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Specific Derivatization of the Vesicle Monoamine Transporter with Novel Carrier-Free Radioiodinated Reserpine and Tetrabenazine Photoaffinity Labels

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Abstract

Two iodophenylazide derivatives of reserpine and one iodophenylazide derivative of tetrabenazine have been synthesized and characterized as photoaffinity labels of the vesicle monoamine transporter (VMAT2). These compounds are: 18-O-[3-(3'-iodo-4'-azidophenyl)-propionyl]methyl reserpate (AIPPMER), 18-O-[N-(3'-iodo-4'-azidophenethyl)glycyl]methyl reserpate (IAPEGlyMER), and 2-N-[(3'-iodo-4'-azidophenyl)-propionyl]tetrabenazine (TBZAIPP). Inhibition of [³H]dopamine uptake into purified chromaffin granule ghosts showed IC₅₀ values of approximately 37 nM for reserpine, 83 nM for AIPPMER, 200 nM for IAPEGlyMER, and 2.1 μM for TBZ-AIPP. Carrier-free radioiodinated [¹²⁵I]IAPEGlyMER and [¹²⁵I]TBZ-AIPP were synthesized and used to photoaffinity label chromaffin granule membranes. SDS-PAGE analysis showed specific [¹²⁵I]IAPEGlyMER labeling of a polypeptide that migrated as a broad band (approximately 55-90 kDa), with the majority of the label located between 70-80 kDa. The labeling by [¹²⁵I]IAPEGlyMER was blocked by 100 nM reserpine, 10 μM tetrabenazine, 1 mM serotonin, and 10 mM (–)-norepinephrine and dopamine. Analysis of [¹²⁵I]TBZ-AIPP labeled chromaffin granule membranes by SDS-PAGE and autoradiography demonstrated specific labeling of a similar polypeptide, which was blocked by 1 μM reserpine and 10 μM tetrabenazine. Incubation of [¹²⁵I]TBZ-AIPP photolabeled chromaffin granule membranes in the presence of the glycosidase N-glycanase shifted the apparent molecular weight of VMAT2 to approximately 51 kDa. These data indicate that [¹²⁵I]IAPEGlyMER and [¹²⁵I]TBZ-AIPP are effective photoaffinity labels for VMAT2.

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¹Abbreviations used are: VMAT, synaptic vesicle monoamine translocator; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11βH-benzo[a]quinolizine); [³H]TBZOH, [2-³H]dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11βH-benzo[a]quinolizine); AIPPMER, 18-O-[3-(3'-iodo-4'-azidophenyl)propionyl]methyl reserpate; IAPEGlyMER, 18-O-[N-(3'-iodo-4'-azidophenethyl)glycyl]methyl reserpate; BAKER, 18-O-bromoacetyl methyl reserpate; TBZ-AIPP, 2-N-[(3'-iodo-4'-azidophenyl)propionyl]tetrabenazine; AIPP, 3-iodo-4-azidophenyl-propionic acid; AIPPS, 3-iodo-4-azidophenyl-propionic acid succinimide ester; THF, tetrahydrofuran; IAPA, 3-iodo-4-azidophenethylamine; TLC, thin-layer chromatography; IR, infra-red spectroscopy; NMR, nuclear magnetic spectroscopy; TMS, tetramethylsilane

Keywords

Vesicle Monoamine Transporter; VMAT2; reserpine; tetrabenazine; photoaffinity labels

Introduction

The vesicular monoamine transporter (VMAT) is an important transport protein for the storage of monoaminergic neurotransmitters into presynaptic vesicles. The storage of monoamines into vesicles allows the cell to recycle neurotransmitters and also protects the cell from potentially harmful monoamine metabolites. Altered VMAT structure/activity has been linked to diseases of the cardiovascular system [1;2], Parkinson's disease [3;4;5], and several psychiatric diseases including schizophrenia [6], depression [7;8], bipolar disorder [9], and addiction [10].

Chromaffin granules are subcellular organelles located within the chromaffin cell of the adrenal medulla that store large quantities of the catecholamines, epinephrine and norepinephrine. Since the chromaffin granule is a secretory vesicle, it has served as a model for studying neurosecretion [11;12;13]. Amines are transported into the lumen by VMAT, located within the chromaffin granule membrane [14;15]. Although both VMAT1 and VMAT2 are present in bovine chromaffin granules, VMAT2 is the predominate isoform [16;17]. The transport reaction is driven by an electrochemical proton gradient that is generated by a proton translocating ATPase via an unknown mechanism [18]. The stoichiometry of transport is one biogenic amine molecule in for two protons out [19].

From cloning studies, the bovine VMAT2 contains 518 amino acids and is predicted to contain 12 transmembrane helices [16], similar to other known transport proteins, both mammalian [20] and bacterial [21;22]. The primary sequence of bovine VMAT2 predicts three potential sites of N-linked glycosylation in a large luminal loop between transmembrane helices 1 and 2. A disulfide bridge has been identified in the human VMAT2 between Cys126 and Cys333 [23]. These cysteines are on the luminal surface of VMAT, but the role of this disulfide bridge on VMAT function is currently unknown.

Several compounds have been shown to inhibit VMAT2, the most potent being the *Rauwolfia* alkaloid, reserpine. The structure of reserpine consists of a five-ring alkaloid system with a trimethoxybenzoyl moiety connected to it by an ester linkage. The five-ring complex includes a portion that is analogous to 5-hydroxytryptamine (serotonin), a substrate of the transport protein. [³H]Reserpine has been observed to bind to the transporter with both a high affinity (30 pM [24]) and a low affinity (25 nM [25]). The high affinity site is dependent upon the presence of an electrochemical proton gradient across the vesicle membrane, whereas the low affinity site is not. In chromaffin granule membranes, Scherman and Henry [25] observed the density of high affinity sites to be about 7 pmole/mg and the low affinity sites to be about 60 pmole/mg. Stern-Bach *et al.* [26] purified the transporter, following bound [³H]reserpine through the purification steps; the molecular weight, as determined by SDS-PAGE, was estimated to be approximately 80 kDa.

Another class of inhibitors of the transporter is tetrabenazine (TBZ) and its derivatives. This class of drugs binds to a single high affinity site on VMAT2. The commonly used radioligand to study this site, [³H]dihydrotetrabenazine ([³H]TBZOH), binds to bovine chromaffin granule membranes with a K_D of about 3 nM and a site density of about 60 pmole/mg. Transporter substrates inhibit [³H]TBZOH binding to this site with K_i values of approximately 12 μ M [27]. Ketanserin, a serotonin 5-HT_{2A} receptor ligand, also binds to this site with a K_D of about

6.1 nM [28]. VMAT1 does not bind TBZ but does bind reserpine and ketanserin [29], suggesting that tetrabenazine might bind to a different site than ketanserin or reserpine.

Although the vesicular monoamine transporters have been purified and cloned from several sources, the ligand binding site(s) of VMAT2 has not been thoroughly characterized. Recent models of ligand binding to VMAT2 indicate three potential binding sites: the substrate/high affinity reserpine site, the ketanserin site, and the tetrabenazine site. These sites may be distinct sites on the transporter, or overlapping regions, with each ligand having somewhat different contact sites. Photoaffinity labeling studies from our laboratory using a ketanserin derivative, [¹²⁵I]AZIK, identified the N-terminal as the insertion site for ketanserin [30]. Additionally, the N-terminus of VMAT2 was also the site of insertion of a TBZ photoaffinity probe, TBZ-AIPP, which also labeled the intracellular loop between transmembrane domains 10 and 11. These data indicate the importance of the N- and C-termini and TMD 5-8 for ligand binding.

Photoaffinity labeling is a powerful technique used to identify binding site peptides from receptors. In this study, we report the synthesis and characterization of several iodophenylazide derivatives of reserpine and tetrabenazine, which inhibit [³H]dopamine uptake into chromaffin granules. Also, a photoactivatable reserpine derivative and a tetrabenazine derivative have been radioiodinated to high specific activity (2200 Ci/mmol) and used successfully as photoaffinity labels for VMAT2.

Materials and Methods

Chemicals

[³H]Dopamine and carrier-free [¹²⁵I]NaI were purchased from New England Nuclear. Dopamine HCl, reserpine, R-(−)-norepinephrine HCl, *p*-aminophenethylamine, oxalyl chloride, reserpine, bromoacetyl bromide, ethylene diamine, phosphorous pentoxide (P₂O₅), and sodium cyanoborohydride (NaCNBH₃) were obtained from Aldrich, and 3-(*p*-aminophenyl)propionic acid was purchased from Pfalz and Bauer, Inc. Thallium trichloride was purchased from Alpha Products. Tetrabenazine was purchased from Fluka. Methyl reserpate was synthesized by the methanolysis of reserpine using sodium methoxide [31]. Other chemicals were from Sigma (serotonin, sodium azide, adenosine triphosphate, and silica gel (70-230 mesh, 60 Å pores)) and Pierce Chemical Co. (dry silylation grade N,N-dimethylformamide, tetrahydrofuran, and pyridine). N-Glycanase was purchased from Boehringer Mannheim. Precoated silica gel, thin layer chromatography plates (GF-254, type 60) were obtained from EM Sciences. The solvents used were of reagent grade from Aldrich. NMR data were obtained in deuterated chloroform using TMS as a standard.

Synthesis of 18-O-[3-(3'-iodo-4'-azidophenyl)propionyl]methyl reserpate (AIPPMER) (IV, Scheme 1)

The starting material 3-iodo-4-azidophenyl-propionic acid (AIPP, **I**) was synthesized according to the procedures of Lowndes *et al* [32]. The acyl chloride of AIPP (**II**) was synthesized using a modified procedure of Adams and Ulich [33]. AIPP (25 mg, 0.0789 mmole) was dissolved in 100 µl of oxalyl chloride (1.165 mmole). This mixture was agitated for 5 min under gentle heat until **II** was totally dissolved. The mixture was allowed to react for 15 min after which it was refluxed at 68°C for 2 hr. The reaction was cooled to room temperature (22°C). To the reaction was added 100 µl of anhydrous diethyl ether that was then removed by rotary evaporation. To the residue was added 16.17 mg (0.03945 mmole) methyl reserpate (**III**) in 200 µl pyridine. This mixture was vortexed until clear and allowed to react at room temperature for 19 hr while stirring. After this time, 3 mL of water was added which resulted in the formation of a sticky brown precipitate. This mixture was vortexed for about 5 min. The precipitate was washed three times with 0.5 mL water and dissolved in 2 mL chloroform. The

chloroform was back-extracted once with 0.5 mL water and removed by rotary evaporation leaving an oily residue. The residue was washed three times with 0.5 mL of anhydrous diethyl ether, which was removed each time by rotary evaporation. This resulted in a brown powder that migrated on thin layer chromatography with an R_f of 0.40 in isopropanol : ethyl acetate : acetic acid (15:10:0.5). This material was purified using a 2×29 cm silica gel column (70-230 mesh, 60 Å pores) and the solvent system described above. The purified product dissolved in 15 mL of cold methanol; 15 mL of cold water was added and the solution lyophilized to yield 17.6 mg of **IV** (69% yield). Analysis by silica gel TLC developed in isopropanol : ethyl acetate : acetic acid (15:10:0.5) yielded one spot (R_f = 0.40). NMR shifts (TMS as standard): 7.25 ppm, doublet; 7.35 ppm, doublet; 7.75 ppm, singlet. IR (KBr), 1710 cm^{-1} (ester), 2100 cm^{-1} (azide). EMS [$\text{M}+\text{H}^+$], Calcd. 714.1789, Found, 714.1806.

Synthesis of 18-O-bromoacetyl methyl reserpate (BAMER) (**V**, Scheme 1)

Bromoacetyl bromide (12 μL , 0.122 mmole) was dissolved in 250 μL of tetrahydrofuran (THF). This solution was added dropwise to 25 mg (0.061 mmole) of methyl reserpate in 250 μL THF containing 0.122 mmole of pyridine. The reaction was stirred vigorously for 15 hr, adding THF as necessary to keep the heavy precipitate in suspension. Water (3 mL) was added to the reaction to dissolve the precipitate. The mixture was then extracted three times with 3 mL dichloromethane which was removed by rotary evaporation. The product was dissolved in ethyl acetate : methanol (15:1) and **V** was isolated using a 2.5×33 cm silica gel column (70-230 mesh, 60 Å pore size) eluted with ethyl acetate : methanol (15:1). The solvent was removed by rotary evaporation. The residue was dissolved in 10 mL cold methanol. Cold water (10 mL) was added and the product was lyophilized to yield 18.9 mg (70%) of bromoacetyl methyl reserpate. This product was homogeneous as determined by silica gel TLC developed in ethyl acetate : methanol (15:1). **V** migrated with an R_f = 0.45 and tested positive to Hartmann's reagent (reacts with acyl halides). IR (KBr), 1740 cm^{-1} (α halogen ester), 1710 cm^{-1} (ester). EMS [$\text{M}+\text{H}^+$], Calcd. 535.1444, Found, 535.1425.

Synthesis of 18-O-[N-(3'-iodo-4'-azidophenethyl)glycyl]methyl reserpate (IAPEGlyMER) (**VII**, Scheme 2)

IAPA (**VI**), 3-iodo-4-azidophenethylamine, (10.55 mg, 0.0366 mmole), synthesized by the procedure of Resek and Ruoho [34], was dissolved in 100 μL THF and added to 5 mg **V** (0.0094 mmole) in 100 μL THF. The reaction was allowed to proceed for 3 hr at room temperature. **VII** was purified using a 2.5×25 cm silica gel column (70-230 mesh, 60 Å pores) eluted with ethyl acetate : methanol (2:3). The solvent was removed by rotary evaporation. The residue was dissolved in 5 mL of cold methanol and 5 mL of cold water was added slowly. The solution was lyophilized to yield 6.3 mg of **VII** (45% yield). The product was homogeneous as determined by silica gel TLC developed in ethyl acetate : methanol (2:3). **VII** migrated with an R_f = 0.40. NMR shifts (TMS as standard): 7.2 ppm, doublet; 7.3 ppm, doublet; 7.70 ppm, singlet. IR (KBr), 1740 cm^{-1} (ester), 2150 cm^{-1} (azide). EMS [$\text{M}+\text{H}^+$], Calcd. 743.2054, Found, 743.2022.

Radiosynthesis of [^{125}I]IAPEGlyMER

Carrier-free [^{125}I]IAPA (2200 Ci/mmmole) was synthesized following the procedure of Resek and Ruoho [34]. The [^{125}I]IAPA was stored in ethyl acetate at a concentration of 1 mCi/mL. To 1 mL of the [^{125}I]IAPA solution was added 50 μL of **V** (2 mg/mL) in dimethylformamide (DMF). The solvent was removed under a stream of argon. Upon complete removal of the solvent, an additional 50 μL of **V** (2 mg/mL in DMF) was added. The reaction vessel was purged with argon and tightly capped. The reaction was allowed to proceed for 19 hr at room temperature. Purification was carried out using a 0.8×18 cm silica gel column (70-230 mesh, 60 Å pores) eluted with ethyl acetate : methanol (2:3). Radiopurity was estimated at 95% as

determined by TLC developed in ethyl acetate : methanol (2:3) and autoradiography. The [125 I]IAPEGlyMER comigrated with IAPEGlyMER (R_f = 0.40).

Synthesis of 2-N-(ethylamine)-tetrabenazine [2-N-(ethylamine)-TBZ] (IX, Scheme 3)

The diacetate salt of ethylenediamine was synthesized by neutralizing 2 mL of ethylenediamine with dilute acetic acid. Water was added (about 50 mL) and the solution lyophilized to give a tan powder. This material was dried in a vacuum dessicator over P_2O_5 for several days together with tetrabenazine and $NaCNBH_3$. Tetrabenazine (**VIII**, 100 mg, 0.315 mmole) was dissolved in 5 mL of dry methanol (dried over 4 Å molecular sieves). To this was added 567.4 mg (3.15 mmole) of the diacetate salt of ethylenediamine. This solution was allowed to stand in the presence of 4 Å molecular sieves for 15 min after which $NaCNBH_3$ (99.0 mg, 1.58 mmole) was added. The reaction was complete after 3 hr as indicated by TLC, monitoring the disappearance of **VIII**. The product migrated with an R_f of 0.32 in methanol:ethylacetate:ammonium hydroxide (1:1:0.1) and was reactive to ninhydrin. The reaction mixture was taken to pH 3 with 1 N HCl to destroy the excess $NaCNBH_3$ and then neutralized with 1 N NaOH. The reaction mixture was then taken to dryness on a rotary evaporator and the pH raised to 9-10 with 1 N NaOH, at which time a white precipitate formed. The solution was extracted four times with 10 mL of ethyl acetate, which was subsequently dried using anhydrous $MgSO_4$. The volume was reduced to approximately 5 mL and streaked onto a 20×20 cm thick layer silica plate. The material that migrated with an R_f of approximately 0.3 was removed from the plate and the silica gel was extracted four times with 10 mL of methanol. The product was homogeneous as indicated by one spot in TLC analysis in the above system. EMS $[M+H^+]$, Calcd. 362.2729, Found, 362.2808.

Synthesis of 2-N-[(3'-iodo-4'-azidophenyl)propionyl]-tetrabenazine (TBZ-AIPP) (XI, Scheme 2)

To 25 mg (0.069 mmole) of **IX** in 2 mL of anhydrous methanol was added 50 mg (0.16 mmole) of AIPPS (**X**), synthesized according to Lowndes *et al.* [32]. The reaction was allowed to proceed to completion (4 hr). The methanol was reduced in volume and the solution streaked onto a 20×20 cm silica gel plate and developed in ethyl acetate:methanol:ammonium hydroxide (1:1:0.05). The isomeric products (*trans*-TBZ-AIPP, R_f 0.35 and *cis*-TBZ-AIPP, R_f 0.40) were extracted from the silica three times with 5 mL of methanol to yield 8 mg *trans*- and 3 mg *cis*-TBZ-AIPP. EMS $[M+H^+]$ (*trans*-TBZAIPP), Calcd. 661.2284, Found, 661.2311.

Radiosynthesis of [125 I]TBZ-AIPP

[125 I]TBZ-AIPP was synthesized by the same procedure as above except that [125 I]AIPPS was used. Carrier-free [125 I]AIPPS was synthesized according to the procedure of Lowndes *et al.* [32]. Briefly, 50 μ L of **IX** (1 mg/mL in methanol) was added to 500 μ Ci of [125 I]AIPPS in ethyl acetate (volume 1-3 mL). This solution was dried under a stream of argon, and an additional 100 μ L of **IX** (1 mg/mL in methanol) was immediately added. This mixture was allowed to react overnight at room temperature. The methanol was streaked onto a 10×20 cm thin layer silica gel plate and developed in ethyl acetate:methanol:ammonium hydroxide (1:1:0.05). The TLC plate was subjected to autoradiography for 10 sec. The autoradiogram showed two products (R_f 0.35 and 0.40), corresponding to the non-radioactive material synthesized above. The products were extracted separately from the silica three times with 1 mL methanol to yield 200 μ Ci and 100 μ Ci of *trans*- and *cis*-[125 I]TBZ-AIPP, respectively. The *trans*-[125 I]TBZ-AIPP (lower isomer) was used for photoaffinity labeling experiments.

Preparation of bovine chromaffin granule ghosts

Chromaffin granule ghosts were prepared from bovine adrenal medulla as described by Smith and Winkler [35], except that 0.3 M sucrose, 10 mM HEPES/NaOH, pH 7.0, was used as the

isolation medium. Ghosts were prepared by lysing and resealing chromaffin granules according to the method of Giraudat *et al.* [36]. Briefly, 50-150 whole bovine adrenals from freshly slaughtered animals were collected at Peck's slaughter house in Milwaukee, WI. The adrenal glands were bisected and the medulla scraped out and put into ice cold 0.3 M sucrose, 10 mM HEPES, pH 7.0. The medullae were minced in a polytron for 3 sec on a high setting and then homogenized with a Teflon pestle. After removal of the unbroken cells and nuclei, the chromaffin granule membranes were pelleted through 1.6 M sucrose at 100,000xg for 60 min. Chromaffin granule ghosts were prepared by osmotically lysing these crude granules in 5 mM HEPES, 2 mM MgSO₄, 10 mM CaCl₂, 0.1 mM DTT, pH 7.5. The membranes were recovered by centrifugation at 100,000xg for 60 min. The granules were resealed by resuspending the pellets in 0.3 M sucrose, 10 mM HEPES, 2 mM MgSO₄, 0.1 mM DTT, pH 7.0 at a concentration of 5-10 mg/mL, snap frozen in liquid N₂, and stored at -80°C until further use.

[³H]Dopamine transport into bovine chromaffin granule ghosts

[³H]Dopamine uptake was assayed by collecting the granule membranes on cellulose acetate filters under vacuum filtration according to the published methods of Knoth *et al.* [37]. Briefly, chromaffin granule ghosts (280 µg protein) were diluted in 1.0 mL of 0.3 M sucrose, 10 mM HEPES, pH 7.0, containing 5 mM ATP, 2.5 mM MgSO₄, and the indicated concentration of reserpine, BAMER, IAPEGlyMER, AIPPMER, TBZ, or *trans*-TBZ-AIPP added from a 100X stock solution in DMSO. Transport was initiated by the addition of 10 µl of 5 mM [³H]dopamine. The chromaffin granule ghosts were incubated for 10 min at 30°C and collected on cellulose acetate filters (Millipore type HAWP, 0.45 µm pore size) by vacuum filtration. The filters were washed three times with 4 mL ice-cold 0.3 M sucrose, 10 mM HEPES, pH 7.0. The [³H]dopamine on the filters was quantitated using liquid scintillation spectroscopy. Uptake in the presence of vehicle (DMSO) alone was defined as 100% and 0% in the presence of 10 µM reserpine in DMSO. Curves were fit to a one-site competitive displacement curve using nonlinear regression with the software application, GraphPad Prism®.

Inhibition of [³H]serotonin transport

Transport of [³H]serotonin was done according to the method of Parti *et al.* [38]. Briefly, chromaffin granule ghosts (265 µg protein) were suspended in 1.0 mL of 0.3 M sucrose, 10 mM HEPES, pH 7.0, containing 5 mM ATP, 2.5 mM MgSO₄, and the indicated concentration of inhibitor (IAPEGlyMER or AIPPMER). Transport was initiated by the addition of [³H]serotonin to the indicated final concentration. Samples were collected by vacuum filtration after incubation at 30°C for 10 min. Filters were washed three times with 4 mL of ice-cold 0.3 M sucrose, 10 mM HEPES pH 7.0 and the [³H]serotonin on the filters was quantitated using liquid scintillation spectroscopy.

Photoaffinity labeling of bovine chromaffin granule ghosts

For photoaffinity labeling with [¹²⁵I]IAPEGlyMER, chromaffin granule membranes (150 to 250 µg of protein) in 0.1 mL of 0.40 M sucrose, 10 mM HEPES at pH 7.0 were incubated in thick-walled Pyrex tubes at 30°C for 15 min with 2.5 nM [¹²⁵I]IAPEGlyMER (2200 Ci/mmol) in the presence of 10 mM ATP, 10 mM MgCl₂, and inhibitor, when required. For photoaffinity labeling with [¹²⁵I]TBZ-AIPP, chromaffin granule membranes (150 to 250 µg of protein) in 0.1 mL of 0.2 M sucrose, and 40 mM HEPES, pH 8.0 were incubated in thick-walled Pyrex tubes at 30°C for 90 min with 2 nM [¹²⁵I]TBZ-AIPP in the presence and absence of 10 µM TBZ. After the incubation, the membranes were diluted with 5 mL of ice cold 0.22 M KCl, 10 mM HEPES at pH 7.0 and immediately photolyzed for 5 sec in ice-water at a distance of 10 cm from a water-jacketed 1-kilowatt high pressure AH-6 mercury lamp [39]. For the experiments using the biogenic amines as inhibitors of photoaffinity labeling, the amines were present in the dilution buffer at their indicated concentration. β-Mercaptoethanol (50 µl) was

added after photolysis to help reduce the nonspecific labeling. The membranes were then collected by centrifugation at 20,000xg for 30 min at 4°C for analysis by SDS-PAGE. Dried, stained gels were subjected to autoradiography using Kodak X-Omat film to visualize photolabel incorporation.

Reserpine inhibition of [¹²⁵I]TBZ-AIPP photolabeling

Chromaffin granule membranes were preincubated in the presence and absence of 1 μM reserpine prior to incubation with 2 nM [¹²⁵I]TBZ-AIPP. Chromaffin granule membranes (100 μg of protein per condition) were suspended in 0.2 M sucrose, 40 mM HEPES, pH 8.0 in the presence and absence of 10 mM ATP and 10 mM MgCl₂ for 15 min at 30°C. After the incubation, reserpine (final concentration 1 μM) was added to one of the -ATP samples and one of the +ATP samples. TBZ was also added to one of the +ATP samples. The samples were incubated again for 15 min at 30°C. At this time [¹²⁵I]TBZ-AIPP was added to a final concentration of 2 nM. All other samples were prepared in an identical manner except that they were not pretreated prior to the addition of reserpine or [¹²⁵I]TBZ-AIPP. The samples were then incubated for 90 min at 30°C and then photolyzed as described above. The membranes were collected by centrifugation at 20,000xg for 30 min at 4°C for analysis by SDS-PAGE.

SDS-PAGE analysis of the membranes

SDS-PAGE (12%) was performed by the method of Laemmli [40] using the following proteins as molecular mass markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Results

BAMER, IAPEGlyMER, AIPPMER, reserpine, tetrabenazine, and TBZ-AIPP inhibit [³H] dopamine uptake

The transport of biogenic amines into chromaffin granules was examined using resealed chromaffin granule ghosts. Reserpine has been shown to be a potent inhibitor of catecholamine uptake into chromaffin granule ghosts with a K_i = 0.5 nM [25]. BAMER, IAPEGlyMER and AIPPMER all inhibit the transport of [³H]dopamine into bovine chromaffin granule ghosts, although at a 10 fold higher IC₅₀ than reserpine (Table 1).

Lineweaver-Burk analysis of the IAPEGlyMER and AIPPMER inhibition of 5-hydroxytryptamine (serotonin) uptake into bovine chromaffin granule ghosts demonstrates that IAPEGlyMER and AIPPMER are both competitive inhibitors of serotonin uptake into chromaffin granule ghosts (Fig. 1). The K_i for IAPEGlyMER was calculated to be approximately 90 nM, and the K_i for AIPPMER was calculated to be approximately 40 nM, 180 and 80 times lower affinity, respectively, than reserpine.

[¹²⁵I]IAPEGlyMER Photoaffinity labeling of the chromaffin granule monoamine transporter

[¹²⁵I]IAPEGlyMER was synthesized carrier-free (2200 Ci/mmol) and was used as a photoaffinity label for the reserpine binding site on VMAT2. A 15 hr autoradiogram (Fig. 2A) of the SDS-polyacrylamide gel showed several bands of labeled proteins; however, only a broad band of apparent molecular mass 55-90 kDa was completely blocked in the presence of 1 μM reserpine, with the majority of the label located at approximately 70-80 kDa. Tetrabenazine at a concentration of 10 μM partially blocked the photolabeling by 2.5 nM [¹²⁵I]IAPEGlyMER; however, if the TBZ concentration was increased to 100 μM, the inhibition of photoaffinity labeling was complete. The specifically labeled protein is not a major

protein in the chromaffin granule membrane as can be observed by a comparison of the SDS-PAGE gel and the autoradiogram.

Inhibition of [¹²⁵I]IAPEGlyMER photoaffinity labeling using the biogenic amines dopamine, (–)-norepinephrine, and serotonin

Blocking of the photoaffinity labeling was also observed with biogenic amine substrates (Fig. 2B). Chromaffin granule membranes were photolabeled with [¹²⁵I]IAPEGlyMER as above in the presence of the biogenic amine substrates. Each of the amines was present at the indicated concentrations in the dilution buffer. Partial blocking was observed with 1 mM serotonin (see inset Fig. 2B, but full inhibition of [¹²⁵I]IAPEGlyMER-labeling required the presence of 10^{−2} M norepinephrine, dopamine, or serotonin.

Photoaffinity labeling of chromaffin granule membranes with [¹²⁵I]TBZ-AIPP

Chromaffin granule membranes were incubated with 2 nM [¹²⁵I]TBZ-AIPP in the dark and then exposed for 5 sec to a high intensity UV light source. Analysis by SDS-PAGE and subsequent autoradiography revealed a specifically labeled polypeptide that migrated with an apparent molecular weight of 55-80 kDa. The majority of the labeling was observed between 70 and 80 kDa (see Fig. 3), similar to the labeling observed with [¹²⁵I]IAPEGlyMER. The specificity of the labeling is shown by blocking with 10 μM TBZ. A polypeptide of similar molecular weight was photolabeled using probes derived from tetrabenazine ([³H]TBA [41]) and ketanserin ([¹²⁵I]AZIK [42]). In addition, the purified transporter has an apparent molecular weight similar to that observed with the [¹²⁵I]TBZ-AIPP labeling [26;43].

In contrast to the results obtained with the [³H]TBA and [¹²⁵I]AZIK photolabels [41;42] the labeling of VMAT2 by [¹²⁵I]TBZ-AIPP is blocked by 1 μM reserpine (Fig. 4). The inhibition of photolabeling is complete regardless of whether the membranes are preincubated with reserpine, or if reserpine and [¹²⁵I]TBZ-AIPP are added simultaneously. Reserpine completely blocks the labeling of VMAT2 by [¹²⁵I]TBZ-AIPP in the absence of ATP.

Discussion

Arylazido derivatives of tetrabenazine ([³H]TBA) [41], ketanserin ([¹²⁵I]AZIK) [42], and serotonin ([³H]ANPA-5HT) [44] have been previously reported as photoaffinity labels for VMAT. The tetrabenazine and ketanserin compounds photolabeled a 70-80 kDa polypeptide in chromaffin granule membranes, while the serotonin photolabel derivatized a 45 kDa polypeptide, also in bovine chromaffin granule membranes, that the authors concluded to be either VMAT or a subunit of the transporter. It is possible that this 45 kDa protein represents a unique or altered form of VMAT (i.e., non-glycosylated or partially proteolyzed) or a VMAT-associated protein. Reserpine photolabels have not been reported to date, and the first successful reserpine photolabel is presented in this report, along with a carrier-free radioiodinated tetrabenazine derivative. Our laboratory has now successfully synthesized several iodophenylazide derivatives of reserpine and tetrabenazine that inhibit the transport of [³H]dopamine into chromaffin granule ghosts. IAPEGlyMER and TBZ-AIPP have been prepared carrier-free (2200 Ci/mmol) in radioiodinated form and both compounds specifically photolabel VMAT in chromaffin granule membranes.

While all the reserpine derivatives reported in this paper inhibited [³H]dopamine uptake into chromaffin granule ghosts, all did so with lower potency than reserpine. From the inhibition of [³H]dopamine uptake into chromaffin granule ghosts, the IC₅₀ values for BAMER, AIPPMER and IAPEGlyMER were approximately 100-200 nM. Additionally, when AIPPMER and IAPEGlyMER were analyzed using a Lineweaver-Burk plot for their inhibition of [³H]serotonin uptake (Fig. 1), the K_i values determined in this experiment were 90 nM for

IAPEGlyMER and 40 nM for AIPPMER. The IC_{50} values for reserpine and the reserpine derivatives are higher than expected, which may be due to the fact that the chromaffin granule ghosts were not preincubated with the inhibitors prior to the addition of [3]dopamine. These data indicate that BAMER, IAPEGlyMER, and AIPPMER are competitive inhibitors of serotonin uptake by bovine VMAT2.

In the presence of either 1 μ M reserpine or 100 μ M tetrabenazine, a polypeptide that migrated with an apparent molecular weight of 55-90 kDa was specifically labeled by [125 I] IAPEGlyMER. From the autoradiogram (Fig. 2), it can be seen that the majority of the labeling is in the molecular weight range of 70-80 kDa, a molecular weight that is consistent with VMAT2. This molecular weight agrees with previous reports of the VMAT photoaffinity labels [125 I]AZIK [42] and [3 H]TBA [41]; however it is larger than the polypeptide labeled by the serotonin photoaffinity label, [3 H]ANPA-5HT [44].

The [125 I]IAPEGlyMER-labeled transporter migrates as a diffuse band on SDSpolyacrylamide gels, consistent with the glycoprotein nature of VMAT. Indeed, when the TBZAIPP-labeled transporter (which also migrates as a diffuse band) is digested by an endoglycosidase, it migrates with an apparent molecular weight of 53 (data not shown). The nonspecific labeling observed with [125 I]IAPEGlyMER is likely due to the hydrophobic nature of the photoaffinity label. Several techniques, including washing the membranes prior to photolysis, washing with bovine serum albumin (BSA), and photolysis in the presence of β -mercaptoethanol were used to try to reduce this nonspecific labeling. None of these techniques increased the specific labeling with respect to the background radioactivity. This nonspecific binding is also seen in [3 H]reserpine binding studies [25] and also with the photolabel [3 H]TBA, a tetrabenazine derivative [41].

The transporter substrates, norepinephrine, dopamine, and serotonin, were also used to block the labeling seen with [125 I]IAPEGlyMER (Fig. 2). Partial inhibition of VMAT2 photoaffinity labeling was observed with 1 mM serotonin and full inhibition of photolabeling required the presence of 10 mM biogenic amines. The observation that high concentrations of the substrates are needed to obtain inhibition of [125 I]IAPEGlyMER-labeling is consistent with reports of the biogenic amines being poor inhibitors of [3 H]reserpine binding [45].

The photolabeling of the monoamine transporter from bovine chromaffin granule membranes with [125 I]TBZ-AIPP was blocked by 10 μ M TBZ and 1 μ M reserpine. Interestingly, reserpine blocked labeling both in the presence and absence of ATP. It has been proposed that there are two conformations of the transporter, R and T, and each conformation binds only one type of ligand, reserpine or TBZ, respectively. The R conformation is dependent on the presence of a proton gradient and is believed to be the conformation that binds reserpine with high affinity and transports substrates across the membrane. The T conformation binds tetrabenazine and ketanserin. The TBZ-AIPP photolabeling in the presence of ATP demonstrates that the R conformation state can bind TBZ. The amount of photolabeling in the presence of ATP is decreased, which may indicate that a population of transporters exists in the T conformation state, even though there is ATP present. The fact that reserpine inhibits [125 I]TBZ-AIPP photolabeling in the absence of ATP indicates that reserpine can bind to the tetrabenazine-binding conformation, in apparent conflict with the proposed model of Darchen *et al.* [24].

Initial experiments have been performed to identify the TBZ binding site peptide [30] utilizing VMAT2 expressed and purified using the baculovirus-expression system [46]. TBZAIPP inserts into two regions of the transporter, the N-terminus and in a region comprised of the cytoplasmic portions of transmembrane domains 10 and 11. The decrease in the amount of [125 I]TBZ-AIPP insertion into VMAT2 in the presence of ATP may indicate a conformational change in either of these regions, which is "sensed" by the photoactive moiety when the vesicles

are charged with a proton gradient. Tetrabenazine binding is not affected by the presence of a proton gradient; therefore, it is possible that a conformational change in VMAT2 in which the [¹²⁵I]TBZ-AIPP photoinsertion site is changed or moved could account for the decrease in labeling in the presence of ATP. This is in contrast to a change in affinity for the photoaffinity label itself. There is a report that the N-terminus of the vesicular GABA transporter, GAT1, is involved in regulating GAT1 transport activity (it activates transport through an intramolecular interaction) [47]. It can be speculated that a similar mechanism may be occurring in VMAT2, whereby the N-terminus, a site of photoinsertion by TBZ-AIPP [30], is restructured upon the generation of a proton gradient, and thus, less TBZ-AIPP is incorporated.

To summarize, we report the synthesis of a reserpine and a tetrabenazine photoaffinity label and demonstrate that these photoaffinity labels specifically derivatize VMAT2 in chromaffin granule membranes. The reserpine photoaffinity label will provide a tool with which to probe the reserpine binding site and determine if conformational changes occur in VMAT2 upon activation by the proton gradient, a hypothesis supported by the fact that TBZ-AIPP labeling is decreased in the presence of ATP.

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Scheme 1. Synthetic scheme for AIPPMER (IV)

AIPP (**I**) was refluxed in oxalyl chloride for 2 hr to produce AIPP-Cl (**II**). Unpurified **II** was reacted overnight at room temperature with methyl reserpate (**III**) to yield AIPPMER (**IV**).

Scheme 2. Synthetic scheme for IAPEGlyMER (VII)

IAPA (**VI**) was dissolved in THF and added to BAMER (**II**) in THF. The reaction was allowed to proceed for 3 hrs at room temperature. **VII** was purified using a 2.5×25 cm silica gel column developed in ethylacetate:methanol (2:3). Carrier-free radioiodinated [¹²⁵I]IAPEGlyMER

Scheme 3. Synthetic scheme for TBZ-AIPP (XI)

2-N-(ethylamine)-TBZ (**IX**) was synthesized by reacting tetrabenazine (**VIII**) with a 10-fold molar excess of ethylene diamine. Purified **IX** was reacted with AIPPS (**X**) in anhydrous methanol to produce **XI**.

Fig. 1. Lineweaver-Burk analysis of [³H]serotonin uptake

Transport of serotonin was inhibited with IAPEGlyMER (A) or AIPPMER (B). Ghosts (265 μg protein) were suspended in 1.0 mL of 0.4 M sucrose, 10 mM HEPES, pH 7.0, containing 5 mM ATP, 2.5 mM MgSO_4 , and the indicated concentration of inhibitor. Transport was initiated by the addition of [³H]serotonin to the indicated final concentration. Samples were collected by filtration after incubation at 30°C for 10 min.

Fig. 2. Photoaffinity labeling of chromaffin granule membranes using [125 I]IAPEGlyMER Chromaffin granule membranes (170 μ g of protein) were photolabeled with 2.5 nM [125 I]IAPEGlyMER (2200 Ci/mmol) in the presence of 0.4 M sucrose, 10 mM HEPES, pH 7.0 containing 10 mM ATP and 10 mM MgCl_2 at 4°C. The membranes were centrifuged at 20,000xg for 30 min at 4°C and then electrophoresed on a 12% SDS-polyacrylamide gel. **(A)** The autoradiogram and the corresponding Coomassie Blue protein staining pattern of the SDS-polyacrylamide gel are shown. **(B)** Inhibition of labeling by 10 mM (—)norepinephrine, dopamine, and serotonin. Inset shows blocking by 1 mM of the biogenic amines.

Fig. 3. Photoaffinity labeling of chromaffin granule membranes using [125 I]TBZ-AIPP
Chromaffin granule membranes (150 μ g of protein) in 0.1 mL of 0.2 M sucrose, and 40 mM HEPES, pH 8.0 were incubated in thick-walled Pyrex tubes at 30°C for 1.5 hr with 2 nM [125 I]TBZ-AIPP (2200 Ci/mmol) in the absence (**a**) or presence (**b**) of 10 μ M TBZ. The autoradiogram (12 hr) of the 12% SDS-polyacrylamide gel is shown.

Fig. 4. Reserpine inhibition of [125 I]TBZ-AIPP photolabeling of chromaffin granule membranes
 Chromaffin granule membranes (100 μ g of protein per condition) were photolabeled with [125 I]TBZ-AIPP after preincubation (a-e) or no preincubation (f-j) with the inhibitors. Lanes a and b contained no MgATP, but were preincubated in the absence (a) or presence (b) of 1 μ M reserpine. Lanes c, d, and e were preincubated in the presence of 10 mM MgATP for 15 min at 30°C prior to the addition of (c) no inhibitor, (d) reserpine (1 μ M final), or (e) tetrabenazine (10 μ M final). The samples were all incubated at 30°C for 15 min prior to the addition of [125 I]TBZ-AIPP (2 nM final concentration). Lanes f-j were not preincubated before the addition of the [125 I]TBZ-AIPP. Lanes f and g contained no MgATP, whereas lanes h, i, and j contained 10 mM MgATP. Lanes g and i contained 1 μ M reserpine and lane j contained

10 μ M tetrabenazine. The samples were incubated for 90 min in the presence of 2 nM [125 I] TBZ-AIPP and photolyzed. The autoradiogram (18 hr) of the 12% SDS-polyacrylamide gel is shown.

Table 1
Inhibition of [³H]dopamine uptake into chromaffin granule

IC₅₀ values were determined from inhibition studies of [³H]dopamine uptake into chromaffin granule ghosts, as described in the *Materials and Methods* section.

Compound IC ₅₀ of [³ H]dopamine transport (nM) +/- SEM	
Reserpine	37 +/- 15
BAMER	73 +/- 57
AIPPMER	83 +/- 16
IAPEGlyMER	200 +/- 67
Tetrabenazine	48 +/- 3
TBZ-AIPP	2100 +/- 450