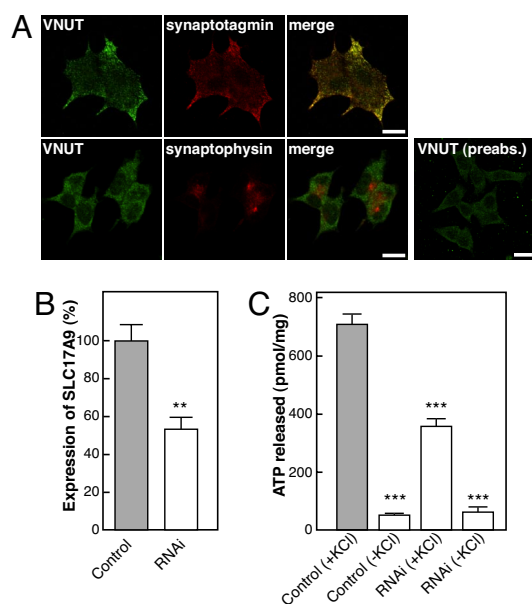


Fig. 5. The impact of rat SLC17A9 siRNA on the endogenous expression of SLC17A9 in in PC12 cells was compared with cells treated with scrambled siRNA (control). (A) Double-labeling immunohistochemistry indicated that SLC17A9 protein was colocalized at least in part with synaptotagmin, a marker of secretory granule, but not with synaptophysin, a marker of synaptic-like microvesicles in cultured PC12 cells. (B and C) Both SLC17A9 mRNA levels (B), as determined by real-time PCR, and the KCl-dependent secretion of ATP after 30 min (C) were assessed. $n = 9$ for three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

immunological reactivity to anti-SLC17A9 antibodies and ability to bind ATP upon UV light illumination (Fig. 3A). The observation of two SLC17A9 protein bands upon PAGE could have been caused by conformational differences or modification.

We then reconstituted SLC17A9 protein into liposomes and investigated whether or not the proteoliposomes took up [α - 32 P]ATP. When an internal inside positive $\Delta\psi$ was established, such as a K^+ -diffusion potential through the addition of valinomycin, the proteoliposomes took up [α - 32 P]ATP in a time-dependent manner (Fig. 3B and C and Table S1). ATP uptake was not observed in liposomes that did not contain SLC17A9 protein even when $\Delta\psi$ was already established (Fig. 3C). In contrast, artificially imposing pH gradient (ΔpH) did not evoke the ATP uptake (Table S1). The valinomycin-evoked ATP uptake exhibited dose dependence with K_m and V_{max} values of 0.8 mM, and 138 nmol/min of mg per protein, respectively (Fig. 3C). These results demonstrated that the SLC17A9 protein was an active transporter of ATP and used $\Delta\psi$ (positive inside) but not ΔpH (acidic inside) as the driving force.

Characterization of Nucleotide Transport. We further characterized the ATP uptake by SLC17A9 protein. Because ATP is a trivalent anion at the physiological pH and behaves as a monovalent anion upon chelation with divalent cations such as Mg^{2+} , we asked which forms were recognized as transport substrates. We found that Mg^{2+} and Ca^{2+} did not affect the ATP uptake (Fig. 4A), suggesting that this transporter recognized ATP as a substrate irrespective of its charge status. Cl^- dependence on the ATP uptake is of interest because VGLUT, another subfamily of SLC17, absolutely requires Cl^- for transport activity (20), and Cl^- stimulates ATP uptake in chromaffin granule membrane vesicles (16). As expected, the presence of Cl^- was an absolute requirement for ATP transport activity in the SLC17A9 protein (Fig. 4B). The transport activity reached steady state at ≈ 4 mM Cl^- , whereas the magnitude of $\Delta\psi$

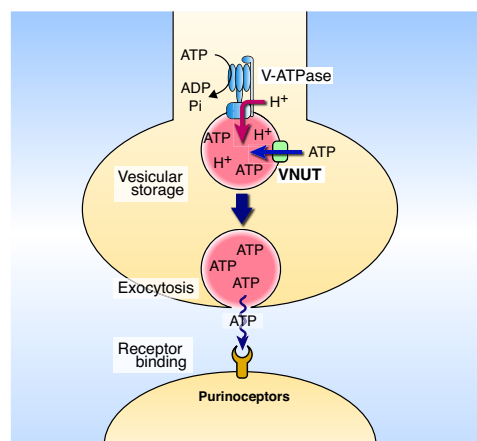


Fig. 6. Schematic presentation of purinergic chemical transmission. VNUT is present in secretory vesicles and is responsible for vesicular storage of nucleotides. The accumulated nucleotides, in particular ATP, are exocytosed upon stimulation and bind to purinoceptors at the surface of target cells, which then triggers intracellular signal transmission.

was unchanged. Br^- also activated ATP transport, whereas sulfate was not effective (Fig. S4). Various anions such as iodine and thiocyanate were rather inhibitory. The anion dependence of the SLC17A9 protein was similar to that of VGLUT (21, 22), suggesting the presence of a similar anion binding site and regulatory mechanism in this transporter family. *Cis* inhibition studies suggested that this transporter preferentially recognized ATP, GTP, and ADP as transport substrates. AMP-PNP and γS -ATP, nonhydrolysable ATP analogues, and diadenosine triphosphate (AP3A), one of the unique physiological ATP derivatives that accumulates in chromaffin granules and synaptic vesicles (9, 18), strongly inhibited the uptake. In contrast, adenosine and adenine were less effective inhibitors (Fig. 4C). The transporter-mediated uptake of GTP and ADP was confirmed by the direct measurement of ADP and GTP uptake (Fig. 4D). The ATP uptake was greatly inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), an inhibitor of VGLUT (23). The concentration required for ID_{50} was 1.5 μM (Fig. 4E). DIDS also inhibited the uptake of GTP and ADP (Fig. 4D). Evans blue, a specific inhibitor of VGLUT (23), also potentially inhibited the ATP uptake with an ID_{50} of 40 nM (Fig. 4F). Atractyloside, an inhibitor of the mitochondrial ATP/ADP exchanger, inhibited the ATP uptake of the SLC17A9 protein only in the presence of Mg^{2+} as reported for ATP uptake in chromaffin granules (16) (Fig. 4G). Thus, the SLC17A9 protein is a Cl^- -dependent nucleotide transporter, whose properties are very similar, if not identical, to those observed for ATP uptake in chromaffin granules (8, 13, 15–17).

Involvement of SLC17A9 Protein in Vesicular Storage and Exocytosis of ATP. Finally, we investigated whether SLC17A9 is responsible for vesicular storage and subsequent exocytosis of ATP in purinergic cells. PC12 cells are known to store and secrete ATP through secretory granule-mediated exocytosis (24). We found that mSLC17A9 protein was associated with secretory granules (Fig. 5A) and that RNAi suppressed $\approx 53\%$ of SLC17A9 expression and $\approx 50\%$ of KCl-stimulated exocytosis of ATP from PC12 cells, whereas the control treated with scrambled siRNA did not show any inhibitory effect (Fig. 5B and C). These results supported the participation of SLC17A9 protein in vesicular storage and exocytosis of ATP.

SLC17A9 Protein as a VNUT. Based on this information, we concluded that SLC17A9 and its orthologues encode the VNUT. SLC17A9 protein may accumulate ATP and other nucleotides in

the secretory vesicles such as adrenal chromaffin granules and synaptic vesicles. SLC17A9 protein is a long-sought nucleotide transporter and seems to be a missing link in purinergic chemical transmission (Fig. 6). Our immunohistochemical studies with anti-mSLC17A9 antibodies indicated that mSLC17A9 protein is expressed in a population of astrocytes, suggesting involvement of this transporter in storage of ATP in ATP-secreting astrocytes (25–27). Thus, identification of SLC17A9 protein as a VNUT may reveal the molecular mechanisms behind how ATP is secreted from purinergic cells and provide a novel molecular target for the pathophysiology of purinergic signaling and its therapeutic potential (9–11). Phylogenetic analysis indicates that the orthologues of SLC17A9 are widely distributed among various invertebrates and vertebrates (Fig. S5). Thus, our findings suggest the occurrence of common molecular mechanisms of the purinergic chemical transmission in animals.

Materials and Methods

Expression and Purification of SLC17A9 Protein. Recombinant baculovirus containing human SLC17A9 cDNA were constructed by using the Bac-to-Bac baculovirus expression systems (Invitrogen) according to the manufacturer's protocol. SLC17A9 cDNA was amplified by PCR using the primers (5'-CACCATGACCCTGACAAGCAGGCGCCAGGA-3' and 5'-CTAGAGTCTCATGGGTAGAGCTC-3'). Sf9 or High Five cells were used for expression of SLC17A9 protein. Insect cells were infected by recombinant baculoviruses at a multiplicity of infection of 2 and cultured an additional 72 h for Sf9 cells and 48 h for High Five cells. The cell ($\approx 1-2 \times 10^8$ cells) were suspended in a buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 M potassium acetate, 10% glycerol, 0.5 mM DTT, 10 μ g/ml pepstatin A, and 10 μ g/ml leupeptin and disrupted by sonication with a TOMY UD200 tip sonifier. Cell lysates were centrifuged at $700 \times g$ for 10 min to remove debris, and the resultant supernatant was centrifuged at $160,000 \times g$ for 1 h. The pellet (membrane fraction) was suspended in buffer containing 20 mM Mops-Tris (pH 7.0), 10% glycerol, 10 μ g/ml pepstatin A, and 10 μ g/ml leupeptin at ≈ 6 mg protein/ml and was solubilized with 2% octyl glucoside. After centrifugation at $260,000 \times g$ for 30 min, the supernatant was taken and applied to 1 ml of Ni-NTA Superflow resin (Qiagen). After incubation for 4 h at 4°C, the resin was washed with 10 ml of 20 mM Mops-Tris (pH 7.0), 5 mM imidazole, 20% glycerol, and 1% octyl glucoside. SLC17A9 protein was eluted from the resin with 3 ml of the same buffer containing 60 mM imidazole and could be stored at -80°C without loss of activity for at least a few months.

Reconstitution. Reconstitution of purified SLC17A9 protein into liposomes was carried out by the freeze-thaw method as described (20). In brief, 10 μ g of SLC17A9 protein was mixed with asolectin liposomes (0.5 mg of lipid), frozen at -80°C , and left at this temperature for at least 5 min. The mixture was thawed quickly by holding the samples tube in hands and diluted 60-fold with reconstitution buffer containing 20 mM Mops-Tris (pH 7.0), 0.5 mM DTT, 0.15 M sodium acetate, and 2 mM magnesium acetate. Reconstituted proteoliposomes were sedimented by centrifugation at $200,000 \times g$ for 1 h at 4°C, suspended in 0.2 ml of 20 mM Mops-Tris (pH 7.0) containing 0.15 M sodium acetate and 2 mM magnesium acetate, and used within 1 day of preparation. Asolectin liposomes were prepared as follows. Soybean lecithin (20 mg; Sigma type IIS) was suspended in 2 ml of 20 mM Mops-NaOH (pH 7.0) containing 0.5 mM DTT. The mixture was sonicated in a bath-type sonicator until clear, divided into small aliquots, and stored at -80°C until use.

Nucleotide Transport. Reconstituted proteoliposomes (0.5 μ g protein per assay) were suspended in 500 μ l of 20 mM Mops-Tris (pH 7.0), 5 mM magnesium acetate, 4 mM KCl, and 0.15 M potassium acetate and incubated for 3 min at 27°C. Valinomycin was added to give a final concentration of 2 μ M, and the mixture was incubated for an additional 3 min. The assay was initiated by addition of 0.1 mM [α - 32 P]ATP (3.7 GBq/mmol), and 130- μ l aliquots were taken at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at $760 \times g$ for 2 min (20). Radioactivity and protein concentration of the eluate were measured. In the case of GTP or ADP transport, [α - 32 P]GTP (3.7 GBq/mmol) or [2,8- 3 H]ADP (0.37 GBq/mmol) were used for the substrates instead of ATP.

Cell Culture and RNAi. PC12 cells were cultured as described (24). HiPerFect transfection reagent (Qiagen) was used for transfection of 25 nM AllStars negative control siRNA or rat SLC17A9 siRNA: UAUCGAGAGAAUGUCACC. KCl-stimulated ATP secretion was assayed as described 3 days later (24).

Data Analysis. All numerical values are shown as the mean \pm SEM for three to six experiments. Statistical significance was determined by Student's *t* test with *, <0.1 ; **, <0.01 ; ***, <0.001 .

Northern blot analysis, cDNA cloning, immunohistochemistry, and other detailed methods are provided in *SI Text*.

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