Mutational Analysis of Aspartate Residues in the Transmembrane Regions and Cytoplasmic Loops of Rat Vesicular Acetylcholine Transporter*

Myung-Hee Kim‡, Mei Lu‡, Eun-Jeong Lim§, Young-Gyu Chai§, and Louis B. Hersh‡¶

From the ‡Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536 and the §Department of Biochemistry, Hanyang University, Ansan 425-791, Korea

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The vesicular acetylcholine transporter (VACHT) is responsible for the transport of the neurotransmitter acetylcholine (ACh) into synaptic vesicles using an electrochemical gradient to drive transport. Rat VACHT has a number of aspartate residues within its predicted transmembrane domains (TM) and cytoplasmic loops, which may play important structural or functional roles in acetylcholine transport. In order to identify functional charged residues, site-directed mutagenesis of rVACHT was undertaken. No effect on ACh transport was observed when any of the five aspartate residues in the cytoplasmic loop were converted to asparagine. Similarly, changing Asp-46 (D46N) in TM1 or Asp-255 (D255N) in TM6 had no effect on ACh transport or vesamicol binding. However, replacement of Asp-398 in TM10 with Asn completely eliminated both ACh transport and vesamicol binding. The conservative mutant D398E retained transport activity, but not vesamicol binding, suggesting this residue is critical for transport. Mutation of Asp-193 in TM4 did not affect ACh transport activity; however, vesamicol binding was dramatically reduced. With mutant D425N of TM11 transport activity for ACh was completely blocked, without an effect on vesamicol binding. Activity was not restored in the conservative mutant D425E, suggesting the side chain as well as the negative charge of Asp-425 is important for substrate binding. These mutants, as well as mutant D193N, clearly dissociated ACh binding and transport from vesamicol binding. These data suggest that Asp-398 in TM10 and Asp-425 in TM11 are important for ACh binding and transport, while Asp-193 and Asp-398 in TM4 and TM10, respectively, are involved in vesamicol binding.

The neurotransmitter acetylcholine (ACh) is synthesized from choline and acetyl-coenzyme A within the cytoplasm of cholinergic nerve terminals in a reaction catalyzed by the enzyme choline acetyltransferase (ChAT). ACh is then transported into synaptic vesicles by a vesicular acetylcholine transporter (VACHT) (1, 2). Transport activity depends on a proton electrochemical gradient generated by a vacuolar type H⁺-ATPase with the exchange of two luminal protons for one cytosolic acetylcholine (1, 3). VACHT is localized to small synaptic vesicles in central cholinergic neurons (4), in PC12 cells (5, 6), and in PC12 cells overexpressing reconstituent human VACHT (7). VACHT appears to be targeted to the small synaptic vesicle via a constitutive vesicle independent pathway, perhaps through the large dense core vesicle from which recruitment to the early endosome occurs (8).

The quaternary cycloalkylpiperidine vesamicol acts as a specific mixed non-competitive, high affinity inhibitor of ACh vesicular transport. Vesamicol inhibits ACh transport with an inhibition constant (Kᵢ) similar to its dissociation constant (Kᵢ), presumably binding to an allosteric site on the transporter (9–12). Vesamicol binding is inhibited by ACh at concentrations ~10 times greater than the Kᵢ for ACh uptake in lysed vesicles (13).

cDNA clones that encode VACHT have been isolated from the nematode Caenorhabditis elegans (14), the electric marine ray Torpedo (15), rat, and human (16–18). Rat VACHT is a 530-amino acid glycoprotein with an apparent molecular mass of 68–70 kDa. Based on hydrophobic moment analysis of the deduced primary amino acid sequence, it appears that VACHT has 12 transmembrane domains and 2 potential N-linked glycosylation sites. Both glycosylation sites are found within the luminal loop between the first and second putative transmembrane domain (16, 17). The sequence of VACHT cDNAs and their predicted structure indicate that VACHT is the two vesicular monoamine transporters VMAT1 and VMAT2, which are responsible for biogenic amine accumulation (19, 20), constitute a new transporter gene family.

Sequence conservation of charged and polar amino acids within the putative hydrophobic transmembrane domains of VACHT from different species suggests functional importance. This could reflect their involvement in maintaining the native conformation of the transporter, an involvement in substrate binding, or a role in H⁺/antiporter activity. Thus, aspartate residues within putative transmembrane domains (TMs) 1, 6, 10, and 11 are conserved between all species of VACHT and both forms of VMAT (14–20). At least three of these conserved aspartates, those in TMs 1, 10 and 11, have been shown to be critical for amine transport in VMAT1 and VMAT2 (21–23), and the conserved aspartate in TM10 of VACHT has been implicated in ACh transport (24). An aspartate residue in TM4, which is found in all species of VACHT, but is absent in VMATs, has been reported as a critical residue for ACh transport (24). A proposed charge-pair interaction between a lysine in TM2 and aspartate in TM11, both conserved in all amine transporters and VACHT, has been demonstrated to be crucial for substrate recognition in VMAT2 (23).
Thus, although transmembrane aspartate residues have been shown to be important for VMATs, these residues have not been systematically studied in VACHT. In this study, we have used a mutant PC12 cell line, which is devoid of endogenous VACHT, but contains the small synaptic vesicles to which VACHT is normally targeted, to study the function of conserved aspartate residues in the cytoplasmic loops and in the hydrophobic transmembrane domains of rat VACHT. The results of this study led to the identification of aspartate residues that are crucial for ACh transport and/or vesamilo binding.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—For these studies, we utilized a mutant PC12 cell line, PC12Δ23,7, which was generated by transfection of PC12 cells with a mutant regulatory subunit of protein kinase A (25). PC12Δ23,7 is deficient in both type I and type II CAMP-dependent protein kinase activity (26) and has been found to express negligible levels of both ChAT (27, 28) and VACHT (28).

**Mutagenesis**—Wild-type rat VACHT was subcloned into the EcoRI and XbaI site of pBluescript SK+ (Invitrogen). Site-directed mutagenesis was performed by the method of Kunkel et al. (29) using the BD2399 strain of *Escherichia coli* to prepare a single-stranded uracil-containing DNA. Mutagenic oligonucleotides, which contain a single base pair change that introduces a wild-type VACHT, were annealed with the parental single-stranded DNA and extended to synthesize the complementary strand. The product was transformed into the JM109 strain of *E. coli* to remove uracil-containing parental DNA. To verify that only the desired mutation was introduced, the mutated portion was sequenced by the dideoxy method (30). The intact VACHT was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). 

**Transient Expression**—PC12Δ23,7 cells were maintained in 10% CO2 at 37 °C in a 1:1 mixture of complete Dulbecco’s modified Eagle’s medium: F-12 Ham’s nutrient mixture containing 10% fetal bovine serum, 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Wild-type and mutant rVACHT cDNAs were transfected into PC12Δ23,7 cells by electroporation. For electroporation, cells were detached from plates with trypsin/EDTA, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in 800 μl of cold PBS at a concentration of ~6 × 106 cells/ml. The resuspended cells were mixed with 50 μg of DNA. After a 10-min incubation on ice, the cell-DNA mixture was transferred to a 0.4-cm gap cuvette (Bio-Rad), electroporated (0.2 kV, 975 microfarads), then replated in Dulbecco’s modified medium:F-12 Ham’s nutrient mixture containing 10% fetal bovine serum, 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Wild-type and mutant rVACHT cDNAs were transfected into PC12Δ23,7 cells by electroporation. For electroporation, cells were detached from plates with trypsin/EDTA, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in 800 μl of cold PBS at a concentration of ~6 × 106 cells/ml. The resuspended cells were mixed with 50 μg of DNA. After a 10-min incubation on ice, the cell-DNA mixture was transferred to a 0.4-cm gap cuvette (Bio-Rad), electroporated (0.2 kV, 975 microfarads), then replated in Dulbecco’s modified Eagle’s medium: F-12 Ham’s nutrient mixture containing 10% fetal bovine serum, 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The transfection mixture was transferred to a 0.4-cm gap cuvette (Bio-Rad), electroporated (0.2 kV, 975 microfarads), then replated in Dulbecco’s modified Eagle’s medium: F-12 Ham’s nutrient mixture containing 10% fetal bovine serum, 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The suspension was incubated for 10 min at 37 °C.

**Cell Fractionation (Linear Sucrose Gradient Centrifugation)**—50 μl of postnuclear supernatant was loaded onto a 10–ml linear sucrose gradient from 0.6 to 1.6 Sucrose in 10 ml HEPES (pH 7.4) and centrifuged at 30,000 rpm for 6 h in an SW40 rotor in a Beckman LE-80 centrifuge at 4 °C. Fractions of 500 μl were collected from the bottom of the tube and stored at −80 °C until use.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—For the detection of mutant protein expression, 5 μl of postnuclear supernatant (50 μg of total protein) from each mutant was diluted to 500 μl with homogenization buffer and then pelleted by centrifugation at 50,000 rpm for 3 h in a TLA-100–3 rotor using a Beckman TL-100 ultracentrifuge. The pellet was resuspended in 20 μl of 1× SDS sample buffer (62 μg Tris-HCl, pH 6.8, 1 ml EDTA, 10% glycerol, 5% SDS, and 5% ß-mercaptoethanol) and separated on a 10% SDS-polyacrylamide gel by electrophoresis. Separated proteins were transferred to an Immobilon P membrane (Millipore) using a semidry electrotransfer apparatus (Altitech). The blot was blocked with Tris-buffered saline containing 10% nonfat dry milk and 0.1% Tween 20 for 1 h at room temperature. The blot was sequentially incubated with primary antibody (goat anti-rVACHT antibody; Chemicon International Co.), then with secondary antibody conjugated to peroxidase for 1 h each at room temperature. Between each reaction, the blot was washed three times with Tris-buffered saline and 0.1% Tween 20 for 10 min. The immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

The same fractions from sucrose gradients were immunoblotted as described above using either a monoclonal antibody against synaptophysin (Boehringer Mannheim) or a rabbit antiserum against secretogranin II. The latter antiserum was a generous gift from Dr. Jonathan Scammell, University of Alabama.

**RESULTS**

**Mutation of Aspartate Residues in the Cytoplasmic Loops**—Based on studies with other transporters, evidence has been obtained that negatively charged residues on the cytoplasmic membrane surface may play a role in substrate-protein interaction at the beginning or end of the transport cycle. We thus used asparagine scanning mutagenesis to investigate the importance of conserved aspartate residues found on cytoplasmic loops of rVACHT. There are two aspartate residues, Asp-147 and Asp-152, in the cytoplasmic loop between TM2 and TM3 of rVACHT. Asp-147 is conserved in all species of VACHT but is absent in both VMAT1 and VMAT2. On the other hand, Asp-152 is not conserved in all species of VACHT, it is absent in *C. elegans* VACHT, and it is absent in VMATs (Fig. 1). As shown in Table I, when these two aspartate residues were substituted by asparagine, there was no significant difference in the ability of transfected medium to transport ACh.

There are three other aspartate residues found in cytoplasmic loops. These include Asp-202, which is located in the cytoplasmic loop between TM4 and TM5 and conserved in all VACHTs sequenced to date but absent in VMATs; and Asp-288, between TM6 and TM7, and Asp-410, between TM10 and TM11, which are conserved in all VACHT species and in VMATs (Fig. 1). Replacement of these residues with Asn also had no effect on acetylcholine transport (Table 1). It should be noted that Asp-202 could reside within a transmembrane domain. It is located at the boundary between a hydrophobic
transmembrane segment and a hydrophilic cytoplasmic loop segment.

Thus, none of the five aspartate residues in cytoplasmic loops between transmembrane domains that, with the exception of Asp-152, which in C. elegans VACHT is a Glu residue, are conserved among VACHT species play a significant role in either ACh binding or ACh transport.

Aspartate Residues in the Hydrophobic Transmembrane Domains—VACHT has 12 transmembrane domains spanning the vesicular membrane. Because of the hydrophobic environment in these transmembrane regions, few charged amino acid residues are present. Those that are found in the transmembrane domain may play an important role in the transport process. We thus applied asparagine scanning mutagenesis to test the functionality of transmembrane aspartate residues.

Asp-46 in TM1 and Asp-255 in TM6—Asp-46 in TM1 and Asp-255 in TM6, are conserved in all VACHTs and in VMATs (Fig. 1B). These residues appear to be located in the middle of the putative transmembrane domains in VACHT (Fig. 1A). The aspartic acid residue in TM1 of VMAT2 plays a critical role in substrate recognition (22), while mutation of the Asp residue in TM6 of VMAT2, although not eliminating serotonin transport, produced a consistent lower rate of transport (23). To elucidate the role of these residues in the transport of acetylcholine, the mutants D46N and D255N were generated. As shown in Fig. 2, removal of the negative charge from these residues has no effect on the transport of acetylcholine or on vesamicol binding. As shown in Fig. 3, each mutant expressed to the same extent as the wild-type VACHT. Furthermore, these mutants produce the same maximal accumulation of ACh as did wild-type VACHT (Fig. 4). Thus, these charged residues do not have a critical role in substrate transport or vesicle targeting.

Asp-193 in TM4, Asp-398 in TM10, and Asp-202—Song et al. (24) mutated Asp-193 (D193N) of TM4 as well as Asp-398 (D398N) in TM10, and found the mutants to be inactive, indicating these residues are essential for ACh transport activity. However, in our hands, cells expressing the D193N mutant of rVACHT transported acetylcholine at the same rate as wild-type transporter (Fig. 2A). As shown in Fig. 3, removal of the negative charge from these residues has no effect on the transport of acetylcholine or on vesamicol binding. As shown in Fig. 5, each mutant expressed to the same extent as the wild-type VACHT. Furthermore, these mutants produce the same maximal accumulation of ACh as did wild-type VACHT (Fig. 4). Thus, these charged residues do not have a critical role in substrate transport or vesicle targeting.

![Figure 1](image)

**TABLE I**

| Mutants | D147 | D152 | D202 | D288 | D410 |
|---------|------|------|------|------|------|
| rat     | 0    | 0    | 0    | 0    | 0    |
| mouse   | 0    | 0    | 0    | 0    | 0    |
| human   | 0    | 0    | 0    | 0    | 0    |
| D. melanogaster | 0 | 0 | 0 | 0 | 0 |
| To      | 0    | 0    | 0    | 0    | 0    |
| unc-17  | 0    | 0    | 0    | 0    | 0    |
| rVMAT1  | 0    | 0    | 0    | 0    | 0    |
| rVMAT2  | 0    | 0    | 0    | 0    | 0    |

![Figure 2](image)

**Figure 2.** A, schematic of rVACHT showing targeted mutation sites. B, sequence homology at each mutant site in VACHT from different species and VMATs. Rat VACHT: GenBank accession no. U09838; mouse VACHT: GenBank accession no. AF019045; human VACHT: GenBank accession no. U10554; Drosophila VACHT; GenBank accession no. AF030197; Torpedo marmorata VACHT (To); GenBank accession no. U05399; C. elegans VACHT (unc-17); GenBank accession no. L19621; rat VMAT1: GenBank accession no. M97380; rat VMAT2: GenBank accession no. L06903.
wild-type VACt. The concentration dependence on acetylcholine ($K_m = 1.8 \pm 0.4 \text{ mM}$, $V_{max} = 108 \pm 16 \text{ pmol/mg}$) as well as the maximal accumulation of ACh by this mutant was identical to wild-type transporter ($K_m = 1.5 \pm 0.3 \text{ mM}$, $V_{max} = 100 \pm 9 \text{ pmol/mg}$) (Fig. 4A). In order to confirm that the transport activity of this mutant is dependent on the H⁺-ATPase, we determined the dependence of the transport assay on Mg-ATP, as well as the effect of the proton ionophore FCCP, or in the specific V-type ATPase inhibitor bafilomycin A1 (31, 32). Since the V-type ATPase is sensitive to low temperature (33, 34), we also examined transport activity at 0 °C. As shown in Fig. 5B, transport activity is completely inhibited in the absence of ATP, by the presence of 2.5 μM FCCP, or by the presence of 1 μM bafilomycin A1. Similarly, no transport was observed at 0 °C.

The D193N mutant exhibited reduced vesamicol binding, while the D398N mutation virtually eliminated vesamicol binding, (Fig. 2B). To confirm this result, the concentration dependence on vesamicol for inhibition of acetylcholine transport was measured. As shown in Fig. 6, with the D193N mutant, vesamicol was much less effective in inhibiting acetylcholine transport, $K_i = 0.3 \text{ mM}$, compared with a $K_i$ of 100 nM for vesamicol inhibition of acetylcholine transport by wild-type transporter. Although not shown, vesamicol had no effect on acetylcholine transport at a concentration of 1 μM with the D398N mutant. In order to evaluate whether the vesamicol binding site might be involved in transporter targeting to synaptic vesicles, the postnuclear supernatants from wild-type transporter and the D193N, D398N, and D425N (see below) mutants were fractionated on a linear sucrose gradient by centrifugation. The result, which is shown in Fig. 7, indicates that both the wild-type transporter and all three mutant transporters co-sedimented with synaptophysin, a marker protein for small synaptic vesicles. In these experiments, the large dense vesicles, identified with goat anti-secretogranin II antiserum, were clearly separated from the small synaptic vesicles. This result indicates that the vesamicol binding site is distinct from the acetylcholine binding site and that its disruption does not affect transporter localization.

Asp-398 in TM10 and Asp-425 in TM11—Asp residues of TM10 and TM11 of VACt1 and VACt2 are critical for substrate recognition and transport (21, 23). The corresponding aspartates, Asp-398 (TM10) and Asp-425 (TM11) are conserved in all VACt species (Fig. 1). Replacement of these residues with asparagine completely abolished transport of acetylcholine (Fig. 2A). The expression level of these mutant proteins was comparable to wild-type VACt (Fig. 3). Fig. 4A shows that at 2 mM ACh, neither D398N nor D425N exhibited detectable ACh transport activity. Based on the sensitivity of the transport assay, this finding means that either the affinity for ACh transport, the $V_{max}$ for transport, or a combination of these parameters must be decreased more than 40-fold for these mutants. To determine whether the loss of transport activity of D398N or D425N results from disruption of transporter localization into the appropriate synaptic vesicles, we determined whether these mutant proteins were localized to the small synaptic vesicle fraction by fractionation of postnuclear supernatants on sucrose gradients. As shown in Fig. 7, both mutant transporters were localized to the same fraction as synaptophysin. Therefore, the loss of transport activity by mutant D398N or D425N is not a consequence of improper
In VMAT2, the functionality of the aspartate residue in TM10 is dependent upon its negative charge, but the functionality of the aspartate residue in TM11 is not (23). In order to determine whether the negative charge on the corresponding aspartates in VAChT are critical, Asp was replaced with Glu. We found that D398E retains significant transport activity (Fig. 8); however, D425E does not. Therefore, as with VMAT, the negative charge on the aspartate residue in TM10 is crucial for either substrate recognition or proton coupling. In contrast, at position Asp-425 in TM11, charge alone is not sufficient for transport activity.

We also assessed the vesamicol binding ability of these mutant transporters. Replacement of Asp-398 with Asn or Glu completely abolished vesamicol binding to the vesicles, whereas D425N or D425E exhibited normal vesamicol binding (Fig. 9). Since vesamicol binding can be inhibited by acetylcholine, this property can be used to determine acetylcholine binding without a requirement for acetylcholine transport. We thus determined whether acetylcholine could inhibit vesamicol binding with the D425N mutant. As shown in Fig. 10, the D425N mutant, although incapable of transporting ACh, did retain the ability to bind ACh, as indicated by the ability of ACh to inhibit vesamicol binding. The IC50 value for ACh is ~100 mM, which can be compared with an IC50 value of 10 mM for the wild-type transporter. This result suggests that aspartate 425, although contributing to ACh binding, is crucial for ACh transport.

**DISCUSSION**

The transport of acetylcholine from the cell cytoplasm into synaptic vesicle involves at least two steps, the initial binding of acetylcholine to the transporter and the actual transfer of acetylcholine across the vesicle membrane. The fact that acetylcholine contains a quaternary ammonium group makes it likely that its binding site would contain charged residues, at least one of which would reside in a transmembrane domain. For the active transport of acetylcholine, two critical sites can be protonated and exchange one ACh for two protons (35).
Vesamicol, which is a specific inhibitor for acetylcholine transport, has been suggested to bind to VAChT at a site other than the ACh binding site (1, 35), although to date this postulate has been based on kinetic evidence. Within the VAChT species that have been sequenced to date, one finds conserved aspartate residues, but not conserved glutamate residues. In order to gain insight into the role of these conserved aspartate residues in energy coupling and substrate recognition or inhibitor binding, we have used asparagine mutagenesis to scan the function of conserved aspartate residues in the cytoplasmic loop and transmembrane domain of rVAChT.

In the tetracycline antiporter encoded by Tn10, Asp-66 in the cytoplasmic loop between TM2 and TM3 is essential for transport function (36). Changing Asp-66 with Asn completely abolished transport activity; however, replacement with Glu retained transport activity. Therefore, a negative charge in position 66 is essential for transport activity. rVAChT has two Asp residues, Asp-147 and Asp-152, in a similar location, which are conserved in all VAChT species but are absent in the related transporters VMAT1 and VMAT2. As shown below, Asp-147 corresponds to Asp-66 of the tetracycline transporter. However, replacement of either these residues in rVAChT with Asn did not affect transport activity. Thus, Asp-66 plays a unique role in the tetracycline antiporter.

Besides the Asp residues in the loop between TM2 and TM3, rVAChT has three more Asp residues in cytoplasmic loops, Asp-202, Asp-288, and Asp-410. Interestingly, all of these residues are conserved in all VAChT species and are located at the boundary between a cytoplasmic loop and a transmembrane domain. Since the precise structures of VAChT or related transporters have not been determined, it is possible that these aspartate residues could be located in the transmembrane domain instead of in the cytoplasm. Therefore, we replaced Asp-147 with Asn to determine if this change did not have any effect on the transport of acetylcholine or on vesamicol binding. Thus, none of the putative aspartate resi-
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Fig. 10. Inhibition of vesamicol binding by acetylcholine in the wild-type (○) or D425N (○) VChT mutant. The postnuclear supernatant from the D425N mutant transfectant was preincubated with various concentration of acetylcholine for 10 min, followed by incubation with [3H]vesamicol for an additional 10 min. Vesamicol binding was determined as described under “Experimental Procedures.”

Neutralization of the charge on Asp-46 in TM1 or Asp-255 in TM6 of rVChT by replacement with Asn did not eliminate acetylcholine transport or vesamicol binding. Previous studies of VMAT2 indicated that the Asp residue in transmembrane domain 1 plays a critical role in the transport of serotonin, especially in substrate recognition (22). Mutagenic analysis of the related plasma membrane dopamine transporter indicated a role for a corresponding Asp residue in substrate recognition (37). The bacterial tetracycline transporter, which is also a transporter containing 12 transmembrane domains and uses a proton for the exchange with substrate, also contains an asparagine residue in the first transmembrane domain that appears to play a critical role in transport (38, 39). Thus, this analysis points out a significant difference between VChT and related transporters and suggests that Asp-46 and Asp-255 may play a unique role in VChT, such as the release of acetylcholine or in synaptic vesicle recycling.

Although VChT and VMATs have a high sequence homology and a similar mechanism for transport, they do not transport each other’s substrates nor do they recognize the same high affinity inhibitors (31, 40). Since Asp-193 is conserved in VChT from a variety of species, but is not found in VMAT, this residue could play a critical role in substrate recognition. Surprisingly, charge removal from this residue by replacement with Asn did not eliminate acetylcholine transport. This result differs from the study of Song et al. (24), who reported this residue is essential for transport activity. To further confirm our results, we demonstrated the dependence of transport activity on ATP and inhibition of transport activity by the proton ionophore FCCP and the specific V-type ATPase inhibitor bafilomycin A1. Furthermore, the kinetics of transport with D193N closely mirrored that of the wild-type transporter. We did not find that the D193N mutant exhibits a dramatically reduced ability to bind vesamicol.

A possible explanation for the difference between our finding of D193N VChT to be fully active in ACh transport assays and the reported inactivity of this mutant by Song et al. (24) is that Asp-193 may function in acetylcholine release. In the studies of Song et al. (24), the quantal release of ACh was measured through the action potential-independent miniature excitatory postsynaptic currents after exocytosis. Therefore, their measurements required two sequential steps, acetylcholine transport and then acetylcholine release. In contrast, we directly measured acetylcholine transport into isolated synaptic vesicles. Although there are no reports for the involvement of VChT in transmitter release, the difference between our results and those of Song et al. indicate that expression of D193N VChT leads to a deficiency in the ability to release acetylcholine. It is tempting to speculate that, since the D193N mutant has a reduced affinity for vesamicol without a change in acetylcholine transport, the vesamicol binding site may be involved in acetylcholine release.

Consistent with recent mutagenesis studies of Asp residues in TM10 and TM11 of VMAT1 and VMAT2 (21, 23), mutagenesis of the corresponding residues, Asp-398 and Asp-425, in rVChT demonstrates important roles for these residues in ACh transport. In VChT, neutralization of these residues by replacement with Asn completely abolished transport activity. Immunoblot analysis revealed that the expression level of these mutant VChTs was comparable to wild-type and their mobility on an SDS-polyacrylamide gel was similar to wild-type protein. Additionally, D398N and D425N are appropriately sorted to small synaptic vesicles. These results eliminate the possibility that the loss of activity of these mutants is due to protein misfolding. On the other hand, the conservative rVChT mutant D398E shows significant transport activity, suggesting the negative charge at position 398 is critical for transport. In contrast, replacement of Asp-425 in TM11 with Glu did not restore transport activity, indicating that at position 425 the Asp side chain as well as charge is important for transport. This finding is consistent with the finding that, in VMAT1, replacement of Asp-404 in TM10 with Ser or Cys eliminated transport activity; however, substitution with Glu showed a slower, but robust, accumulation of serotonin with an altered sensitivity to pH (21).

In VMAT2, replacement of the aspartates in TM10 (Asp-400) and TM11 (Asp-427) with Asn eliminated transport of serotonin. In addition, changing a basic residue, Lys-139, in TM3 to Ala eliminated transport activity. However, the double mutant K139A/D427N showed substantial transport activity in contrast to the K139A/D400N double mutant. This was interpreted as suggesting an electrostatic interaction between Lys-139 and Asp-427 in VMAT2 (23). VChT has a corresponding Lys, Lys-131, in TM2, which also is required for transport activity. Replacement of Asp-425 in TM11 with Asn or Glu eliminated transport activity. This suggests that VChT also may have charge pairs between Lys-131 in TM2 and Asp-425 in TM11. In contrast to VMAT2, however, VChT has other basic transmembrane residues, His-338 in TM8 and His-444 in TM12. His-338 has been shown to be involved in ACh transport. Therefore, in VChT, there are other possible ion pairs: one between Lys-131 and Asp-425 and another between His-338 and Asp-425.

Our Asn scanning mutagenesis also revealed effects on vesamicol binding. Replacement of Asp-398 with Asn or Glu completely abolished vesamicol binding, while the Asp-193 mutant

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had significantly reduced vesamicol binding. Thus, in terms of ACh transport and vesamicol binding, three distinct classes of mutants were observed in this study. In the D398N mutant, both ACh transport and vesamicol binding are eliminated. In contrast, the D425N mutant was blocked in ACh transport, but retained vesamicol binding. The third type of mutant, seen with D193N, had full ACh transport activity, but severely compromised vesamicol binding. These mutants unequivocally demonstrate that the vesamicol binding site is not the same as the substrate recognition site, and further that an intact vesamicol binding site is not required for acetylcholine transport.

Acetylcholine can inhibit vesamicol binding in the absence of Mg-ATP or in the absence of acetylcholine transport, as seen with the D425N mutant. This can be interpreted to suggest that either there are two sites for ACh binding, the transport site and the vesamicol site, or that ACh binding to the transport site causes a conformational change which blocks vesamicol binding. Since vesamicol can also block ACh transport, but has a separable binding site, the latter alternative is favored. The data further suggest that Asp-425 is important, but not required, for ACh binding, but is required for ACh transport. Thus, Asp-425 might be required for proton coupling or it might be required for the conformational change that moves ACh from facing the cytoplasm to facing the inside of the vesicle.

Taken together, these studies begin to unravel the topology of the vesicular acetylcholine transporter and identify critical functional residues.

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