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Identification of the Substrate Binding Region of Vesicular Monoamine Transporter-2 (VMAT-2) Using Iodoaminoflisopolol as a Novel Photoprobe

Anupama Gopalakrishnan, Michael Sievert, and Arnold E. Ruoho

Department of Pharmacology, University of Wisconsin Madison, Madison, Wisconsin

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ABSTRACT

Monoamines, such as serotonin, dopamine, and norepinephrine, are sequestered into synaptic vesicles by specific transporters (vesicular monoamine transporter-2; VMAT2) using energy from an electrochemical proton gradient across the vesicle membranes. Based on our previous studies using photoaffinity-labeling techniques in characterizing the VMAT2-specific ligands ketanserin and tetrabenazine, this study describes the synthesis and characterization of a fluorenone-based compound, iodoaminoflisopolol (IAmF), as a photoprobe to identify the substrate binding site(s) of VMAT2. Using vesicles prepared from rat VMAT2 containing recombinant baculovirus-infected Sf9 cells, we show the inhibition of [^{3}H]5-hydroxytryptamine (5-HT) uptake and [^{3}H]dihydrotetrabenazine (TBZOH) binding by aminoflisopolol and iodoaminoflisopolol. The interaction of [^{125}I]IAmF with VMAT2 is highly dependent on the presence of ATP and an intact proton gradient. We report a simple and novel method to distinguish between a ligand and substrate using classic compounds such as [^{3}H]5-HT and [^{3}H]TBZOH by incubating the compound with the vesicles followed by washes with isotonic and hypotonic solutions. Using this method, we confirm the characterization of IAmF as a novel VMAT2 substrate. Sf9 vesicles expressing VMAT2 show reserpine- and tetrabenazine-protectable photolabeling by [^{125}I]IAmF. [^{125}I]IAmF photolabeling of recombinant VMAT2, expressed in SH-SY5Y cells with an engineered thrombin site between transmembranes 6 and 7, followed by thrombin digestion, retained photolabel in a 22-kDa fragment, indicating that iodoaminoflisopolol binds to the C-terminal half of the VMAT2 molecule. Thus, IAmF possesses a unique combination of VMAT2 substrate properties and a photoprobe and is, therefore, useful to identify the substrate binding site of the vesicular transporter.

The vesicular monoamine transporter-2 (VMAT2) is the main transporter protein involved in sequestration of cytoplasmic neurotransmitters such as dopamine, serotonin, and norepinephrine into vesicles for storage and subsequent release (Henry et al., 1994; Peter et al., 1995; Erickson et al., 1996; Erickson and Varoqui, 2000) and is inhibited by reserpine, tetrabenazine (Scherman et al., 1983), and ketanserin (Darchen et al., 1988). The energy for amine transport is derived from a proton gradient generated by ATP hydrolysis. The proton gradient is coupled, by an unknown mechanism, to the transport of biogenic amines into the synaptic vesicle against a steep concentration gradient (Schuldiner, 1994; Rudnick, 1998; Schuldiner et al., 1998). Two protons are released from the storage vesicle in exchange for one substrate molecule transported to the inside of the vesicle (Kanner and Schuldiner, 1987). The rat synaptic vesicular monoamine transporter (rVMAT2) contains 515 amino acids, and a hydrophobic analysis of rat VMAT2 predicts 12 α-helical transmembrane segments, with a predicted large lumenal loop between TM1 and TM2 (Erickson et al., 1992).

Photoaffinity labeling is an extremely useful technique that enables the direct probing of a target protein through a covalent bond between a ligand and its binding protein. Using photoprobes, previous studies from our laboratory identified the ketanserin and tetrabenazine (TBZ) binding regions of VMAT2 (Sievert and Ruoho, 1997). Analyses of the binding site peptides showed that although the ketanserin photoprobe [^{125}I]7-iodo-8 azido ketanserin ([^{125}I]AZIK) derivatized mainly the N-terminal region, the TBZ photoprobe [^{125}I]2-*N*-[(3′-iodo-4′-azidophenyl)propionyl]tetrabenazine labeled both the N- and C-terminal portions of the VMAT2 molecule. The binding of both ketanserin and TBZ are not dependent on the proton gradient, indicating that they bind

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ABBREVIATIONS: VMAT2, vesicular monoamine transporter-2; IAmF, iodoaminoflisopolol; AZIK, 7-iodo-8-azido ketanserin; TBZ, tetrabenazine; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; TBZOH, dihydrotetrabenazine; 5-HT, 5-hydroxytryptamine; β_2AR, β_2-adrenergic receptor; SH, sucrose-HEPES; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; TM, transmembrane.

to similar conformational states of the VMAT2 molecule (Darchen et al., 1988). Another important inhibitor of VMAT2, reserpine, is believed to have a low-affinity binding site (which does not require the proton gradient) and a high-affinity site (which requires the presence of a proton gradient) (Weaver and Deupree, 1982). It is hypothesized that reserpine begins to be transported in the same way as substrates such as serotonin or dopamine; however, because of the bulkiness of the compound, reserpine becomes trapped in the protein such that it can be neither transported nor readily released. This leads to the formation of a "dead-end" complex, which may explain the "irreversible" nature of this inhibitor (Rudnick et al., 1990).

Our previous studies have successfully used photoprobes to identify specific binding regions of interacting proteins and drug interaction with receptors (Sievert and Ruoho, 1997; Wu et al., 2001; Sievert et al., 2002; Guo et al., 2005, 2006). In a previous study, in an attempt to develop compounds capable of directly probing the catechol binding region of the β_2-adrenergic receptor (β_2AR), our laboratory synthesized novel benzophenone- and fluorenone-based β_2AR antagonists as photoaffinity probes (Wu and Ruoho, 2000; Wu et al., 2001). Although the benzophenone-containing ligands bound with relatively modest affinity, one of the fluorenone-based compounds, 4-(2-hydroxy-3-isopropylaminopropoxy)-7-amino-6-iodofluorenone (iodoaminoflisopolol, IAmF), showed very high affinity for the β_2AR, inhibiting [^{125}I]ICYP binding with an apparent K_i value of approximately 1×10^{-9} M (Wu and Ruoho, 2000). In comparison with the benzophenone ligands, the fluorenone ligands have one additional carbon-carbon bond that creates a planar unsaturated ring system that leads to a large increase in receptor binding affinity. Figure 1, A and B, shows the chemical structures of serotonin, ketanserin, reserpine, tetrabenazine, and iodoaminoflisopolol (with the pharmacophore region in boldface type). Unlike previous photoaffinity ligands, an attractive and unique feature of the fluorenone derivative IAmF is that the large planar unsaturated ring, which is similar to the indole ring of serotonin and reserpine, serves as both the binding pharmacophore and the photoreaction center for this molecule (Fig. 1E). Therefore, based on the characteristics of this compound, we tested and found that aminoflisopolol inhibits [^{3}H]5-HT uptake in chromaffin granules with a low micromolar K_i value. In this study, we present data demonstrating that iodoaminoflisopolol shows VMAT2-specific substrate-like properties and is a photoprobe and, therefore, is ideally suited to probe the substrate-binding site on the VMAT2 molecule.

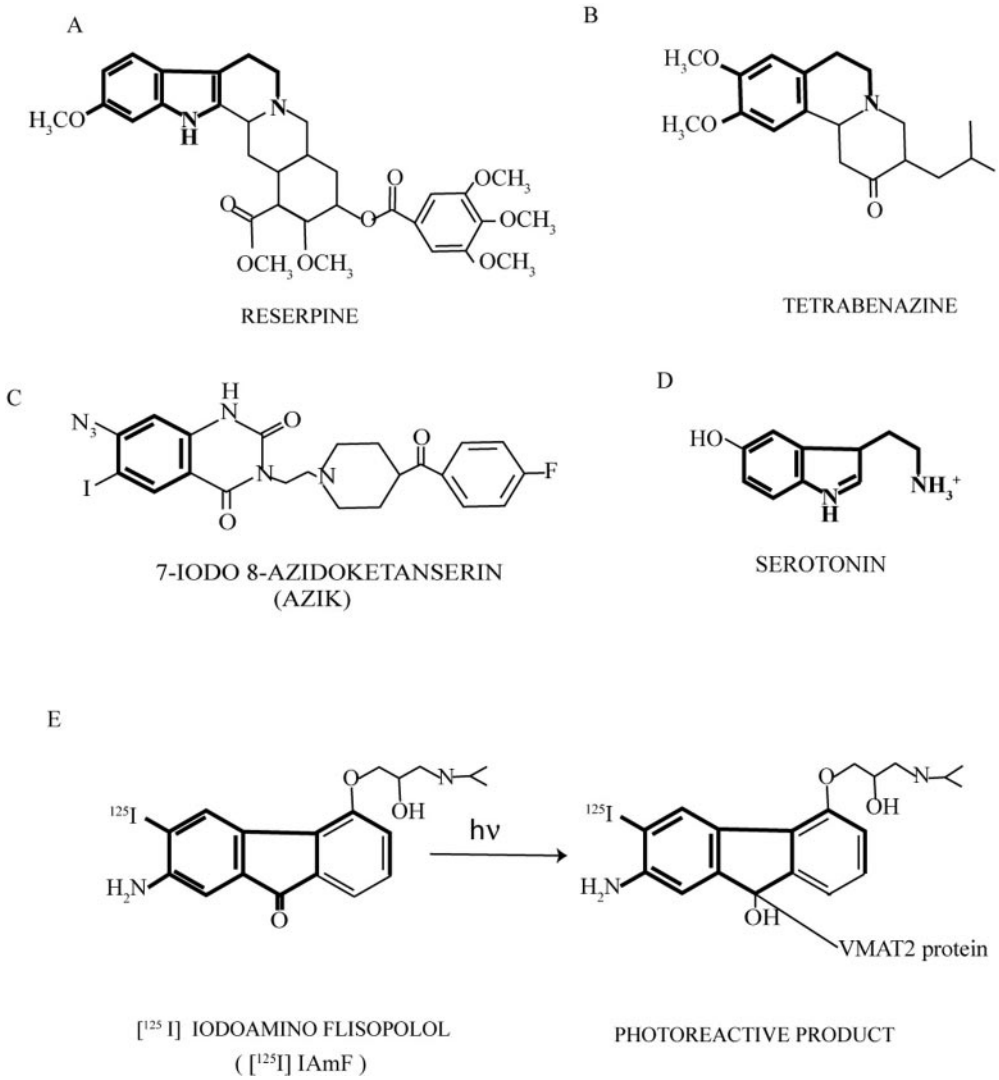

Fig. 1. The chemical structures of reserpine, tetrabenazine, 7-iodo 8-azido ketanserin, serotonin, and IAmF with the pharmacophore (in boldface type). Also shown is the photochemical reaction of IAmF with VMAT2 and the proposed mechanism of covalent bond formation during the photolysis reaction (E).

Materials and Methods

Expression of rVMAT2 in Sf9 Cells. The full-length cDNA for the rVMAT2 containing a C-terminal polyhistidine epitope was engineered into baculovirus DNA for expression in *Spodoptera frugiperda* (Sf9) insect cells (Sievert et al., 1998). Sf9 cells were grown in suspension cultures in Ex-cell 420 media (SAFC Biosciences, Lenexa, KS) at 25°C in flasks maintained at 110 rpm. Plaque-purified viral lysate was used for infection of log-phase Sf9 cells (density, 1×10^6 cells/ml), and cells were harvested 48 to 60 h later. Cells typically looked large and infected with no increase in cell number and showed less than 5% lysis.

[^{3}H]5-HT Uptake. Sf9 cells infected with rVMAT2 containing baculovirus were harvested and suspended in 0.32 M sucrose and 10 mM HEPES, pH 7.6, passed through a cell cracker 20 times, and centrifuged at a low speed (3000 rpm/5 min) to obtain a crude vesicle preparation, which was used to assess [^{3}H]serotonin uptake. Uptake assays were performed by incubating the vesicle preparation in SH buffer at 32°C for 10 min in the presence of ATP-Mg (10 mM), thus allowing for the formation of a proton gradient followed by the addition of [^{3}H]5-HT (20 nM; 30 Ci/mmol) (PerkinElmer Life and Analytical Sciences, Waltham, MA). The reaction was terminated after 7 min followed by a rapid vacuum filtration through GF-B filters (Brandel, Gaithersburg, MD) using a cell harvester. The filters were washed three times with SH buffer, and the radioactivity retained on the filter disks was determined in a liquid scintillation counter. The nonspecific uptake of [^{3}H]5-HT was subtracted from total uptake by including samples containing reserpine (10 μM) (Sigma-Aldrich, St. Louis, MO).

[^{3}H]TBZOH Binding. Specific [^{3}H]TBZOH binding was determined by incubating the vesicular preparations with [^{3}H]TBZOH (20 nM) (20 Ci/mmol) (Sievert et al., 1998) in the absence or presence of TBZ (10 μM) (Sigma-Aldrich) at 32°C for 60 min. After the incubation, the samples were rapidly filtered through GF-B filters using a cell harvester. The filters were washed three times with SH buffer, and the radioactivity retained on the filter disks was determined in a liquid scintillation counter.

To assess the nature of the interaction of [^{125}I]IAmF with VMAT2, the compound was incubated with vesicle preparation obtained from infected Sf9 cells in the presence or absence of ATP. In addition, the effects of the V-type ATPase inhibitor, bafilomycin A1 (10 μM) (Sigma-Aldrich), or a proton gradient releaser carbonylcyanide-*m*-chlorophenylhydrazone (CCCP; 1 μM) (Sigma-Aldrich) on the interaction between [^{125}I]IAmF and the VMAT2 protein were studied. Specificity was determined by tetrabenazine inhibition. Similar experiments were performed with [^{3}H]5-HT and [^{3}H]TBZOH to obtain typical results for the known substrate (serotonin) and known inhibitor (TBZ). Binding and uptake experiments were performed in triplicate, and statistical analysis of data was performed using Prism 4 software (GraphPad Software Inc., San Diego, CA).

Iodination of Aminoflisopolol. The fluorenone compound aminoflisopolol, originally synthesized and identified as a β-2 adrenergic antagonist (Wu and Ruoho, 2000), was radioiodinated using carrier-free [^{125}I]NaI (PerkinElmer) in the presence of Chloramine T (Sigma-Aldrich). The compound was extracted using ethyl acetate and streaked on a thin-layer chromatography plate. Upon chromatography in solvent system containing methanol/ethyl acetate/triethyl amine (10:10:1), the plate was exposed to an X-ray film, and the radioactive band of the corresponding area was scraped off the plate. The product was extracted in methanol and estimated for radioactivity. Carrier-free [^{125}I]AZIK was synthesized by following a protocol described earlier (Sievert et al., 1998). In brief, 7-aminoketanserin was iodinated with [^{125}I]NaI in the presence of Chloramine T, and purified 7-amino-8-[^{125}I]iodoketanserin was converted to 7-azido-8-[^{125}I]iodoketanserin by reaction with ice-cold $NaNO_2$ and 1 M NaN_3 in the dark and extracted with ethyl acetate.

Photolabeling VMAT2 Using [^{125}I]IAmF and [^{125}I]AZIK. Sf9 cells infected with recombinant baculovirus were used to prepare vesicles as described above. The tubes containing vesicle preparations were transferred to ice, and [^{125}I]IAmF (1.5 nM) or [^{125}I]AZIK (1 nM) was added. After incubation on ice for 5 min, the tubes were moved to a waterbath at 32°C for 2 min and were photolyzed for 5 s in ice water at a distance of 10 cm from a water-jacketed 1-kW high-pressure mercury vapor lamp (AH-6 bulb; Advanced Radiation Corporation, Santa Clara, CA). Figure 1E shows the proposed photoreaction and covalent modification mechanism of transporter derivatization by [^{125}I]IAmF during photolysis. After photolabeling, vesicles were solubilized with equal volumes of 2% digitonin (Gallard Schlesinger, Carle Place, NY) for 1 h in a cold room, and the soluble fraction was collected by centrifugation at 14,000*g*/30 min at 4°C. Soluble fractions were electrophoresed on a 12% SDS-PAGE gel and exposed to a PhosphorImager (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Because the rVMAT2 possessed a 6× His-tag at its C terminus, the transporter was partially purified using a nickel-nitrilotriacetic acid resin (Sigma-Aldrich). In some experiments, VMAT2-specific antibody (or IgG as control) and Protein A beads were used sequentially to pull down VMAT2 from the digitonin-solubilized fraction (Sigma-Aldrich) following the manufacturer's protocols. The beads were washed, and bound proteins were eluted with 1× Laemmli buffer, electrophoresed on a 12% SDS-PAGE, and exposed to a PhosphorImager.

Photolabeling of Recombinant VMAT2 in SH-SY5Y Vesicles. SH-SY5Y cells (American Type Culture Collection number CRL-2266) were grown in 10-cm dishes in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% cosmic calf serum and penicillin-streptomycin to 50 to 70% confluence in a 37°C/5% CO_2 incubator. Plasmid (25 μg) encoding deglycosylated, HA-tagged hVMAT2 containing an engineered thrombin site between putative TM6 and TM7 (Thiriot et al., 2002) was transfected using the *Trans*IT-LT1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's recommendations and harvested 42 h later. The cells were suspended in 1 ml of sucrose-HEPES buffer, pH 7.6, passed through a 27-gauge needle three times, and centrifuged at 1200*g* for 5 min to obtain a crude vesicle preparation. This vesicle preparation was used for photolabeling using the procedure outlined above.

Thrombin Digestion. Photolabeled vesicle preparations from SH-SY5Y cells, transfected with VMAT2 construct, containing thrombin site in between TM6 and TM7 were digested with 0.2 units of thrombin (Promega, Madison, WI) at 37°C for various times. Reactions were stopped with protease inhibitor cocktail (Sigma Aldrich) and snap-frozen in dry ice. The samples were denatured with 50 mM dithiothreitol in 1× Laemmli buffer and separated by electrophoresis on a 12% SDS-PAGE to observe thrombin digestion pattern. In another experiment, the electrophoresed samples were transferred to polyvinylidene difluoride membrane and immunoblotted with C-terminal anti-His antibody (Invitrogen, Carlsbad, CA) using a Western blot protocol.

Results

Baculovirus-Infected Sf9 Cells Express Functional VMAT2. Vesicles prepared from recombinant VMAT2-expressing baculovirus-infected Sf9 cells show increased binding of [^{3}H]TBZOH with increasing concentrations of the ligand. The nonspecific binding (in the presence of 10 μM TBZ) was typically less than 10% of the total ligand bound to the vesicles. K_d and B_{max} values were determined by nonlinear regression of the saturation curves as 28 nM and 119 pmol/mg of protein, respectively. Vesicle preparation from 2- or 3-day baculovirus-infected Sf9 cells expressing VMAT2 showed uptake of [^{3}H]serotonin, which was dependent on the presence of ATP-Mg in the assay buffer (Fig. 3A). This uptake was specifically inhibited by reserpine, thus confirming the presence of functional VMAT2 in the Sf9 cells. Nonin-

fected Sf9 cells did not show any reserpine-protectable substrate uptake or TBZ-protectable ligand binding (data not shown). This is the first report of an ATP-dependent, reserpine-protectable VMAT2-mediated uptake in vesicles using the baculovirus expression system.

[^{125}I]IAmF Shows VMAT2 Substrate-Like Properties. In a displacement study, increasing concentrations of aminoflisopolol or iodoaminoflisopolol were used to inhibit [^{3}H]TBZOH (20 nM) binding or [^{3}H]5-HT uptake (30 nM) in vesicle preparations obtained from Sf9 cells 3 days after baculovirus infections. Nonspecific binding (in the presence of 10 μM TBZ or 10 μM reserpine) was subtracted from the respective values. Figure 2, A and B, shows the inhibition of [^{3}H]TBZOH binding and [^{3}H]5-HT uptake by IAmF with K_i values of 8 and 0.8 μM, respectively. On the other hand, the highest concentration of aminoflisopolol (the noniodinated precursor of IAmF) showed less than 50% inhibition of [^{3}H]TBZOH binding (Fig. 2C) but showed a K_i value of 0.3 μM when assayed for inhibition of [^{3}H]5-HT uptake (Fig. 2D).

The low micromolar K_i value of IAmF inhibition of [^{3}H]5-HT uptake is very similar to K_i values of other established VMAT2 substrates (Erickson et al., 1996) In addition, the ratio of K_i binding to K_i uptake is 10, supporting the substrate nature of IAmF. It has been observed in previous studies by Rothman's group that whereas substrates show an increased values of the ratio of K_i binding to K_i uptake, inhibitors show values less than 3 (Rothman et al., 1999). Similar experiments in our lab established a value of 0.1 for a known inhibitor of VMAT2 ketanserin and 205 for a known substrate, norepinephrine (data not shown). These observations led us to further address the possibility that IAmF is a substrate for VMAT2.

To assess the substrate characteristics of [^{125}I]IAmF, we tested the interaction of [^{125}I]IAmF with VMAT2 by incubating the compound with vesicles prepared from infected Sf9 cells in the absence or presence of ATP, bafilomycin A1, and CCCP. As seen in Fig. 3A, a typical substrate such as [^{3}H]5-HT showed almost no specific uptake in the absence of ATP and in the absence of a proton gradient. [^{3}H]TBZOH binding, on the other hand, was not significantly affected under any condition. [^{125}I]IAmF interaction with VMAT2, like [^{3}H]5-HT, was highly dependent on the presence of ATP and also on the proton gradient, as observed by loss the of specific interaction in the absence of ATP or in the presence of ATP along with the V-type ATPase inhibitor bafilomycin (10 μM) or CCCP, a proton gradient releaser (1 μM).

As further proof, we used a simple experiment to distinguish between a substrate (defined as a compound that is taken up into the lumen of the vesicle by an uptake mechanism) and an antagonist (i.e., a compound that only binds to the VMAT2 protein) using classic compounds such as [^{3}H]5-HT and [^{3}H]TBZOH. These two scenarios could be distinguished by comparing the radiolabel retention after washes with isotonic (SH buffer: 0.32 M sucrose and 10 mM HEPES, pH 7.6) or hypotonic (lysis buffer: 1 mM HEPES, pH 7.6). In the case of a substrate, wash with hypotonic buffer would cause lysis of the vesicles, leading to loss of the radiolabeled compound sequestered during the assay. However, little or no loss of the photolabel is expected by washing with hypotonic buffer if the compound in question is only bound to the VMAT2 in the membrane. As expected, treating the VMAT2-expressing Sf9 vesicles with hypotonic buffer after [^{3}H]5-HT uptake led to more than 80% loss of radiolabeled 5-HT, whereas less than 15% loss of radiolabel was observed when similar vesicles were treated with hypotonic buffer after [^{3}H]TBZOH binding (Fig. 3B). To determine whether [^{125}I]IAmF is a substrate or an antagonist, vesicles expressing VMAT2 were incubated with [^{125}I]IAmF in the presence of ATP-Mg and in the presence or absence of reserpine. One set of vesicles was washed with SH buffer during filtration,

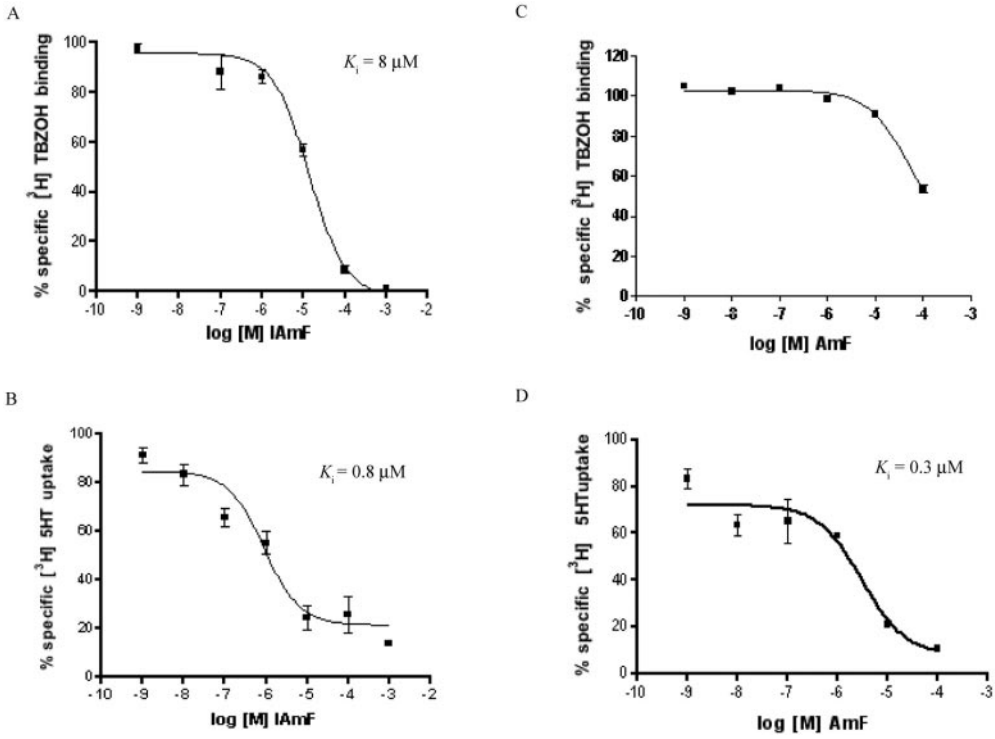

Fig. 2. A, binding of [^{3}H]TBZOH (20 nM) was assayed in triplicate in 2-day postinfected Sf9 cell vesicle preparation in the presence of increasing concentrations of IAmF (A) and AmF (C). Binding in the presence of TBZ (10 μM) was subtracted from each of the values. Uptake of [^{3}H]5-HT (30 nM) was assayed in triplicate in a Sf9 cell vesicle preparation, 2 days after baculovirus infection, in the presence of increasing concentrations of IAmF (B) and AmF (D) The assay buffer contained 0.3 M sucrose and 10 mM HEPES, pH 7.6, and 10 mM ATP-Mg. Uptake in the presence of reserpine (10 μM) was subtracted from each of the values.

whereas another set was washed with lysis buffer. Reserpine-protectable uptake was measured and compared from filters washed with SH buffer and from lysis buffer in three separate experiments (Fig. 3B). Significant loss of radiolabel (77%) after washing of vesicles with lysis buffer indicated that [^{125}I]IAmF interacted with VMAT2 as a substrate.

Because our studies showed that [^{125}I]IAmF behaved as a substrate (i.e., is actively transported into the lumen of the vesicle) instead of binding to VMAT2 as an antagonist, we determined the K_m value of VMAT2 for [^{125}I]IAmF in a transport assay. In this case, the carrier-free hot compound was supplemented with unlabeled IAmF to obtain concentrations from 250 nM to 200 μM, and the corrected specific activity values were used to calculate uptake in Sf9 vesicles expressing VMAT2 as picomoles per milligram of protein per min. The data obtained were fitted to a nonlinear curve fit using GraphPad Prism software to obtain a K_m and V_{max} values of 122 μM and 292 pmol/mg protein/min, respectively (Fig. 4). In the case of serotonin, saturation curves showed that VMAT2 in these vesicle preparations transport [^{3}H]5-HT with a K_m value of 359 nM and V_{max} value of 101 nmol/mg protein/min. Similar studies performed in the presence of 10 μM aminoflisopolol showed a significant decrease in K_m value (583 nM), with a small decrease in V_{max} value (81 nmol/mg protein/min).

[^{125}I]IAmF is a VMAT2 Photoprobe. Vesicles obtained from baculovirus-infected Sf9 cells were photolabeled with carrier-free [^{125}I]IAmF (1.5 nM) in the presence or absence of reserpine and TBZ (10 μM). Figure 5A(i) shows a PhosphorImager scan of reserpine and TBZ-protectable [^{125}I]IAmF-photolabeled VMAT2 as seen on a 12% gel. Figure 5A(ii) shows a scan of a parallel experiment using [^{125}I]AZIK as a photoprobe. [^{125}I]AZIK had been shown previously to photolabel VMAT2 specifically (Sievert et al., 1998) and had been determined to bind at the TBZ binding site (Darchen et al., 1988).

As represented in Fig. 5A(iii), we used vesicles prepared from SH-SY5Y cells, transfected previously with hVMAT2, which contain a thrombin cleavage site between putative TM6 and TM7. This construct had been shown to express a functional protein capable of substrate uptake and ligand binding (Thiriot et al., 2002). In Fig. 5A(iii), we also show that [^{125}I]IAmF is capable of photolabeling this protein in a reserpine- and TBZ-protectable manner.

Because the rVMAT2 expressed in Sf9 cells has a 6× His tag, the photolabeled soluble fraction was passed through a

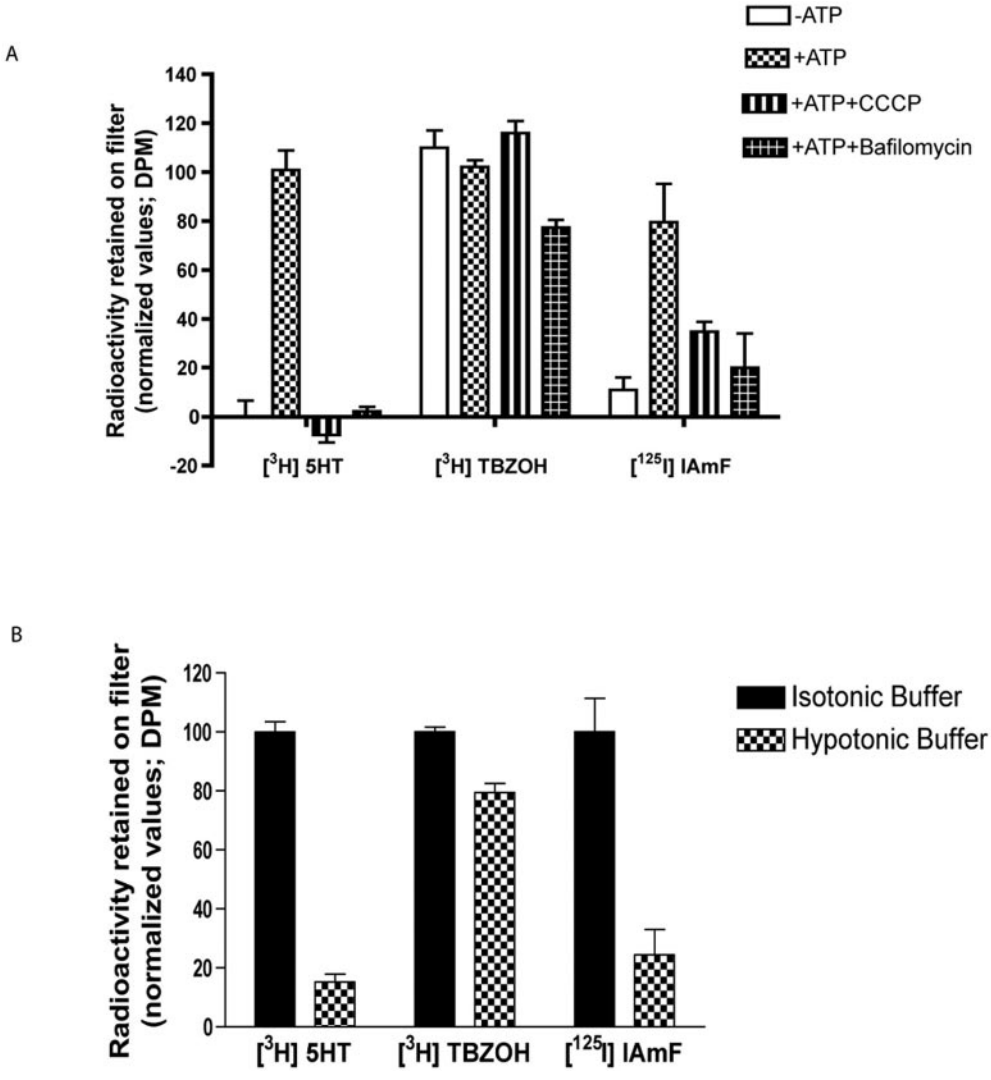

Fig. 3. A, radioactive 5-HT, TBZOH, and IAmF were allowed to interact with vesicles from infected Sf9 cells in the absence or presence of ATP (10 mM) or in the presence of bafilomycin A1 (10 μM) or CCCP (1 μM). VMAT2-specific radioactivity retained on the filter is shown normalized to values obtained in the presence of 10 mM ATP-Mg. B, demonstration of a method to distinguish between a substrate and ligand by allowing the compounds to interact with the Sf9 vesicles expressing rVMAT2, followed by isotonic or hypotonic buffer washes. Radioactivity retained on the filters is shown as normalized DPM values to the values obtained upon isotonic washes.

nickel resin, and rVMAT2 was partially purified by eluting with an imidazole (500 mM)-containing buffer. Figure 5B shows a PhosphorImager scan of photolabeled VMAT2 protein in a nickel column eluate. The presence of photolabeling in the nickel-imidazole elution further showed that [^{125}I]IAmF is able to specifically photolabel VMAT2. Fig. 5B, right, shows the lack of photolabeling in a vesicle preparation from uninfected Sf9 cells.

Photolabeled vesicle preparations, which were solubilized with equal volumes of 2% digitonin and then incubated sequentially with VMAT2 antibodies (or IgG) and Protein A-bound Sepharose, immunoprecipitated VMAT2 from the solubilized fraction. Bound proteins were eluted with SDS sample buffer and separated on a 12% gel. A PhosphorImager scan (Fig. 5C) shows that VMAT2 antibodies (and not IgG antibodies) are able to immunoprecipitate photolabeled VMAT2, thus further confirming that [^{125}I]IAmF specifically photolabeled VMAT2.

Thrombin Digestion of Photolabeled VMAT2. An [^{125}I]IAmF photolabeling experiment was performed in vesicles prepared from SH-SY5Y cells transfected with recombinant VMAT2 containing an engineered thrombin site in the loop between TMs 6 and 7 (Thiriot et al., 2002). Thrombin digestion of the photolabeled vesicle preparation retained the photolabel in a 22-kDa fragment, indicating that [^{125}I]IAmF cross-linked within the C-terminal half of the VMAT2 molecule [Fig. 6A, (i)]. The image in Fig. 6A, (ii) shows a PhosphorImager scan of reserpine protection of 22-kDa fragment obtained after thrombin cleavage, confirming that this fragment was obtained from the VMAT2 protein. Fig. 6(iii) shows a C-terminal anti-His Western blot of an SDS-PAGE gel, showing the presence of 6× His epitope in the full-length and 22-kDa fragment of photolabeled VMAT2 after thrombin digestion.

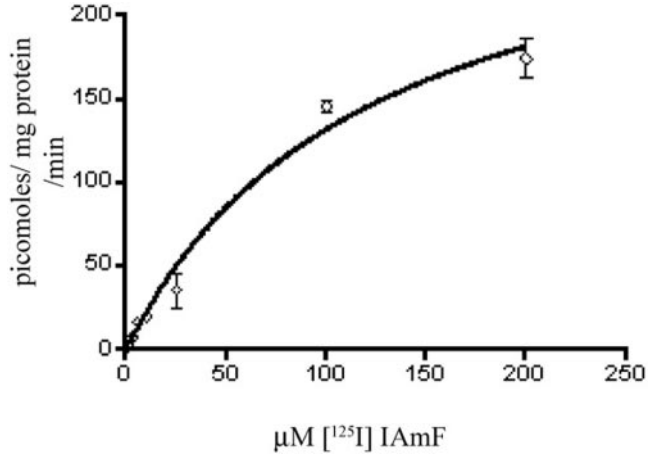

Fig. 4. Saturation uptake of [^{125}I]IAmF into Sf9 vesicles expressing VMAT2. The specific activity of carrier-free [^{125}I]IAmF was reduced by adding appropriate amounts of unlabeled IAmF to obtain high concentrations of the substrate. Vesicles were incubated with increasing concentrations of [^{125}I]IAmF in the presence of ATP-Mg for 15 min at 32°C and filtered using vacuum filtration on to GF-B disks. Radioactivity retained on the filter was counted in a γ-counter. Uptake was calculated as picomoles per milligram of protein per minute after correction of specific activity.

Discussion

Vesicular monoamine transporters are proteins that transport biogenic amines into storage vesicles so that neurotransmitters are available for exocytosis upon stimulation. This study describes the characterization of [^{125}I]IAmF as a tool to identify substrate binding sites on VMAT2 expressed in Sf9 cells. Specific [^{3}H]TBZOH binding and [^{3}H]5-HT uptake is entirely due to the infected baculovirus-expressed VMAT2, because uninfected Sf9 cells show negligible specific binding or uptake. Having established an efficient expression system in Sf9 cells, we characterized iodoaminoflisopolol as a possible ligand/substrate for VMAT2. This fluorenone-based compound has been successfully used as a photoprobe in our laboratory (Wu and Ruoho, 2000), and a similar compound was used to covalently modify the N terminus of parathyroid hormone receptor stably expressed in human embryonic kidney-293 cells (Han et al., 2000). Initial studies in our lab showed that AmF could inhibit [^{3}H]5-HT uptake in chromaffin granules with a K_i value of 0.4 μM.

In the current study, we used increasing concentrations of IAmF and/or AmF to inhibit [^{3}H]5-HT uptake and [^{3}H]T-

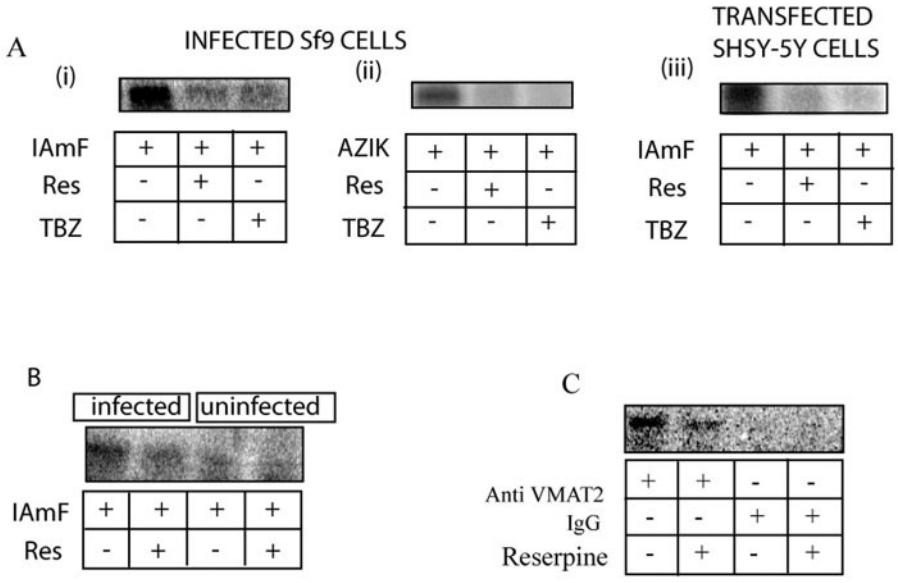

Fig. 5. A, protectable photolabeling of VMAT2 by [^{125}I]IAmF (i) and [^{125}I]AZIK (ii) in Sf9 vesicles. iii, protectable photolabeling of VMAT2 by [^{125}I]IAmF in vesicles from transfected SH-SY5Y cells. B, vesicles from infected and uninfected Sf9 cells were photolabeled with [^{125}I]IAmF, digitonin-solubilized, and purified through a nickel-nitrilotriacetic acid resin. Retention of [^{125}I]IAmF photolabeling of VMAT2 is shown by preincubation with TBZ or reserpine (10 μM). C, immunoprecipitation of photolabeled VMAT2 from digitonin solubilized Sf9 vesicles using rVMAT2 specific antibodies (and not IgG) and Protein A beads.

BZOH binding in VMAT2-containing Sf9 vesicles. IAmF and AmF showed K_i values for inhibition of substrate uptake in the same range as the K_m values for serotonin. However, whereas the highest concentration of AmF did not inhibit more than 50% of [^{3}H]TBZOH binding, an inhibition curve of [^{3}H]TBZOH binding with the iodinated form of IAmF yielded a K_i value of 8 μM. Previous studies have shown that known VMAT2 inhibitors such as tetrabenazine, ketanserin, and reserpine are potent inhibitors of both [^{3}H]TBZOH binding and [^{3}H]dopamine uptake, whereas known VMAT2 substrates (dopamine, norepinephrine, and serotonin) inhibit [^{3}H]TBZOH binding very poorly but show inhibition of [^{3}H]dopamine uptake in the high nanomolar to low micromolar range (Rothman et al., 1999; Partilla et al., 2006). A comparison of K_i values of AmF and IAmF in inhibition studies indicated that the addition of an iodine group adds bulk and renders the compound a poorer substrate compared with the noniodinated precursor compound, AmF.

The substrate characteristic of [^{125}I]IAmF is evident in its specific interaction with the VMAT2 protein (as evidenced by TBZ-protectable interaction) only in the presence of ATP and an intact proton gradient. In addition, after isotonic or hypotonic washes, [^{125}I]AmF behaved similarly to [^{3}H]5-HT, as

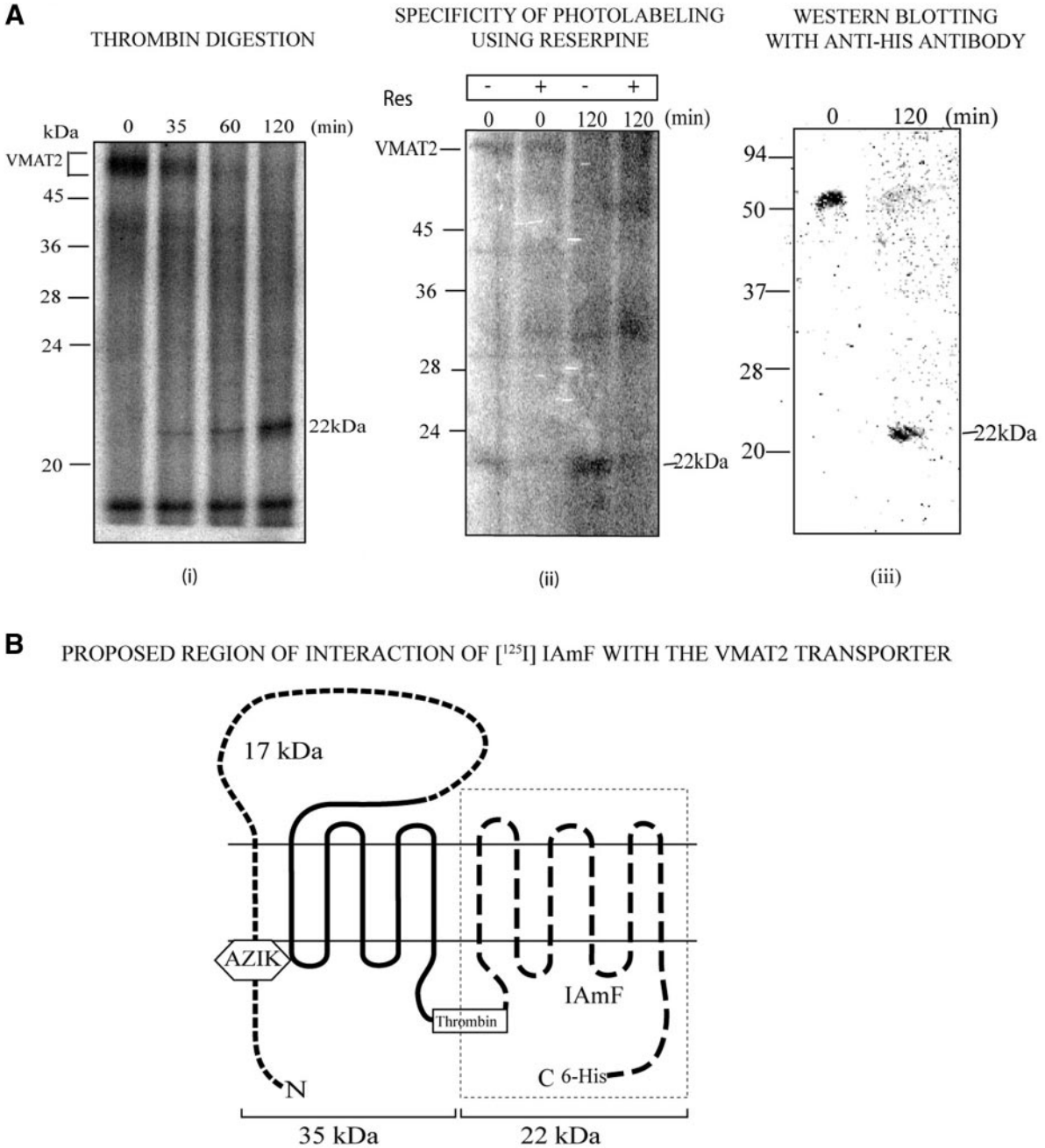

Fig. 6. Ai, time course of thrombin digestion of a [^{125}I]IAmF photolabeled vesicle preparation from SH-SY5Y cells transfected with hVMAT2 with an engineered thrombin site between TM6 and TM7 (Thiriot et al., 2002). ii, reserpine protection of the 22-kDa fragment, generated from thrombin digestion of photolabeled transporter. iii, Western immunoblotting of undigested and thrombin-digested photolabeled transporter with C-terminal anti-His antibody. B, the proposed sites of interaction of [^{125}I]IAmF with the VMAT2 molecule; also shown is the site of interaction of the inhibitor photolabel [^{125}I]AZIK, which derivatized Lys-20 of VMAT2 (Sievert and Ruoho, 1997).

77% of reserpine-protectable uptake was hypotonic lysis-sensitive, whereas a known antagonist/ligand ([^{3}H]TBZOH) maintained approximately 80% of bound radioactivity after washes in hypotonic buffer. These results agree with the hypothesis that [^{125}I]IAmF behaves as a substrate for VMAT2.

An earlier report showed stereoselective uptake of another β_2 receptor antagonist, atenolol, into storage granules isolated from PC12 cells and chromaffin granules. It is interesting that atenolol was also shown to be a substrate for VMAT2 (Bagwell et al., 1989) because the uptake was ATP-dependent, reserpine-protectable, and nigericin-sensitive. In previous studies, propranolol ((Street et al., 1984; Bright et al., 1985) and atenolol (Bright et al., 1985) had been shown to be accumulated in rat cortical synaptosomes and released upon stimulation by elevated K^+, Rb^+, or Cs^+ ions. These observations, together with the results of this study, indicate that low levels of β-receptor antagonists may, as a rule, be accumulated in synaptic vesicles and subsequently released into the synaptic cleft to further inhibit β-adrenergic receptors.

Reserpine-protectable uptake was studied in Sf9 vesicles using increasing concentrations of [^{125}I]IAmF. This nonlinear saturation curve yielded a K_m value of 122 μM, which further indicates that this compound has a relatively lower affinity for the transporter compared with the classic substrates such as serotonin. The apparent discrepancy between the K_m value for [^{125}I]IAmF uptake and the K_i value for [^{3}H]5-HT inhibition indicates that whereas mere binding of the [^{125}I]IAmF to the transporter is sufficient to inhibit [^{3}H]5-HT uptake, the larger K_m value indicates a slower transport across the vesicular membrane, thereby suggesting that the binding of the compound to the transporter is not the rate-limiting step of the transport process. The V_{max} value of [^{3}H]5-HT uptake (nanomolar values) is much higher than the V_{max} value of [^{125}I]IAmF (picomolar values), indicating that the latter is transported more slowly across the vesicular membrane. Saturation experiments performed in the presence of 10 μM aminoflisopolol showed a small change in V_{max} value but an increase in the K_m value of [^{3}H]5-HT uptake, indicating that aminoflisopolol inhibits [^{3}H]5-HT in a competitive manner and, therefore, is likely to bind to the same or to similar site(s) on the VMAT2 protein as does the substrate serotonin.

Photolabeling experiments demonstrated that [^{125}I]IAmF bound specifically to the transporter, because only by close proximity of the photolabel and transporter can there be a covalent modification. The specificity of the photoprobe was confirmed by the absence of photolabeling in the presence of reserpine (10 μM) and TBZ (10 μM). In addition, photolabeled VMAT2 in vesicle preparations from infected Sf9 cells (but not uninfected cells) could be partially purified through a nickel resin or immunoprecipitated by VMAT2-specific antibodies, which further confirmed the specific interaction of the photolabel with VMAT2.

The native VMAT2 molecule is not cleaved by thrombin because it does not possess a thrombin cleavage site (Thiriot et al., 2002). The construct used in these experiments has an HA epitope in the large loop between TM1 and TM2 and 6× His epitope at the C terminus apart from a thrombin site in between putative TM6 and TM7. Therefore a thrombin site, which was engineered midway into the VMAT2 sequence between putative TM6 and TM7, upon thrombin digestion yields two unequally sized fragments of 35 and 22 kDa. [^{125}I]IAmF photolabeling of VMAT2 expressed in SH-SY5Y cells followed by thrombin digestion retained radioactivity in the 22-kDa fragment, thus indicating that the C-terminal half of the molecule contained the [^{125}I]IAmF interaction site, which is different from the region modified by the antagonist photoprobe [^{125}I]AZIK. Thiriot et al. (2002) showed that HA epitope was detectable in the 35-kDa fragment after Western immunoblotting with anti-HA antibody. Therefore, the presence of HA epitope in the 35-kDa fragment and the presence of photoprobe and 6× His epitope in the 22-kDa fragment supports the conclusion that the photoprobe [^{125}I]IAmF interacts with the C-terminal region of the transporter. A small amount of radioactivity was detected at the 22-kDa region in the 0 time point, which may be due to an immediate partial cleavage of VMAT2 by thrombin.

Previous studies have used site-specific mutagenesis to determine the critical residues important for substrate and transport activity. These residues were chosen based on their importance in other related transporters such as the tetracycline transporter Tn10 (Merickel et al., 1995), because vesicular transporters are evolutionarily related to multidrug transporters of the Major Facilitator Superfamily (Schuldiner et al., 1995; Saier and Paulsen, 2001). Random mutations in BMR, a member of large family of H^+/substrate antiporters, gave rise to four independent mutants exhibiting altered spectra of cross-resistance to various drugs. All of these mutations were clustered in the region of TMs 9 to 11 (Klyachko et al., 1997) (the homologous proposed TMs are in the 22-kDa region of VMAT2). Likewise, residues important for drug specificity and recognition lie in TMs 10 and 11 (P-glycoprotein) (Hafkemeyer et al., 1998), TMs 8 and 9 and TMs 1 to 3 (TetA) (Yamaguchi et al., 1993), TMs 9 and 10 (lactose permease) (Kaback, 1992). Merickel et al. (1994) mutated several charged residues that were predicted to reside in transmembrane domains of VMAT2 and showed that Asp-33 was essential for substrate recognition. In addition, Lys-139 and Asp-427 in TM2 and TM11, respectively, form an ion pair, which promotes high-affinity interaction with the substrate. Schuldiner et al. (1995) speculated that the region of TMs 9 to 11 is involved in substrate binding more than any other region, but not exclusively. In VMAT2, most of the mutations affecting apparent substrate binding were found to lie in the region of TMs 1 to 3, 9, and 12 (Parsons, 2000). Based on these studies, in the future, we will test the hypothesis that [^{125}I]IAmF as a substrate photoprobe would derivatize one or more residues within the region of TMs 9 and 12.

Lactose permease, the most well-studied member of the major facilitator superfamily, has 12 TMs, most of which pack circumferentially around TM 7 to form a central pore region. Based on a lactose permease model, Parsons (2000) proposed similar packing of 12 TMs of VMAT2 around TM7 and proposed a rotating domain model, in which a proton and neurotransmitter bind to opposite sites of the rotating domain formed by TM7 and TM10 after entering separate dead-end chambers. This model would favor our proposed substrate binding region (TMs 7–12) based on the results of this study.

Our previous studies show that human VMAT2 Cys-126 in loop 1/2 and Cys-333 in loop 7/8 form a disulfide bond, which contributes to efficient monoamine transport (Thiriot et al., 2002). Therefore, based on mutagenesis and photolabeling

studies, TMs 1, 2, 7, 8, 10, and 11 are speculated to be in close proximity. Therefore, multiple TMs are likely to contribute to the formation of the substrate binding site. Because IAmF is a substrate for VMAT2 (as opposed to TBZ and AZIK, which are inhibitors), this specific unique photoprobe, therefore, provides an excellent tool to study and identify the substrate binding site on the VMAT2 protein to understand the mechanism of reuptake and sequestration of monoamines in storage vesicles.

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Address correspondence to: Dr. Arnold E. Ruoho, Department of Pharmacology, 4775 SMI, 1300 University Avenue, Madison WI 53705. E-mail: aeruoho@wisc.edu