Mutational Analysis of Basic Residues in the Rat Vesicular Acetylcholine Transporter

IDENTIFICATION OF A TRANSMEMBRANE ION PAIR AND EVIDENCE THAT HISTIDINE IS NOT INVOLVED IN PROTON TRANSLOCATION

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The function of positively charged residues and the interaction of positively and negatively charged residues of the rat vesicular acetylcholine transporter (rVAChT) were studied. Changing Lys-131 in transmembrane domain helix 2 (TM2) to Ala or Leu eliminated transport activity, with no effect on vesamicol binding. However, replacement by His or Arg retained transport activity, suggesting a positive charge in this position is critical. Mutation of His-444 in TM12 or His-413 in the cytoplasmic loop between TM10 and TM11 was without effect on ACh transport, but vesamicol binding was reduced with His-413 mutants. Changing His-338 in TM8 to Ala or Lys did not effect ACh transport, however replacement with Cys or Arg abolished activity. Mutation of both of the transmembrane histidines or all three of the luminal loop histidines showed no change in acetylcholine transport. The mutant H338A/D398N between oppositely charged residues in transmembrane domains showed no vesamicol binding, however the charge reversal mutant H338D/D398H restored binding. This suggests that His-338 forms an ion pair with Asp-398. The charge neutralizing mutant K131A/D425N or the charge exchanged mutant K131D/D425K did not restore ACh transport. Taken together these results provide new insights into the tertiary structure in VACHT.

The vesicular acetylcholine transporter (VACHT) catalyzes the transport of acetylcholine (ACh) from the cytoplasm into small synaptic vesicles of cholinergic neurons. Transport of ACh by VACHT is dependent on a proton electrochemical gradient across the synaptic membrane involving the exchange of two protons from inside the vesicle for one cytoplasmic substrate (1). For the transport of ACh, at least two sequential steps are required: binding of ACh to the transporter and the actual transfer of ACh across the vesicle membrane. In this process, it is suggested that the loss of a proton leads to a conformational change which makes the transporter active (2). Vesamicol (-)-trans-2-(4-phenylpiperidino)cyclohexanol has been shown to be a specific inhibitor of ACh transport by VACHT (3, 4). This compound acts in a noncompetitive manner; however ACh blocks vesamicol binding to the transporter. Recent mutagenesis studies have separated the vesamicol binding site from the ACh binding site (5).

Hydropathy analysis predicts that VACHT contain 12 transmembrane domains, and within these putative hydrophobic domains are found a number of both positively and negatively charged residues (6, 7). Because of the large energy requirement for placing a single charged amino acid residue in the hydrophobic environment of a membrane, such residues are a relatively infrequent occurrence in membrane spanning α-helices. On the other hand when present, these charged residues frequently have specific physiological functions. In the case of VACHT, charged residues could function in maintaining the native conformation of the transporter, they could be involved in substrate binding, or they could play a role in the H⁺/antiporter activity. Sequence conservation of charged and polar amino acids within the putative hydrophobic transmembrane domains of VACHT suggests functional importance. In a previous report (5), Asp-398 in TM10 and Asp-425 in TM11 were shown to possess important roles in acetylcholine transport.

The placing of a charged amino acid residue in membrane-spanning regions of a membrane protein is thermodynamically unfavorable. However, Honig and Hubbell (8) have pointed out that the transfer of an ion pair from water to a region of low dielectric constant is not so energetically unfavorable. Indeed such an ion pair could make additional hydrogen bonds in the membrane. In the case of voltage-sensitive ion channels, charged residues are a prominent feature of membrane-spanning α-helices and may provide an essential binding site for transported ions or for the gating mechanism (9). In such channel proteins, the positively charged amino acids in amphipathic helices probably line the aqueous channel. It is believed that these positive charges are ion paired with negatively charged residues on adjacent α-helices (9). A salt bridge between oppositely charged amino acid residues in membrane-spanning regions would greatly reduce the energy required to stabilize two individual charged residues in a hydrophobic environment and may also provide a proton relay system for the H⁺/substrate transporter (10).

Rat VACHT has three positively charged residues within its transmembrane domains, Lys-131 in TM2, His-338 in TM8, and His-444 in TM12, and has three histidines located on luminal loops. In this paper, we have investigated the role of these positively charged residues and their potential interaction with the negatively charged residues in the transmembrane domains.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis was performed as described previously (5). Briefly, rVACHT was subcloned into pBluescript SK⁺ and transformed into the BD2399 strain of Escherichia coli with R408 helper phage yielding single-stranded uracil containing DNA. Mutagenic oligonucleotides, which contain a one- or two-base mismatch with
Fig. 1. (A) Scheme showing the membrane topology of rVAChT based on hydrophathy analysis. Targeted mutagenesis sites are indicated. (B) Sequence homology among different species of VAChT and VMATs at sites subjected to mutagenesis. See reference 5 for ascension numbers.

wild type VAChT, were annealed to 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 (11). The mutant VAChT was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Insertions of fragments into PsI or XhoI restriction enzyme sites were used for double or triple mutant construction.

**Transient Expression**—PC1281237 cells (12) were cultured at 37 °C and 10% CO2 in complete Dulbecco’s modified Eagle’s medium nutrient mixed 1:1 with Ham’s F-12 medium. The culture medium contained 10% fetal bovine serum, 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For electroporation, trypsin/EDTA was used to first detach cells from culture plates. After washing with cold phosphate-buffered saline, cells were resuspended in cold phosphate-buffered saline at a concentration of 8 × 107 cells/ml. Resuspended cells (800 μl) were mixed with DNA (50 μg), incubated on ice for 10 min, and then transferred to a 0.4-cm gap electroporation cuvette (Bio-Rad). Electroporation was carried out at 0.2 kV and 975 microfarads. Cells were replated in the above medium with fresh medium added at 24 h. Cells were cultured for an additional 24–48 h, harvested, and vesicles prepared. The expression level of wild type and of mutant VAChTs was determined through Western blotting. It was noted that with a given batch of cells, VAChT expression did not vary significantly between replicates or between different constructs.

**Preparation of Postnuclear Supernatant**—A postnuclear supernatant fraction was prepared as described previously (5). Briefly, transfected cells were removed from culture plates with trypsin, washed with phosphate-buffered saline containing 10 mM EDTA (pH 7.4), and resuspended in a homogenization buffer (0.32 M sucrose, 10 mM Hepes-KOH [pH 7.4], 4% of a protease inhibitor mixture [Roche Molecular Biochemicals], and 5% Pefabloc SC [Roche Molecular Biochemicals]). Cells were broken by several up and down strokes in a Potter-Elvehjem homogenizer. Centrifugation at 800 × g for 10 min yielded the postnuclear supernatant fraction. Protein was determined with the Coomassie Plus Protein Assay Reagent (Pierce).

**Acetylcholine Transport and Vesamicol Binding Assays**—Transport of acetylcholine into synaptic vesicles and vesamicol binding to the vesicles were performed according to Kim et al. (5). For ACh transport assays, 50 μl of postnuclear supernatants containing ~500 μg of protein was mixed with 50 μl of uptake/binding buffer (110 mM potassium tartrate, 20 mM HEPES [pH 7.4], and 1 mM ascorbic acid) and preincubated at 37 °C for 15 min in the presence or absence of l-vesamicol (Research Biochemicals Inc.). Uptake was initiated by the addition of 10 μM Mg2+ -ATP and 0.5 μM [3H]ACh (40–50 μCi/mmoll, NEN Life Science Products) and allowed to proceed for 30 min at 37 °C. For vesamicol binding assays, an aliquot of postnuclear supernatant containing ~250 μg of protein was mixed with uptake/binding buffer containing 35 mM L-11Hvesamicol (31 Ci/mmol, NEN Life Science Products). The total volume was adjusted to 200 μl with uptake and binding buffer. The suspension was incubated for 10 min at 37 °C.

Both uptake and binding reactions were terminated by vacuum filtration through an HAWP filter (Millipore) pre-wetted with cold ice-cold uptake/binding buffer. Radioactivity bound to the filters was solubilized in 0.5 ml of 1% SDS and measured by liquid scintillation counting. Nonspecific binding was determined in the absence of ATP but in the presence of 4 μM vesamicol at 0 °C and was subtracted from total binding. Each experiment was performed in duplicate and repeated at least twice.

**Cell Fractionation**—The postnuclear supernatant containing ~5 mg of protein in 0.5 ml was loaded onto a 10-ml linear sucrose gradient from 0.6–1.6 M sucrose in 10 mM 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**—Western blot analysis was performed as described previously (5) to assess the expression of mutant VAChTs. An aliquot of postnuclear supernatant, containing ~50 μg of protein was diluted 1000-fold with homogenization buffer and then pelleted by centrifugation. The pellet was resuspended in SDS sample buffer (62 mM Tris-HCl, pH 6.8, 1 mM EDTA, 10% glycerol, 5% SDS, and 5% β-mercaptoethanol) and electrophoresed on a 10% SDS-polyacrylamide gel. A semi-dry electrotransfer apparatus (Alltech) was used to transfer proteins to an Immobilon P membrane (Millipore). The membrane was blocked with Tris-buffere saline containing 10% non-fat dry milk and 0.1% Tween 20 and then incubated first with a goat anti-rVAChT antisera (Chemicon International Co.) and then with peroxidase-conjugated rabbit anti-goat antisera. Immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Fractions from sucrose density gradients were immunoblotted in a similar manner and included additional Western blot analysis using either a monoclonal antibody against synaptophysin (Roche Molecular Biochemicals) or a rabbit antisera against secretogranin II. The secretogranin II antisera was a generous gift from Dr. Jonathan Scammell of the University of Alabama.

**RESULTS**

Fig. 1 is a schematic showing the position of the positively charged amino acid residues of rat VAChT and their amino acid sequence homology among different species of VAChT and the related transporters VMAT1 and VMAT2. In rat VAChT, there
are eight histidine residues. Two of these, His-338 and His-444, are located in a transmembrane domain; three histidines, His-283, His-354, and His-413, are found in cytoplasmic loops; and three, His-63, His-318, and His-441, are located on luminal loops. His-338, found in TM8 is conserved among all VACHTs but is absent in VMAT. His-413 found in the cytoplasmic loop between TM10 and TM11 is conserved among mammalian VACHTs and VMAT, whereas His-444 found in TM12 is conserved among all VACHTs except the <i>Drosophila</i> and <i>Caenorhabditis elegans</i> proteins and is absent from the VMATs. There is one lysine residue in a transmembrane domain, Lys-131 in TM2, which is conserved among all cloned VACHTs as well as in the VMATs.

**Characterization of Mutants of Lys-131**—Using site-directed mutagenesis, Lys-131 was replaced with alanine or leucine to remove the positive charge from this position. The mutant VACHTs were expressed in PC12<sup>Δ123.7</sup> cells, and their ability to transport acetylcholine and bind vesamicol was determined. As shown in Fig. 2 replacement of Lys-131 with alanine or leucine led to a severe transport defect, but vesamicol binding activity remained at a level comparable with the wild type transporter. The decreased transport activity of these mutants could be because of several factors unrelated to transport activity. The concentration of mutant transporter could be low because of reduced production or reduced stability of the mutant protein, or the targeting of the mutant transporter to the small synaptic vesicles could be impaired. The first possibility seems unlikely because vesamicol binding appeared normal and was further ruled out by comparing VACHT<sup>K131A</sup> and VACHT<sup>K131L</sup> expression levels to that of the wild type transporter by Western blot analysis. As shown in Fig. 3, the mutant and wild type transporters were expressed at comparable levels and exhibited the same subunit molecular weight. Therefore, the impairment of the transport activity in VACHT<sup>K131A</sup> and VACHT<sup>K131L</sup> is not attributable to reduced levels of transporter protein. Although the Lys-131 VACHT mutants were expressed at wild type levels, it is possible that they were inefficiently targeted to the synaptic vesicle. To rule out this possibility, post-nuclear supernatants were fractionated on a 0.6–1.6 M linear sucrose gradient using synaptophysin as a marker for synaptic vesicles and secretogranin II as a marker for dense core vesicles (5). The Lys-131 mutant proteins were found to co-localize with synaptophysin as did the wild type transporter as illustrated in Fig. 4 for K131A. Therefore, the loss of transport activity in VACHT<sup>K131A</sup> and VACHT<sup>K131L</sup> is not a consequence of improper localization. In contrast to the results obtained with the Lys-131 alanine or leucine mutants, replacement of Lys-131 with a histidine or arginine residue reduced, but did not eliminate, ACh transport activity, Fig. 2.

**Characterization of Mutants of Histidine Residues**—A histidine residue in the cytoplasmic loop between TM10 and TM11 has been shown to be important for energy coupling in VMAT1 (13). Rat VACHT has three His residues in cytoplasmic loops: His-283 between TM6 and TM7, His-354 between TM8 and TM9, and His-413 between TM10 and TM11. Because His-283 and His-354 are not conserved among VACHT species, see Fig. 4, it is unlikely they would be functionally important. On the other hand, His-413 is conserved among mammalian VACHTs, although it is absent in <i>Drosophila</i> and <i>Torpedo</i> VACHT, and corresponds to His-419 in VMAT1. Thus although His-413 might be anticipated to be functionally important in acetylcho-

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**Fig. 2.** ACh uptake (A) and vesamicol binding (B) by wild type (WT) and Lys-131 mutant rVACHTs. Wild type and mutant VACHTs were transiently expressed in PC12<sup>Δ123.7</sup> cells. A, acetylcholine transport assays were conducted as described under “Experimental Procedures” using 0.5 nM [3H]ACh and 5 mM Mg-ATP for 30 min at 37 °C with 500 μg of post-nuclear supernatant from cell homogenates. B, vesamicol binding assays were conducted as described under “Experimental Procedures” by incubating 250 μg of post-nuclear supernatant and 35 nM [3H]vesamicol for 10 min at 37 °C. Nonspecific binding was determined at 0 °C in the presence of 4 μM unlabeled vesamicol and the absence of ATP and was subtracted from total binding. Values are given with their standard deviation indicated as error bars determined from three independent experiments.

**Fig. 3.** Western blot analysis of wild type (WT) and mutant rVACHT proteins. Post-nuclear supernatants from wild type (WT) and mutant VACHT transfected PC12<sup>Δ123.7</sup> cells were pelleted by centrifugation at 100,000 × g for 1 h and then separated by 10% SDS-polyacrylamide gel electrophoresis. Western blot analysis was then conducted as described under “Experimental Procedures.” The rVACHT proteins appeared as a diffuse band with a molecular mass of approximately 68–70 kDa.
line transport, its replacement by Ala, Arg, or Cys showed no significant affect on the transport of acetylcholine. However, vesamicol binding was reduced to half of wild type levels, Table I. Thus, His-413 must be functionally different between VMAT and VAChT, and its lack of conservation in C. elegans and Drosophila and its nonessential role.

There are two histidine residues located in transmembrane domains of rVAChT, His-338 in TM8 and His-444 in TM12. His-338 is conserved in all known species of VChT but is not found in VMATs. This histidine, which is located in the center of the putative transmembrane domain 8, has been suggested to serve as the proton exchanger which drives ACh transport (1). Neither of the two transmembrane histidines are required for ACh transport except His-338, which was not conserved in VMATs. This histidine, which is located near the luminal surface of the 12th putative transmembrane domain.

Replacement of His-338 with alanine (H338A) or lysine (H338K) had no effect on ACh transport; however, replacement with arginine (H338R) or cysteine (H338C) completely abolished transport activity, Fig. 5. The difference in transport activity between H338A and H338K on the one hand and H338R and H338C on the other hand were not because of differences in the expression level of the recombinant transporters (Fig. 3). As illustrated for H338A in Fig. 4, sucrose gradient centrifugation showed that all of the mutant proteins localized to the same synaptic vesicle fraction as did wild type VAChT. Although His-338 mutants exhibited differential effects on ACh transport dependent on the specific substitution, all of the His-338 mutants lacked vesamicol binding activity.

In contrast to the results obtained with mutations at His-338, changing His-444 in TM12 to alanine, arginine, or cysteine had no effect on the transport of acetylcholine or vesamicol binding. The finding that neither of the transmembrane histidines were required for acetylcholine transport seemed to eliminate a histidine as the proton exchanger. However, these results do not rule out the possibility that mutation of the histidine involved in proton coupling (i.e. His-338) was compensated for by the second transmembrane histidine (i.e., His-444) taking over this function. Therefore, we constructed the double mutant H338A/H444A and examined its transport activity. As shown in Table I, this double mutant exhibited approximately the same rate of ACh transport activity as did the wild type transporter.

As acetylcholine is transported from the cytoplasm into the vesicle, protons are exchanged from inside the vesicle to the cytoplasm (4, 14), and a histidine has been suggested to be the proton carrier (1). Neither of the two transmembrane histidines are required for this proton coupling nor could one
envison how a histidine in the cytoplasmic loop could fulfill this role. The remaining possibility is that one of the three histidine residues in the luminal loop of rVAChT functions in proton coupling. To test this possibility, we constructed a triple mutant in which each of these histidines was replaced with alanine. As shown in Table I, this mutant, VAChT\textsubscript{H338A/H338R/H444A}, showed the same rate of acetylcholine transport activity as did wild type VAChT.

Analysis of Double Mutants between Oppositely Charged Residues in Transmembrane Domains—A number of transporters have been found to have ion pairs formed between transmembrane positively charged and negatively charged residues (15–19) as a means of avoiding an energetically unfavorable environment. If two oppositely charged residues form such a salt bridge, replacement of either charged residue with a neutral residue creates an unpaired charge which can lead to functional defects. On the other hand, neutral substitutions for both residues may not cause inactivation nor might charge reversal of the ion pairing residues. For example, studies of the E. coli lactose permease have demonstrated that neutralization of residues forming a charge pair can restore the function lost in single mutants, as could reversal of the polarity (16, 18, 20). It has also been proposed that in the lactose permease of E. coli, positively and negatively charged residues in the TM interact and form a proton relay system that is postulated to function as a chemical pathway for proton movement through the lactose permease (10).

Because mutations of Lys-131 affect ACh transport activity, but not vesamicol binding, we sought to determine whether this residue might form an ion pair with either of the two transmembrane aspartate residues, Asp-398 and Asp-425, whose mutations also showed effects on ACh transport activity (5). We constructed mutants in which Lys-131 and either Asp-398 or Asp-425 were simultaneously replaced with neutral residues. Neither the K131A/D398N mutant nor the K131A/D425N mutant recovered acetylcholine transport activity. We also constructed the charge reversal mutants K131D/D398K and K131D/D425K. Despite normal levels of transporter expression with these mutants (Fig. 3), ACh transport activity was not detected, Fig. 6.

Although His-338 had no known function in acetylcholine transport, mutants H338A, H338R, and H338C were almost completely blocked in vesamicol binding. Similarly, replacement of Asp-398 in TM10 by Asn abolished vesamicol binding (5). We therefore examined the possibility that His-338 and Asp-398 might make an ion pair. Neutralization of charge at positions 338 and 398 in the mutant H338A/D398N did not restore vesamicol binding; however, the charge reversal mutant H338D/D398H showed almost the same vesamicol binding activity as the wild type transporter, Fig. 7. To rule out the possibility that vesamicol binding activity would be recovered by just a single mutation, we examined vesamicol binding with the H338D and D398K single mutants. As expected, there was no activity with either of these single mutants, Table I.

DISCUSSION

The energy requirement for placing a charged amino acid residue in a hydrophobic environment makes such residues infrequent in protein membrane spanning α-helices. When present, they frequently play a role in substrate binding and translocation (15, 16, 18, 21, 22) and are often found as ion pairs (15, 16, 18, 21). We have investigated the three putative transmembrane domain basic residues of rat VAChT, Lys-131 in TM2, His-338 in TM6, and His-444 in TM12. At position 131 in TM2, a positive charge rather than Lys is required for ACh transport activity. However, neutral residues at position 131 retain vesamicol binding. This finding functionally distinguishes the site of vesamicol binding from ACh transport.

In VMAT1, His-419 in the cytoplasmic loop between TM10 and TM11 is suggested to play a role in energy coupling (13). However, the corresponding His in VACHT, His-413, is not conserved in Drosophila or Torpedo VAChTs. The finding that a variety of amino acid substitutions for His-413 of rVAChT were without effect is in keeping with the lack of conservation of this residue. This raises the question as to whether VMAT His-419 plays a functional role in monoamine transport or whether this is a structural residue. Alternatively, the mechanism of neurotransmitter transport for VMAT and VAChT may differ more than anticipated.

Several transport systems for which a proton is a co-transported ion are known to contain essential histidyl residues which most likely participate in the binding and translocation of protons. Examples include the Na+/H+ coupled glutamate transporter (23), the organic cation/H+ antiporter (24), lactose permease (25, 26), the folate transporter (27), VMAT (13, 28), and the metal-tetracycline/H+ antiporter (19, 29, 30). In the human H+/peptide co-transporters PEPT1 and PEPT2, the obligatory histidyl residues are located in an almost identical topological position near the extracellular surface of the second putative transmembrane domain (31). Rat VAChT has two His residues in transmembrane do-

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**FIG. 6.** ACh uptake by double mutants made between oppositely charged residues K131A/D398N and K131A/D425N and the charge reversal mutants K131D/D398K and K131D/D425K. All double mutants were constructed from single mutants using subcloning of restriction fragments. Uptake of ACh by mutants was performed as described in Fig. 2. Values are given with their standard deviation indicated as error bars determined from three independent experiments.

**FIG. 7.** Vesamicol binding by mutant H338A/D398N and H338D/D398H. All double mutants were constructed from single mutants as noted in Fig. 6. Vesamicol binding was measured as described in Fig. 2. Values are given with their standard deviation indicated as error bars determined from three independent experiments.
mains; His-338 in TM8 and His-444 in TM11. His-338 was suggested to function as a proton carrier, moving a proton from inside the vesicle to the outside as acetylcholine entered the vesicle (1). The finding that H338A is transport competent argues that it cannot be involved in proton translocation. Some, but not all, His-338 mutants are defective in ACh transport, and yet all are defective in vesamicol binding. This suggests that substitutions at His-338 result in conformational changes. The nature of the conformational change is residue-dependent, some affecting ACh transport, and all affecting vesamicol binding.

None of the substitutions at His-444 affected ACh transport or vesamicol binding. This is in keeping with the lack of conservation of this residue in Drosophila and C. elegans VAChT. The finding that ACh transport is unaffected when both of the transmembrane histidines were mutated together definitively rules out the involvement of a transmembrane histidine in proton translocation. Histidine residues in the luminal loop of rVAChT were also ruled out as having a functional role in proton coupling. We are left to conclude that VAChT differs from other H+-coupled neurotransmitter transporters in that a His residue does not play a role in substrate binding or proton translocation.

We reported that mutation of Asp-398 of TM10 or Asp-425 of TM11 to glutamine abolished ACh transport (5). The D398N mutant was additionally defective in vesamicol binding. The finding of two functional TM positively charged residues, Lys-131 and His-338, and the above two functional acidic residues suggests the possibility of ion pairing. However, double mutants, in which interhelical combinations of positively and negatively charged residues were replaced pairwise with neutral residues, did not recover ACh transport activity nor did a H338A/D398N mutant recover vesamicol binding. These results indicate either there are no ion pairs between these charged residues or that ion pairs exist, but a specific charged residue contributes to the transport of acetylcholine and/or vesamicol binding.

If a charged residue of an ion pair is important for the structure and function of the transporter, charge reversal might restore function. Of the charge reversal mutants constructed, H338D/D398H recovered vesamicol binding activity suggesting that His-338 in TM8 and Asp-398 in TM10 are connected via a salt bridge. Because polarity of the salt bridge is not important, this ion pair most likely serves a structural role. The ion pair between His-338 and Asp-398, eliminates an ion pair between Lys-131 and Asp-398. However, an ion pair between Lys-131 and Asp-425 cannot be ruled out. Charge reversal between these positions did not recover transport activity, consistent with the finding that a positive charge is required at position 131 for transport activity. Thus, it cannot be determined at this time whether or not an ion pair exists between Lys-131 and Asp-425. However the finding that, in VMAT2, the corresponding Lys-139 in TM2 and Asp-427 in TM11 form a charge pair whose polarity is critical for function (15) would support the contention that an ion pair exists between Lys-131 and Asp-425 of VAChT.

The results of this study provide the first insights into the tertiary structure of VAChT. The ion pair between His-338 in TM8 and Asp-398 in TM10 indicates TM8 and TM10 must exist in close proximity. This charge pair may anchor these helices together and help form the vesamicol binding site. We propose that His-413 in the cytosol between TM helix 10 and TM helix 11 participates directly in vesamicol binding, and that vesamicol binding to His-413 induces a conformational change transmitted through the His-338/Asp-398 salt bridge. This conformational change leads to the vesamicol-dependent inhibition of ACh transport. It appears probable that Lys-131 of TM2 forms an ion pair with Asp-425 in TM11, bringing these two TM domains in close proximity. Importantly, we have provided evidence that histidine residues do not play a role in acetylcholine binding or proton translocation. This seems to mechanistically distinguish VAChT from VMAT.
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