THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 50, pp. 42881–42887, December 16, 2011

Divalent Cation Transport by Vesicular Nucleotide Transporter*

Received for publication, June 29, 2011, and in revised form, October 19, 2011 Published, JBC Papers in Press, November 3, 2011, DOI 10.1074/jbc.M111.277269

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Background: The vesicular nucleotide transporter (VNUT) transports nucleotides in the presence of Mg^{2+}.
Results: VNUT transports divalent cations in a membrane potential- and nucleotide-dependent manner.
Conclusion: VNUT transports divalent cations as a nucleotide complex.
Significance: VNUT functions as a divalent cation importer in secretory vesicles.

The vesicular nucleotide transporter (VNUT) is a secretory vesicle protein that is responsible for the vesicular storage and subsequent exocytosis of ATP (Sawada, K., Echigo, N., Juge, N., Miyaji, T., Otsuka, M., Omote, H., and Moriyama, Y. (2008) *Proc. Natl. Acad. Sci. U.S.A.* 105, 5683–5686). Because VNUT actively transports ATP in a membrane potential ($\Delta\psi$)-dependent manner irrespective of divalent cations such as Mg^{2+} and Ca^{2+}, VNUT recognizes free ATP as a transport substrate. However, whether or not VNUT transports chelating complexes with divalent cations remains unknown. Here, we show that proteoliposomes containing purified VNUT actively took up Mg^{2+} when ATP was present, as detected by atomic absorption spectroscopy. The VNUT-containing proteoliposomes also took up radioactive Ca^{2+} upon imposing $\Delta\psi$ (positive-inside) but not ΔpH. The $\Delta\psi$-driven Ca^{2+} uptake required ATP and a millimolar concentration of Cl^-, which was inhibited by Evans blue, a specific inhibitor of SLC17-type transporters. VNUT in which Arg-119 was specifically mutated to alanine, the counterpart of the essential amino acid residue of the SLC17 family, lost the ability to take up both ATP and Ca^{2+}. Ca^{2+} uptake was also inhibited in the presence of various divalent cations such as Mg^{2+}. Kinetic analysis indicated that Ca^{2+} or Mg^{2+} did not affect the apparent affinity for ATP. RNAi of the VNUT gene in PC12 cells decreased the vesicular Mg^{2+} concentration to 67.7%. These results indicate that VNUT transports both nucleotides and divalent cations probably as chelating complexes and suggest that VNUT functions as a divalent cation importer in secretory vesicles under physiological conditions.

Like other classical neurotransmitters, ATP is stored in secretory vesicles in neurons and some endocrine cells and is exocytosed upon stimulation (1). The vesicular nucleotide transporter (VNUT)[3] is one of the class of vesicular neurotransmitter transporters such as the vesicular glutamate and vesicular monoamine transporters and is responsible for the vesicular storage of ATP through an active transport mechanism, which is driven primarily by vacuolar proton ATPase (2, 3). Besides ATP, VNUT can transport various nucleotides such as GTP and UTP. Suppression of the gene expression of VNUT in pheochromocytoma PC12 cells and neurons decreases vesicular ATP release, indicating its essential role in the occurrence of purinergic chemical transmission (2).

VNUT is the latest identified SLC17 type I phosphate transporter family member and may share some structural and functional properties with other members (2). All members comprise ~400–500 amino acid residues with 12 putative transmembrane helices, exhibiting 23–29% identity and 41–48% similarity to each other, and transport various organic anions in a membrane potential ($\Delta\psi$)-dependent manner (2–4). All members require Cl^- as an allosteric activator for their activity and exhibit similar inhibitor sensitivities: Evans blue, DIDS, and ketone bodies are common inhibitors (2, 3, 5–8). However, VNUT is unique in its substrate recognition capability. VNUT can transport ATP and GTP in the presence of EDTA, indicating that VNUT can recognize tri- or tetravalent anions as transport substrates, whereas other family members may recognize monovalent anions as transport substrates. Simultaneously, however, we also found that VNUT can transport ATP even in the presence of excess Mg^{2+} or Ca^{2+}. Because ATP forms chelating complexes with divalent cations and behaves as a divalent anion under physiological conditions, it is possible that VNUT also recognizes chelating nucleotide-divalent cation complexes as transport substrates and acts as a divalent cation importer in the ATP-accumulating secretory vesicles.

It is well known that secretory granules accumulate relatively high concentrations of Mg^{2+} and Ca^{2+}. For instance, chromaffin granules contain 20–40 mM Ca^{2+} and ~6 mM Mg^{2+} (9, 10). Such secretory granules are recognized as one of the sources of internal stores of Ca^{2+} and Mg^{2+}. Based mainly on biochemical

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (to Y. M., T. M., and H. O.) and for salt science (to Y. M.).
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[3] The abbreviations used are: VNUT, vesicular nucleotide transporter; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; VEAT, vesicular excitatory amino acid transporter.

pharmacological evidence, several transport systems such as the Ca^{2+}-ATPase, Ca^{2+}/H^{+} antiporter, and Mg^{2+} transporter have been postulated to explain the mechanism underlying the vesicular accumulation of divalent cations (11–13). However, such studies have been limited, and the transporter molecule(s) responsible for this accumulation process remains to be identified.

In this study, using proteoliposomes containing purified VNUT as the only protein source, we investigated the hypothesis that VNUT transports divalent cations as nucleotide-divalent cation chelating complexes. We present evidence that VNUT is a divalent cation importer.

EXPERIMENTAL PROCEDURES

Expression—Recombinant baculoviruses containing wild-type and mutant human VNUT cDNAs were constructed using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's protocol. VNUT cDNA was amplified by PCR using primers 5′-CACCATGACCCTGACAAGCAGGCGCCAGGA-3′ and 5′-CTAGAGGTCCTCATGGGTAGAGCTC-3′ and ligated into the pENTR/D-TOPO vector. VNUT cDNA was transferred from the pENTR/D-TOPO vector to a destination vector that was named pDEST10-VNUT. The resulting cloned VNUT gene also encoded an N-terminal His_6 tag. DH10Bac cells carrying bacmid DNA were transformed with pDEST10-VNUT. Recombinant bacmid was isolated from DH10Bac cells and used to transfect High Five cells for expression of the VNUT protein. High Five cells (6×10^6 cells/10-cm dish) were grown in Express Five medium (Invitrogen) supplemented with 2 mM L-glutamine and 10 μg/ml gentamycin at 27 °C. The High Five cells were infected with recombinant baculoviruses at a multiplicity of infection of 2 and grown for an additional 48 h. Afterward, the cells were harvested for membrane preparation. Upon infection, the insect cells expressed His-tagged VNUT as revealed by Western blot analysis. Maximum expression was observed 48 h after infection (2).

Purification—Insect cells ($1\sim2 \times 10^8$ cells) were suspended in buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 M potassium acetate, 10% glycerol, 0.5 mM dithiothreitol, 10 μg/ml pepstatin A, and 10 μg/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 supernatants were centrifuged at $160{,}000 \times g$ for 1 h. The pellet (membrane fraction) was suspended at ~1.5 mg/ml protein in buffer containing 20 mM MOPS/Tris (pH 7.0), 10% glycerol, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The membrane fraction was solubilized with 2% octyl glucoside. After centrifugation at $260{,}000 \times g$ for 30 min, the supernatant was added to 1 ml of nickel-nitrilotriacetic acid Superflow resin (Qiagen), followed by incubation for 4 h at 4 °C. The resin was washed with 20 ml of 20 mM MOPS/Tris (pH 7.0), 5 mM imidazole, 10% glycerol, and 1% octyl glucoside in a column. VNUT was eluted from the resin with 3 ml of the same buffer containing 60 mM imidazole. The eluate containing purified VNUT was stored at −80 °C, at which it was stable without loss of activity for at least a few months.

Reconstitution—Reconstitution of purified recombinant VNUT into liposomes was carried out by the freeze-thaw method described previously (2, 5–8). In brief, 10 μg of VNUT (50 μl) was mixed with 0.5 mg of liposomes (50 μl), frozen at −80 °C, and then left at that temperature for at least 10 min. The mixture was thawed quickly by holding the sample tube in the hand and then diluted 60-fold with reconstitution buffer (20 mM MOPS/Tris (pH 7.0), 0.5 mM dithiothreitol, and 0.15 M sodium acetate). Reconstituted proteoliposomes were pelleted by centrifugation at $200{,}000 \times g$ for 1 h at 4 °C and then suspended in 0.2 ml of reconstitution buffer. The resultant proteoliposomes were further subjected to sucrose density gradient centrifugation at $200{,}000 \times g$ for 1 h for estimating the amount of VNUT aggregates. Approximately 76.5% of VNUT was recovered in the liposome fraction, and the remaining protein (23.5%) was pelleted as VNUT aggregates.

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 it became clear, divided into small aliquots, and then stored at −80 °C until used.

Transport Assays—Assays were carried out by the gel permeation procedure as described previously (2, 5–8). Proteoliposomes containing VNUT (0.4 μg of protein/assay) were suspended in 20 mM MOPS/Tris (pH 7.0), 4 mM KCl, 0.15 M potassium acetate, and 100 μM ATP, followed by incubation for 2 min at 27 °C. Radiolabeled substrate Ca^{45} (3 GBq/mmol) was then added to a final concentration of 1 mM, and the mixture was incubated for an additional 2 min. Valinomycin was added to a final concentration of 2 μM to initiate the reaction. Aliquots (130 μl) were taken at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at $760 \times g$ for 2 min. The radioactivity of the eluate was measured. For magnesium transport, proteoliposomes containing VNUT (0.4 μg of protein/assay) were suspended in 20 mM MOPS/Tris (pH 7.0), 4 mM KCl, 0.15 M potassium acetate, and 100 μM ATP, followed by incubation for 2 min at 27 °C. Magnesium acetate was then added to a final concentration of 1 mM, and the mixture was incubated for an additional 2 min. Valinomycin was added to a final concentration of 2 μM to initiate the assay. Aliquots (130 μl) were taken at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at $760 \times g$ for 2 min. The amount of magnesium in the eluate was determined by atomic absorption spectroscopy. For ATP transport, proteoliposomes containing VNUT (0.4 μg of protein/assay) were suspended in 20 mM MOPS/Tris (pH 7.0), 4 mM KCl, 0.15 M potassium acetate, and 1 mM magnesium acetate, followed by incubation for 2 min at 27 °C. Radiolabeled substrate [α-^{32}P]ATP (3.7 GBq/mmol) was then added to a final concentration of 100 μM, and the mixture was incubated for an additional 2 min. Valinomycin was added to a final concentration of 2 μM to initiate the assay. Aliquots (130 μl) were taken at the times indicated and centrifuged through a Sephadex G-50 (fine) spin column at $760 \times g$ for 2 min. The radioactivity of the eluate was measured.

Purification, Reconstitution, and Transport Assay of NPT1, Vesicular Excitatory Amino Acid Transporter (VEAT), and VGLUT2—Mouse NPT1 (Na$^+$/P$_i$ cotransporter 1), mouse VEAT, and rat VGLUT2 (vesicular glutamate transporter 2) were purified and reconstituted as described previously (5–8).

Cell Culture and RNAi—PC12 cells were cultured as described (2, 14). HiPerFect transfection reagent (Qiagen) was used for transfection of 25 nM AllStars negative control siRNA or rat SLC17A9 siRNA (UAUUCGAGAGAAUGUCACG). Isolation of the secretory granule fraction was performed as described 3 days later (14).

Isolation of Secretory Granule Fraction from PC12 Cells—PC12 cells were harvested and disrupted by homogenization. The particle fractions were fractionated by sucrose density gradient centrifugation (14). Enrichment of the secretory granules in the fraction was 5–10-fold greater compared with the postnuclear supernatant, which was verified by Western blot analysis with anti-VNUT and anti-synaptotagmin antibodies.

Atomic Absorption Spectrometry—Quantitative analysis of magnesium was performed using a graphite furnace atomic absorption spectrophotometer (Shimadzu AA-6200) equipped with an L733-202NU hollow cathode lamp and WizAArd software. The absorbance was monitored at 285.2 nm.

Protein Concentration Determination—Bovine serum albumin was used as the standard (15).

Data Analysis—All numerical values are shown as the mean ± S.E. ($n = 3–6$). Statistical significance was analyzed by Student's unpaired t test. The concentrations of the ionic species of nucleotides and metal complexes were calculated according to Fabiato and Fabiato (16).

RESULTS

VNUT-mediated Mg^{2+} Uptake—Upon the addition of valinomycin to VNUT-containing proteoliposomes in a reaction mixture containing potassium acetate, ~90 mV $\Delta\psi$ (positive-inside) was established and triggered ATP uptake in the presence of Mg^{2+} (2). As the first step of the study, we examined whether or not proteoliposomes take up Mg^{2+} under the assay conditions used. Atomic absorption spectroscopy revealed that valinomycin treatment stimulated the uptake of Mg^{2+} by proteoliposomes containing purified VNUT (Fig. 1). Mg^{2+} uptake was significantly reduced when either VNUT or ATP was omitted from the assay mixture. These results indicate that VNUT transports Mg^{2+} in the presence of positive-inside $\Delta\psi$ and ATP.

VNUT-mediated Ca^{2+} Uptake—To study the mechanism in the VNUT-mediated uptake of divalent cations more precisely, we measured the uptake of radiolabeled Ca^{2+}. Upon the addition of valinomycin, inside-positive $\Delta\psi$ (88.0 ± 0.10 mV, $n = 3$) was established through electrogenic entry of K^+, and Ca^{2+} uptake by VNUT-containing proteoliposomes was facilitated (Fig. 2). As in the case of Mg^{2+} uptake, valinomycin, VNUT, and ATP were necessary for the full uptake. Only background level uptake of Ca^{2+} was observed when either valinomycin or ATP was omitted. Liposomes without VNUT did not take up radiolabeled Ca^{2+} even in the presence of ATP and $\Delta\psi$. With 1 mM Ca^{2+} and 100 μM ATP, the $\Delta\psi$-dependent uptake of Ca^{2+} and ATP was 5.3 and 6.8 nmol/min/mg, respectively (Fig. 2*B*),

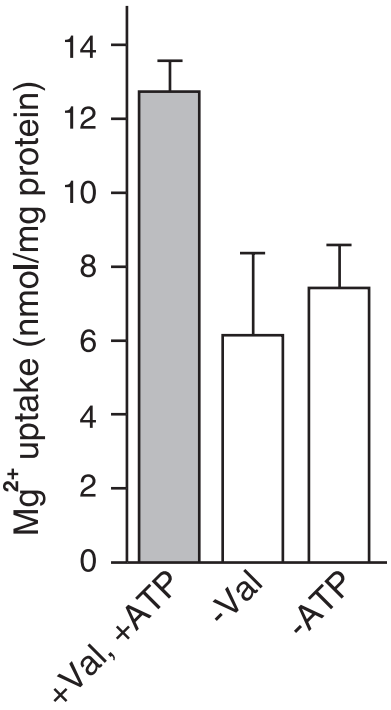

FIGURE 1. **Mg^{2+} uptake by VNUT-containing proteoliposomes as assessed by atomic absorption spectrometry.** Purified VNUT and reconstituted VNUT were suspended in a reaction mixture containing 100 μM ATP and 1 mM magnesium acetate. Uptake was measured at 2 min after the addition of valinomycin (*Val*). Mg^{2+} uptake was expressed after subtraction of the value for liposomes without VNUT. *Error bars* represent means ± S.D. ($n = 3–5$).

indicating that the stoichiometry of Ca^{2+} and ATP uptake was close to 1.

We then examined whether or not other SLC17 type I phosphate transporter family members possess Ca^{2+} uptake ability. As shown in Fig. 3, $\Delta\psi$-dependent Ca^{2+} uptake was not observed with proteoliposomes containing other purified SLC17 family members, even in the presence of ATP or their own transport substrates. These results indicate that Ca^{2+} uptake is a particular property of VNUT.

We further characterized the VNUT-mediated Ca^{2+} transport. Detailed bioenergetics under a defined $\Delta\psi$ and/or ΔpH indicated that inside-positive $\Delta\psi$ but not ΔpH primarily triggered Ca^{2+} uptake (Table 1). We next assessed the effects of Cl^- on valinomycin- and ATP-mediated Ca^{2+} uptake because VNUT-mediated ATP uptake absolutely requires Cl^- (2). As shown in Fig. 4, background level Ca^{2+} uptake was observed in the absence of Cl^-; the uptake increased upon the addition of Cl^-; and the full activity was observed with >4 mM Cl^-, indicating that Cl^- is also essential for the VNUT-mediated Ca^{2+} transport. As to inhibitor sensitivity, we found that Evans blue at 1 μM and DIDS at 10 μM, potent inhibitors of VNUT, also completely inhibited VNUT-mediated Ca^{2+} uptake. In contrast, ruthenium red, an inhibitor of the Ca^{2+}/H^+ exchanger, and thapsigargin, an inhibitor of Ca^{2+}-ATPase, were both only slightly effective, and both inhibited by 25% (Fig. 5) (17, 18). Thus, the properties of VNUT-mediated Ca^{2+} uptake are consistent with those of VNUT-mediated ATP uptake regarding energetics, requirement of Cl^-, and sensitivities to inhibitors.

In addition, we examined the effects of various divalent and trivalent cations on valinomycin- and ATP-mediated Ca^{2+} uptake

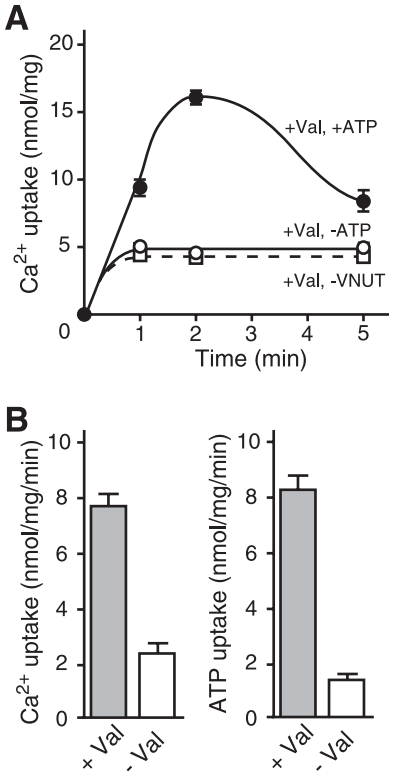

FIGURE 2. **VNUT is responsible for radioactive Ca^{2+} uptake by reconstituted proteoliposomes.** *A*, time course. The assay mixture was incubated with radioactive Ca^{2+} at a final concentration of 1 mM in the presence (●) or absence (○) of 100 μM ATP. The reaction was started by the addition of valinomycin (*Val*). Ca^{2+} uptake by liposomes lacking VNUT is also shown (□). *B*, stoichiometry of Ca^{2+} and ATP uptake. Ca^{2+} and ATP uptake was measured in the presence of 100 μM ATP and 1 mM calcium acetate. *Error bars* represent means ± S.D. (n = 3–5).

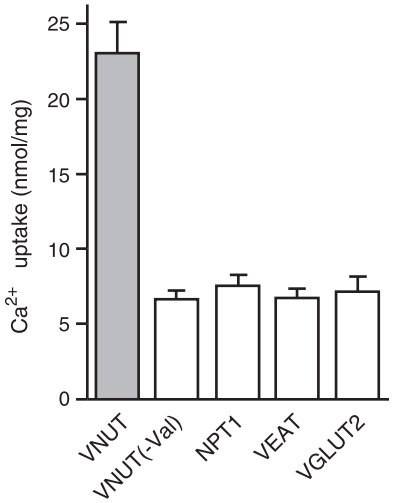

FIGURE 3. **Other SLC17 family members do not exhibit Ca^{2+} uptake activity.** NPT1, VEAT, and VGLUT2 were purified and reconstituted into liposomes. The valinomycin-evoked Ca^{2+} uptake at 2 min was examined in the presence of 100 μM p-aminohippuric acid for NPT1, 100 μM aspartate for VEAT, and 100 μM glutamate for VGLUT2 and valinomycin (*Val*). The ATP-dependent Ca^{2+} uptake by VNUT at 2 min is also shown as a positive control. *Error bars* represent means ± S.D. (n = 3–5).

(Fig. 6*A*). Among the cations tested, Mg^{2+} and La^{3+} strongly inhibited Ca^{2+} uptake, with 50% inhibition at 0.42 and 0.62 mM, respectively (Fig. 6*B*). Zn^{2+} also potently inhibited Ca^{2+} uptake, whereas other divalent and trivalent cations were less effective.

TABLE 1

Energetics of VNUT-mediated Ca^{2+} uptake

Proteoliposomes containing Na+ or K^+ were prepared and incubated in buffer containing K^+ or Na^+ as indicated. Ca^{2+} uptake was measured at 2 min after the addition of valinomycin (2 μM) or nigericin (2 μM). For some experiments, proteoliposomes were prepared at pH 5.5 and incubated in buffer at either pH 7.0 or 5.5. Ca^{2+} uptake was then assayed after 1 min. Uptake is expressed as relative activity. Control activity (100%) corresponds to 11.7 ± 1.1 nmol/mg/min. Values represent means ± S.D. (n = 3–5).

Inside	Outside	Ionophores	Inside pH	Outside pH	Ca^{2+} uptake
					%
Na^+	K^+	None	7.0	7.0	21.9 ± 3.5
		Valinomycin			100.0 ± 4.6
		Nigericin			17.5 ± 3.9
Na^+	Na^+	None	7.0	7.0	15.4 ± 3.0
		Valinomycin			17.1 ± 4.1
		Nigericin			21.5 ± 4.9
K^+	K^+	None	7.0	7.0	15.7 ± 3.6
		Valinomycin			16.6 ± 4.6
		Nigericin			20.2 ± 4.2
K^+	Na^+	None	7.0	7.0	19.6 ± 5.2
		Valinomycin			21.1 ± 5.3
		Nigericin			18.5 ± 5.4
K^+	K^+	None	7.0	7.0	15.7 ± 3.6
		None	5.5	7.0	16.6 ± 4.6

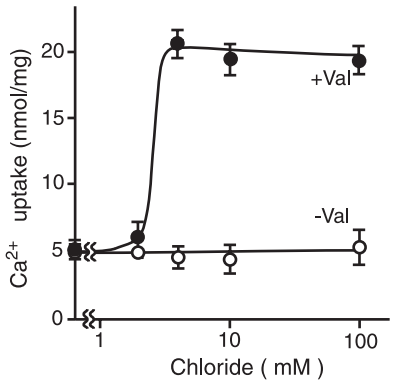

FIGURE 4. **Effect of Cl^- on Ca^{2+} uptake.** Ca^{2+} uptake was assayed in the presence of different concentrations of Cl^- in the presence (●) or absence (○) of valinomycin (*Val*). *Error bars* represent means ± S.D. (n = 3–5).

Effects of Mutations on ATP and Ca^{2+} Uptake—To examine the relationship between VNUT-mediated ATP uptake and Ca^{2+} uptake in more detail, we measured these activities of VNUT mutants. We mutated the charged amino acid residues in the transmembrane regions (Arg-35, Arg-119, and Gln-126) to alanine, which correspond to important amino acid residues of NPT1 and VGLUT2, members of the SLC17 type I phosphate transporter family. The VNUT mutants were expressed in the insect cells and then purified to near homogeneity (Fig. 7, *left panel*, *inset*), and their Ca^{2+} and ATP transport activities were measured after reconstitution. This protocol enabled us to determine the effects of mutations on the transport activity quantitatively. Arg-119 is an essential amino acid residue for all other SLC17 family members. As expected, R119A VNUT lost both transport activities almost completely. Arg-35 and Gln-126 are located near the substrate-binding region for VGLUT2 and NPT1, and alanine substitution of them caused a loss of the transport activity accordingly. Likewise, the R35A and Q126A mutants exhibited decreased ATP transport by 44.5 and 40.0%, respectively. We also found that these mutants lost $\Delta\psi$-dependent Ca^{2+} uptake ability in the same order and to the same

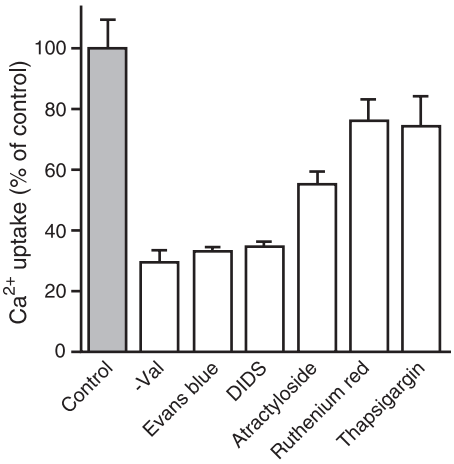

FIGURE 5. **Effects of inhibitors of VGLUT and various Ca^{2+}-transporting proteins on Ca^{2+} uptake.** The additions were 1 μM Evans blue, 10 μM DIDS, 200 μM atractyloside, 100 μM ruthenium red, and 200 μM thapsigargin. Ca^{2+} uptake was measured at 2 min after the addition of valinomycin (*Val*). Control activity (100%) corresponds to 20.7 ± 1.8 nmol/mg. *Error bars* represent means ± S.D. (n = 3–5).

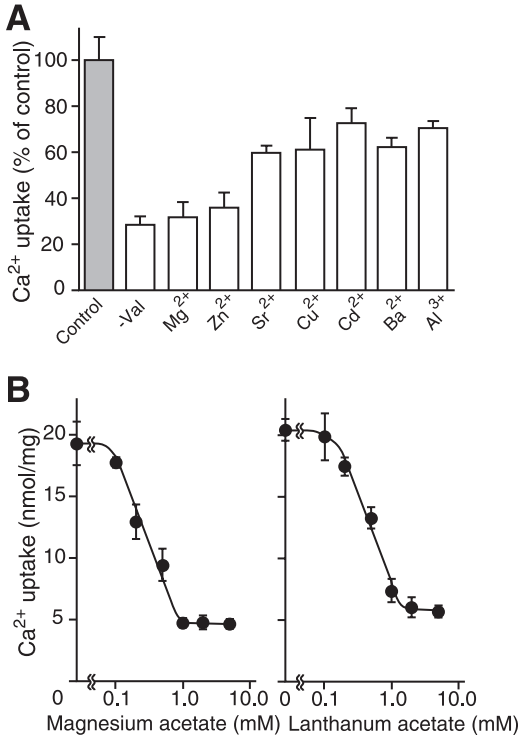

FIGURE 6. **Effects of divalent cations on Ca^{2+} uptake.** *A*, Ca^{2+} uptake at 2 min was measured in the presence of various cations at 1 mM. Control activity (100%) corresponds to 21.8 ± 2.4 nmol/mg. *B*, Ca^{2+} uptake was assayed in the presence of different concentrations of magnesium or lanthanum. Ca^{2+} uptake was measured at 2 min after the addition of valinomycin (*Val*). *Error bars* represent means ± S.D. (n = 3–5).

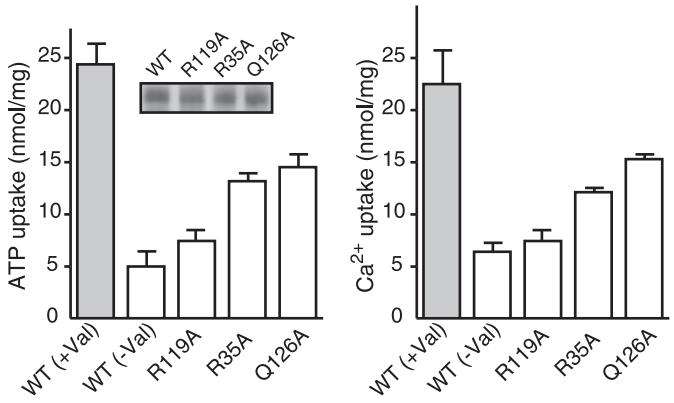

FIGURE 7. **Effects of mutations on ATP and Ca^{2+} uptake.** The VNUT mutants were purified, and ATP uptake (*left panel*) and Ca^{2+} uptake (*right panel*) were measured at 2 min after the addition of valinomycin (*Val*). Coomassie Brilliant Blue staining of the purified wild-type and mutant VNUT-containing samples is shown in the *inset*. *Error bars* represent means ± S.D. (n = 3–5).

degree (Fig. 7). These results further support that VNUT-mediated ATP and Ca^{2+} transport is tightly coupled.

Effects of Divalent Cations on Kinetics of Nucleotide Uptake—Subsequently, we measured the effects of Ca^{2+} and Mg^{2+} on the ATP and ADP uptake kinetics to evaluate the mode of substrate recognition. The results so far obtained supported the idea that VNUT recognizes both free nucleotides and their chelating complexes as transport substrates. Because they have different total charges and structures, modification of the K_m value by divalent metal ions is expected. As shown in Fig. 8, free ATP was transported with K_m and V_{max} values of 0.61 mM and 94 nmol/min/mg of protein, respectively. In the presence of excess Ca^{2+} and/or Mg^{2+}, however, ATP transport showed a similar K_m with a slightly lower V_{max}. Under these assay conditions, >90% of ATP formed an Mg-ATP complex, and <10% of ATP was present as the free nucleotide. Thus, these results further support the idea that VNUT can recognize divalent ion complexes of nucleotides. In the case of ADP as a substrate, the same was true. In the presence of Ca^{2+} and/or Mg^{2+}, ADP was transported with slightly lower K_m and V_{max} values compared with free ADP. AMP was not transported in the presence or absence of Ca^{2+} and Mg^{2+}. Thus, the mode of substrate recognition by VNUT is more complicated than expected (see "Discussion").

Effects of RNAi of VNUT Gene Expression on Vesicular ATP and Mg^{2+} Concentrations in PC12 Cells—Finally, we assessed whether suppression of VNUT gene expression affects the vesicular Mg^{2+} concentration in PC12 cells. As shown previously (2), RNAi suppressed ~53% of VNUT gene expression and inhibited ~50% of ATP exocytosis. Accordingly, the amount of VNUT protein was reduced to 61% by RNAi. The secretory granules were isolated by sucrose density gradient centrifugation, and vesicular ATP and Mg^{2+} contents were quantified: 140.8 ± 1.6 and 21.0 ± 0.3 nmol/mg, respectively, for negative control siRNA-treated cells. When cells were treated with VNUT siRNA, vesicular ATP and Mg^{2+} contents were reduced to 114.1 ± 1.3 and 14.2 ± 1.7 nmol/mg, respectively. The significance of the siRNA-dependent decrease was $p < 0.005$ for both ATP and Mg^{2+}. These results suggest that VNUT-mediated divalent cation uptake functions in PC12 cells.

DISCUSSION

In this study, we characterized the transport properties of VNUT purified and reconstituted into proteoliposomes with special reference to divalent cation transport. We found that the proteoliposomes took up Mg^{2+} and Ca^{2+} during ATP transport. The transport requires VNUT, ATP, and Cl^-; is

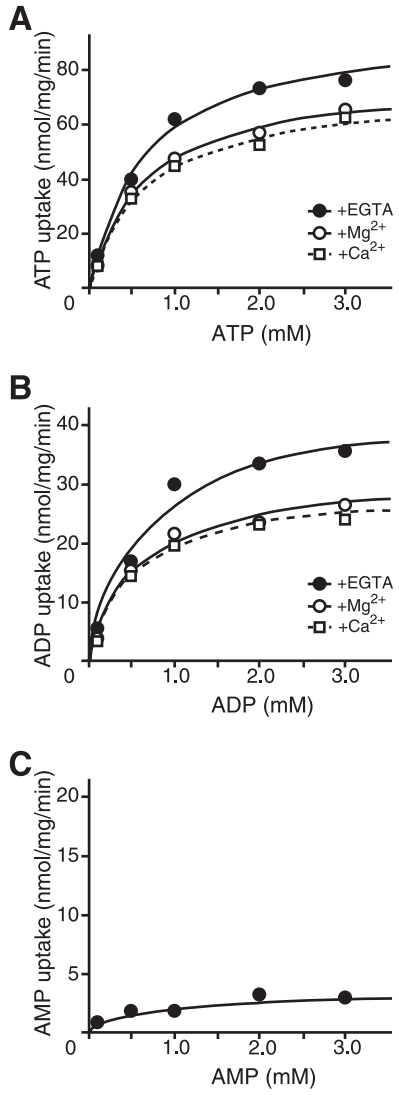

FIGURE 8. **Effects of divalent cations on kinetics of nucleotide uptake.** The dose dependence of nucleotide uptake in the presence of 5 mM magnesium acetate (○), 5 mM calcium acetate (□), or 1 mM EGTA (●) was measured at 1 min after the addition of valinomycin. The nucleotide transport activities after background subtraction are shown.

inhibited by inhibitors; and exhibits essentially the same properties as those of VNUT-mediated ATP uptake, leading to the conclusion that VNUT transports divalent cations during ATP transport.

Subsequently, we prepared VNUT harboring mutations by site-directed mutagenesis. The previous mutational study on VGLUT2 in our laboratory revealed that His-128, Arg-184, and Glu-191 in the transmembrane region are essential and involved in substrate binding (5). Among them, Arg-184 seems to be the most important because this residue is conserved in all SLC17 family members. The importance of this residue was also confirmed by mutagenic studies on NPT1 (7) and VEAT.[4] In this study, we found that Arg-119, corresponding to Arg-184 of VGLUT2, is essential for ATP uptake. In contrast, replacement of Arg-35 and Gln-126 had only moderately effects on ATP transport. The full conservation and indispensability of Arg-119 indicate that VNUT has a transport mechanism similar to those of other SLC17 family transporters. We assume that Arg-119 is involved in the common role(s) of all SLC17 transporters such as Cl^- activation, voltage-driven transport, and anionic substrate recognition. We should emphasize that Ca^{2+} uptake by these mutants exhibits the same tendency as ATP uptake, further supporting that Ca^{2+} and ATP are simultaneously transported via VNUT through the same mechanism.

VNUT seems to be unique regarding substrate recognition. In the absence of divalent cations, most ATP is in the ATP^{4-}, ATP^{3-}, and ATP^{-}-K^+ forms, and in the presence of excess divalent cations, ATP should be present as the Mg^{2+}-ATP^{4-} form in a chelating complex. Stoichiometric transport of ATP and divalent cations suggests that VNUT transports ATP as a divalent cation complex. This study and VNUT-mediated ATP uptake in the presence of EGTA indicated that VNUT can transport multivalent anionic forms of ATP. In this respect, it is noteworthy that SLC17 transporters other than VNUT behave as $\Delta\psi$-dependent monovalent anion transporters. In addition, different total charges of substrates in the presence or absence of divalent cation suggest that VNUT can recognize various ionic species of ATP.

Another unique aspect of VNUT regarding substrate recognition is the effect of Mg^{2+} (or Ca^{2+}). Our previous study indicated that the nucleobase is not a critical part for substrate recognition by VNUT because ATP transport is inhibited by various nucleotides such as GTP, ITP, and UTP (2). The weak AMP inhibition and low AMP uptake suggest that the primary substrate recognition site is located near the pyrophosphate moiety of the nucleotide. Mg^{2+} complex formation at the pyrophosphate moiety of ATP changes the shape and charge distribution of the pyrophosphate moiety. Thus, different substrate affinity is expected in the absence and presence of Mg^{2+}. We found, however, that the K_m values for ATP uptake in the absence and presence of excess divalent metal ions were not greatly different, suggesting that VNUT recognizes free ATP and the Mg^{2+}-ATP^{4-} complex with a similar apparent affinity even though K_m can be affected by the transport rate constant and is not equal to the substrate affinity. In this respect, it is also noteworthy that other ATP transporters can distinguish free ATP and the Mg-ATP complex: the ATP/ADP exchanger uses free ATP as a substrate, and the Mg-ATP/P_i transporter requires Mg-ATP (19, 20). Ion-transporting ATPases also discriminate between free ATP and its metal complex. In the case of F_1-ATPase, the enzyme exhibits 1000-fold higher affinity for Mg-ATP than for free ATP, indicating the critical role of Mg^{2+} in ATP binding (21). Thus, VNUT possesses kinetic properties that are quite different from those of other ATP-binding proteins. The details of such a unique nucleotide-binding mode of VNUT can be solved by future structural analysis and will provide new insights into nucleotide recognition.

Finally, our results provide new insights into the mechanism of divalent cation storage in various ATP-containing secretory vesicles. Because the cytoplasmic concentration of free Mg^{2+} is in the approximately millimolar range, the major fraction of ATP transported by VNUT is expected to be Mg-ATP. Thus, under physiological conditions, approximately equimolar concentrations of Mg^{2+} and ATP are transported by VNUT into

[4] T. Miyaji, H. Omote, and Y. Moriyama, manuscript in preparation.

secretory granules. Suppression of VNUT gene expression reduced the vesicular content of both ATP and Mg^{2+} to a similar extent, strongly suggesting the involvement of VNUT in Mg^{2+} accumulation in secretory granules. In chromaffin granules, ~0.1 M ATP and only <10% Mg^{2+} are present (9, 10). Thus, Mg-ATP should be dissociated within the granules and somehow released from the granules through an unidentified pathway. The internal pH is maintained acidic-inside by vacuolar ATPase. Mg-ATP exhibits a lower stability constant for trivalent ATP, which is the major form under acidic luminal pH conditions (22).

In contrast to Mg^{2+}, various molecular machineries have been reported to explain the mechanism responsible for Ca^{2+} storage, including Mg^{2+}- and Ca^{2+}-transporting ATPases and the Ca^{2+}/Na^{+} and Ca^{2+}/H^{+} antiporters (11, 13). We demonstrated that VNUT transports Ca^{2+}. However, the free Ca^{2+} concentration is less than micromolar under physiological conditions, which is far lower than the free Mg^{2+} concentration, ~0.5 mM (23, 24). Thus, the major fraction of intracellular ATP is present as a chelating complex with Mg^{2+}, but not with Ca^{2+}, and the Ca-ATP complex should account for <0.1% of the ATP species in cells (25). Such a small fraction of Ca-ATP cannot explain the high accumulation of Ca^{2+} in secretory granules. The participation of VNUT and other transporters in the vesicular concentrations of Mg^{2+} and Ca^{2+} can be estimated with the use of VNUT knock-out mice. An experiment along these lines is in progress in our laboratory. In conclusion, VNUT transports Ca^{2+} and Mg^{2+} probably as chelating complexes with ATP. VNUT may act as an Mg^{2+} importer in ATP-containing secretory granules.

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