Multiple Residues Contribute Independently to Differences in Ligand Recognition between Vesicular Monoamine Transporters 1 and 2

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J. Patrick Finn III‡§ and Robert H. Edwards¶

From the ‡Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024 and §Departments of Neurology and Physiology, University of California School of Medicine, San Francisco, California 94143-0435

The two closely related vesicular monoamine transporters (VMATs) 1 and 2 differ substantially in ligand recognition. The neuronal VMAT2 exhibits a higher affinity for monoamine substrates and in particular for histamine as well as a greater sensitivity to the inhibitor tetrabenazine than the nonneuronal VMAT1. The analysis of chimeric transport proteins has previously shown that two major domains, one spanning transmembrane domains (TMDs) 5–8 (TMD5–8) and the other, TMDs 9–12 (TMD9–12), are required for the high affinity interactions characteristic of VMAT2. Using site-directed mutagenesis to replace residues in TMD5–8 of VMAT2 with the equivalent residues from VMAT1, we now show that the sensitivity of VMAT2 to tetrabenazine requires Ala-315, and this interaction occurs independently of the interaction with residues in TMD9–12. The ability to recognize histamine as a substrate depends on Pro-237, and the contribution of TMD9–12 to histamine recognition appears to involve a common mechanism. In contrast, the replacement of many residues in TMD5–8 of VMAT2 with equivalent residues from VMAT1 improves the recognition of both serotonin and tryptamine, and these mutations show a dominant effect on the recognition of both tryptamine and serotonin over mutations in TMD9–12. The results indicate that different ligands interact through distinct mechanisms with the VMATs and that the recognition of each ligand involves multiple, independent interactions with the transport protein.

Neurotransmitters are packaged into secretory vesicles so that their release can be regulated by exocytosis. In the case of monoamine transporters, two closely related vesicular transport proteins have been identified (VMAT1 and VMAT2) (1, 2). VMAT1 is expressed in adrenal medulla and other nonneuronal cells, whereas VMAT2 appears in multiple monoamine populations of brain as well as sympathetic neurons, mast cells, and histamine-containing cells in the gut (3, 4). In addition to their differences in distribution, the two VMATs differ in ligand recognition and pharmacology. VMAT2 has a higher apparent affinity for dopamine, norepinephrine, and serotonin than VMAT1 (5, 6). Furthermore, VMAT2 transports histamine, whereas VMAT1 does not, presumably accounting for the expression of VMAT2 but not VMAT1 in histamine-containing cells of the gut as well as in mast cells (7). VMAT2 also shows 10-fold greater sensitivity to the classical inhibitor tetrabenazine than VMAT1 (5, 6).

The existence of two closely related proteins that differ substantially in their substrate affinity and pharmacology provides an opportunity to dissect the structural basis for these differences. To identify domains responsible for these differences, we first expressed and analyzed a series of chimeric transport proteins (8). These chimeras retained function, and analysis showed that two major regions from VMAT2, one extending from transmembrane domain (TMD) 5 to the beginning of TMD8 (TMD5–8) and the other from the end of TMD9 through TMD12 (TMD9–12), are apparently both required for the high affinity interactions characteristic of VMAT2 (8). Replacement of individual VMAT2 residues from either region with corresponding VMAT1 residues may therefore reduce the affinity of VMAT2 interactions to those characteristic of VMAT1, whereas multiple replacements in both TMD5–8 and TMD9–12 of VMAT1 would be required to increase the affinity of the interactions toward that of VMAT2. Focusing first on TMD9–12, we introduced mutations that convert the VMAT2 sequence into VMAT1 and found that many residues in this region have no effect on recognition (9). However, substitution of Tyr-434 with Phe (mutant Y434F) and Asp-461 with Asn (D461N) significantly reduced the affinity of VMAT2 for tetrabenazine, histamine, and serotonin. Replacement of Lys-446 with Gln (K446Q) reduced the affinity of VMAT2 for tetrabenazine and serotonin but not histamine, whereas substitution of Phe-464 with Tyr (F464Y) reduced serotonin affinity but not tetrabenazine or histamine recognition. The analysis of multiple mutants indicated that Tyr-434 and Asp-461 account for essentially all of the difference in histamine recognition between the two VMATs attributable to TMD9–12. In addition, Tyr-434, Lys-446, and Asp-461 account for essentially all of the differences in serotonin and tetrabenazine recognition attributable to this region. To identify the features of the ligand recognized by these residues, we also examined the interaction of these mutants with structurally related compounds.

In contrast to other ligands recognized with higher affinity by VMAT2 than VMAT1, tryptamine inhibits transport of [3H]serotonin by VMAT1 much more potently than VMAT2 (9). Replacement of Tyr-434 with Phe (Y434F) alone increases the affinity of VMAT2 for tryptamine to that of VMAT1 (9). Thus, the same residue that contributes to the high affinity interactions of VMAT2 with tetrabenazine, histamine, and serotonin...
accounts for the lower affinity interaction of VMAT2 with tryptamine, suggesting that this residue discriminates between serotonin and tryptamine.

The analysis of chimeras has suggested that TMD5–8 is required along with TMD9–12 for most of the high affinity interactions characteristic of VMAT2. In the present study, we have substituted amino acids in TMD5–8 of VMAT2 with the equivalent residues from VMAT1. Functional analysis of these mutants implicates specific residues in the interaction of VMAT2 with serotonin, histamine, tetrabenazine, and tryptamine. The comparison of single and multiple mutants also indicates that these residues interact differently with the ligands from residues in TMD9–12.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—Mutations were introduced into wild-type VMAT2 using single-stranded, uracil-containing DNA as described previously (9, 10). VMAT2 double, triple, and quadruple mutant combinations were produced through the sequential subcloning of single mutants. In contrast, VMAT1 triple and quadruple mutants were each produced in a single reaction containing multiple mutagenic oligonucleotide primers. In all cases, a restriction fragment containing the desired mutation(s) was sequenced in its entirety by the dideoxyn method both to confirm the desired mutation and to exclude unwanted mutations (11). After sequence analysis, the fragments were subcloned back into appropriate wild-type cDNAs.

**Transient Expression and Membrane Preparation**—Plasmid DNA was prepared, COS1 cells were transfected, and membranes were isolated for analysis as described previously (9). Briefly, transfected cells were collected by centrifugation and resuspended in cold 0.32 M sucrose, 10 mM HEPES-KOH, pH 7.4 (SH buffer) containing 2 mM tryptamine (NEN Life Science Products), or 29 nM [2,5-3H]histamine from three separate transfections. The protein concentration of each replicate was determined on a total of three separate occasions using membranes prepared with the scint scintillation reagent scint 29 (ICN). For each mutant, transport activity was measured in duplicate in triplicate for analysis as described previously (9). Briefly, a frozen aliquot of membranes was thawed on ice, and 10 ml (50–100 g/ml) of protein was added to 200 ml of SH buffer at 29 °C containing 4 mM KCl, 2 mM MgSO₄, 2.5 mM ATP, and either 20 mM diisopropyl fluorophosphate. Cell debris were removed by centrifugation at 16,000 g for 5 min. The resulting supernatant was divided into aliquots and stored at –80 °C.

**Transport Assay**—The transport assay was performed as described previously (9). Briefly, a frozen aliquot of membranes was thawed on ice, and 10 ml (50–100 g/ml of protein) was added to 200 ml of SH buffer at 29 °C containing 4 mM KCl, 2 mM MgSO₄, 2.5 mM ATP, and either 20 mM 1,2-[3H]serotonin (NEN Life Science Products), 20 mM [2,5,6-3H]dopamine (NEN Life Science Products), or 29 mM [2,5,6-3H]histamine (Amersham Life Science, Inc.). To determine the Km for dopamine and serotonin, a range of nonradioactive substrate concentrations was used to determine the concentration of compounds needed to inhibit serotonin transport by 50% (IC50), increasing concentrations of tetrabenazine (Fluka), histamine (Sigma), and tryptamine (Sigma) were added to the reaction solution. After incubation for 1 min at 29 °C, the transport reaction was terminated by rapid dilution with 3 ml of cold SH buffer and filtration through 0.2-μm Super 200 membranes (Gelman Instrument Co.). The filters were then dried, and the bound radioactivity was quantitated by scintillation counting in a liquid scintillation counter (ICN). For each mutant, transport activity was measured in duplicate for analysis as described above. The unpaired two-tailed t test was used for all statistical comparisons.

**RESULTS AND DISCUSSION**

The analysis of chimeric transporters has suggested that replacing individual residues in VMAT2 with the equivalent residues from VMAT1 may suffice to reduce the affinity with which VMAT2 interacts with multiple ligands (8). Indeed, TMD9–12 of VMAT2 contains several residues that when replaced with the equivalent residues from VMAT1 reduce the high affinity of VMAT2 interactions toward those of VMAT1 (9). In the present study, we have extended the analysis to TMD5–8 in VMAT2. Of 27 residues in TMD5–8 that differ between the two VMATs, we focused on 18 that show nonconservative differences (Fig. 1).

**Tetrabenazine Sensitivity**—The majority of VMAT1 substitutions introduced into TMD5–8 of VMAT2 do not influence the sensitivity to tetrabenazine as measured by the IC50 for inhibition of [3H]serotonin transport (Table I). However, replacement of Ala-315 from VMAT2 with Thr from VMAT1 (A315T) significantly reduces tetrabenazine sensitivity. The addition of A315T to the triple mutant Y434F/K446Q/D461N previously shown to account for all of the reduced tetrabenazine sensitivity attributable to TMD9–12 (9) further reduces tetrabenazine sensitivity to a level similar to that of wild-type VMAT1 (Table I). Taken together with the previous results (9), Ala-315, Tyr-434, Lys-446, and Asp-461 thus each contribute independently to the high affinity recognition of tetrabenazine by VMAT2 (Fig. 2). These residues may interact directly with tetrabenazine, but they may also affect ligand recognition indirectly through changes in protein conformation.

**Histamine Recognition**—To determine whether specific residues from TMD5–8 of VMAT2 contribute to the high affinity recognition of histamine, we measured the effect of VMAT1 substitutions in this region on the ability of histamine to inhibit [3H]serotonin transport. Similar to the analysis of tetrabenazine sensitivity, most of these mutations, including A315T which influences tetrabenazine sensitivity, do not affect histamine recognition. However, the substitution of Pro-237 from VMAT2 with Ala from VMAT1 (P237A) significantly reduces histamine recognition (Table I). Thus, different residues in TMD5–8 account for the differences between the two VMATs in tetrabenazine sensitivity and histamine recognition, consistent with previous results from the analysis of mutants in TMD9–12 that also suggest distinct recognition sites (9). In addition, the involvement of a proline in histamine recognition suggests an indirect effect of this residue on protein structure rather than a direct interaction with the ligand.

Since Tyr-434 and Asp-461 were the only residues in TMD9–12 of VMAT2 previously found to influence histamine recognition (9), we combined the P237A mutation from VMAT5–8 with Y434F and D461N (Table II). Analysis of this triple mutant (P237A/Y434F/D461N) showed that the combination of mutations still does not suffice to completely convert the histamine recognition of VMAT2 into that of VMAT1. Thus, other residues in VMAT2 may also contribute to the high affinity recognition of histamine. Further, P237A/Y434F/D461N has an IC50 for histamine identical to that of either P237A or Y434F/D461N alone (Table II), indicating that these residues do not function independently and hence contribute to a single site of interaction with histamine (Fig. 2). As a control for these multiple mutations, we also combined K328E in TMD5–8, which alone has no effect on histamine recognition, with the Y434F/K446Q/D461N triple mutation in TMD9–12 previously shown to reduce histamine recognition (K328E/Y434F/K446Q/D461N). Surprisingly, this combination restores the affinity of the triple mutant to that of wild-type VMAT2, suggesting that the regions spanning TMD5–8 and TMD9–12 interact to confer high affinity histamine recognition.

**Serotonin Affinity**—The recognition of serotonin by VMAT2 appears more complex than the recognition of tetrabenazine and histamine. The analysis of chimeras has shown that TMD9–12 of VMAT1 reduces serotonin affinity when introduced into VMAT2; however, TMD5–8 of VMAT1 increases serotonin affinity in the context of C-terminal VMAT1 sequences (8). Thus, although TMD5–8 of VMAT1 decreases the affinity of VMAT2 for tetrabenazine and histamine, the same region reverses the loss of affinity for serotonin produced by TMD9–12 from VMAT1. To determine whether the same residues in TMD5–8 of VMAT1 responsible for low affinity interactions with tetrabenazine and histamine contribute to the high affinity for serotonin, we analyzed the VMAT1 substitutions into VMAT2 described above. Surprisingly, many of the VMAT1 substitutions into TMD5–8 of VMAT2, including mu-
tations A315T and P237A that, respectively, impair tetrabenazine and histamine recognition, increase apparent affinity for serotonin (Table I).

The analysis of chimeras suggested that TMD5–8 of VMAT1 has a dominant effect on serotonin recognition over TMD9–12 from VMAT1 (8). To determine whether specific residues in this region also have a dominant effect, we have focused on mutation A315T (because it influences tetrabenazine and serotonin recognition), P237A (because it influences histamine and serotonin recognition), and K328E (because it does not substantially influence any of these interactions). The introduction of A315T into Y434F/K446Q/D461N (A315T/Y434F/K446Q/D461N) and P237A into Y434F/D461N (P237A/Y434F/D461N), both, increase the apparent affinity for serotonin (Table II). Interestingly, the K328E mutation that does not alone substantially affect serotonin recognition does increase the apparent affinity for serotonin when combined with Y434F/K446Q/D461N (K328E/Y434F/K446Q/D461N) and with F464Y (K328E/F464Y) (Table II). Taken together, these results indicate that residues from TMD5–8 of VMAT1 have a dominant effect on serotonin affinity over VMAT1 residues from TMD9–12. In addition, the $V_{\text{max}}$ for serotonin transport by all the individual and multiple mutants is equivalent to that of the wild-type VMATs (100–200 pmol/min/mg; data not shown), suggesting comparable levels of expression and turnover. Importantly, the combination of P237A with A440T, a mutation from TMD9–12 that increases serotonin affinity, further enhances serotonin recognition to produce a transport protein with an apparent affinity for serotonin ($K_m$; 40 nM) seven times greater than that of wild-type VMAT2 ($K_m$; 270 nM), suggesting that the two residues independently contribute to substrate recognition (Fig. 2). However, the ability of multiple residues in TMD5–8 of VMAT1 to increase substrate recognition makes it more likely that these substitutions act indirectly through changes in protein conformation rather than through direct interactions with the ligand.

**Tryptamine Recognition**—Previous analysis of residues in TMD9–12 has shown that the replacement of Tyr-434 in VMAT2 with Phe from VMAT1 increases the recognition of tryptamine, and this single residue accounts for all of the difference in tryptamine recognition between the two VMATs (9). Since Pro-237 and Tyr-434 both contribute to histamine recognition, we considered that Pro-237 may also influence tryptamine recognition. Indeed, like Y434F, mutation P237A completely converts VMAT2 into VMAT1 with respect to the recognition of tryptamine (Table III). Since P237A increases
Individual residues from VMAT2 were converted into the corresponding VMAT1 residue using site-directed mutagenesis. Membranes were prepared from COS1 cells transfected with the indicated cDNAs and incubated at 29°C for 1 min in [3H]serotonin. The reaction was terminated by rapid dilution followed by filtration, and the bound radioactivity was measured by scintillation counting. Tetrabenazine sensitivity and histamine recognition were assessed by measuring the amount of ligand required to inhibit serotonin transport by 50% (IC$_{50}$). A range of serotonin concentrations was used to determine the $K_a$ values for serotonin from Lineweaver-Burk analysis. Uptake for zero time at 0°C was subtracted as background. The values shown were calculated from three separate experiments and are expressed as mean ± S.D. All values were compared to the value obtained from wild-type VMAT2 using the unpaired two-tailed $t$ test.

The combination of mutations from TMD5–8 and TMD9–12 influence the recognition of serotonin, histamine, and tryptamine by VMAT2. Membranes were prepared, transport was assayed, and the results were displayed as described in Table I. Each value was compared to wild-type VMAT2 using the unpaired two-tailed $t$ test.

The apparent affinity for serotonin and tryptamine differs from serotonin only in the absence of the 5-hydroxyl group, alanine at position 237 appears to promote the recognition of some feature in common between these two ligands. Other residues that increase apparent affinity for serotonin may therefore also promote tryptamine recognition. Indeed, the A313T mutation significantly increases tryptamine as well as serotonin recognition. Furthermore, if these substitutions recognize a feature common to both serotonin and tryptamine and Tyr-434 recognizes the 5-hydroxyl group present on serotonin but absent from tryptamine as suggested in previous studies (9), then the combination of either P237A or A313T with Y434F should further increase tryptamine recognition. Indeed, the recognition of tryptamine by triple mutant P237A/Y434F/K446Q/D461N (IC$_{50}$: 71 nM) significantly exceeded that of P237A (IC$_{50}$: 470 nM) and Y434F (IC$_{50}$: 350 nM) alone ($p < 0.01$). Triplicate mutant A313T/Y434F/K446Q/D461N also recognizes tryptamine with higher affinity than A313T and Y434F alone ($p < 0.05$). Thus, VMAT1 residues in TMD5–8 interact with a feature of the indoleamino ligand distinct from that recognized by the phenylalanine residue in TMD11 of VMAT1. Conversely, Pro-237 of VMAT2 appears to hinder the interaction with a feature of
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The assays were performed, and the results are displayed as described in Table I. The values for wild-type VMAT2 mutants (Y434F/K446Q/D461N and P237A/Y434F/D461N) were compared to wild-type VMAT2 using the unpaired two-tailed t test, whereas the values for the VMAT1 mutants (F434Y/Q446K/N461 and A237P/F434Y/Q446K/N461D) were compared to wild-type VMAT1.

**Table IV**

| Ligand | VMAT1 (µM) | VMAT2 (µM) |
|--------|------------|------------|
| Tetrabenazine | 4.27 ± 0.72 | 0.52 ± 0.21 |
| Histamine | 305 ± 92 | 33 ± 14 |
| Serotonin | 0.47 ± 0.17 | 0.27 ± 0.04 |

| Ligand | VMAT2 substitutions into VMAT1 | VMAT2 substitutions into VMAT1 |
|--------|--------------------------------|--------------------------------|
| Tetrabenazine | 2.39 ± 0.18 | 159 ± 9 |
| Histamine | 0.67 ± 0.12 | 0.06 ± 0.01 |
| Serotonin | 119 ± 29 | 0.06 ± 0.01 |

The ligand common to both serotonin and tryptamine, whereas Tyr-434 hinders the recognition of tryptamine but not serotonin. Tyr-434 presumably interacts with the hydroxyl group on serotonin and the residues in TMD5–8 with some other feature common to tryptamine and serotonin (Fig. 2).

**VMAT1 Mutations**—To determine whether the residues required for the high affinity interactions characteristic of VMAT2 also suffice to confer high affinity, we introduced these residues from VMAT2 into the equivalent positions of VMAT1. We first constructed the triple mutant F434Y/Q446K/N461D that contains three major residues from TMD9–12 of VMAT2, since this residue influences histamine, serotonin, and tryptamine recognition. Analysis of these triple and quadruple mutants shows that these replacements do not confer high affinity interactions with tetrabenazine, histamine, or serotonin (Table IV). Indeed, both VMAT1 mutants recognize these ligands very poorly, suggesting that, rather than simply failing to restore the VMAT2 phenotype, the mutations have interfered with some aspect of protein structure. Further, neither VMAT1 mutant transports [3H]histamine, and the apparent affinity of these mutants for dopamine is also greatly reduced (data not shown), suggesting a general defect in ligand recognition. Nonetheless, the mutant A237P/F434Y/Q446K/N461D, which lacks A237P (Table IV), supporting a dominant role for TMD5–8 of VMAT1 in substrate recognition. In addition, A237P/F434Y/Q446K/N461D recognizes tryptamine very poorly, whereas the recognition of tryptamine by F434Y/Q446K/N461D resembles wild-type VMAT2 (Table III), consistent with a role for residues in TMD5–8 and specifically, the alanine (in TMD5) in tryptamine recognition.

**Conclusion**—In summary, multiple residues contribute to the high affinity interaction of VMAT2 with ligands. The increased sensitivity to tetrabenazine appears to derive independently from Ala-315 in TMD7 and Tyr-434, Lys-446, and Asp-461 in TMD9–12. The ability of VMAT2 to recognize histamine requires Pro-237 in TMD5 as well as Tyr-434 and Asp-461 in TMD9–12, and the comparison of single and multiple mutants suggests that although not all the residues involved in histamine recognition have been identified, Pro-237 and TMD9–12 contribute to a single recognition site. In contrast, essentially all of the residues from TMD5–8 of VMAT1 increase serotonin affinity relative to VMAT2, and this effect dominates over mutations in TMD9–12, strongly supporting distinct roles for the two regions in substrate recognition. Similar to Tyr-434 in TMD9–12, replacement of Pro-237 and other residues in TMD5–8 of VMAT2 with equivalent residues from VMAT1 improves tryptamine recognition. However, unlike VMAT1 substitutions into TMD5–8 of VMAT2 that increase serotonin recognition, Y434F impairs the apparent affinity for serotonin, further indicating that the two sites interact with distinct features of the ligand. Thus, although the recognition of histamine appears to involve a single recognition site contributed by both major domains, VMAT2 recognizes the other ligands through multiple independent interactions.

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