Photoaffinity Labeling of the Vesamicol Receptor of Cholinergic Synaptic Vesicles†

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Received March 1, 1993; Revised Manuscript Received June 2, 1993

ABSTRACT: On the basis of the high-affinity vesamicol analog 4-aminobenzovesamicol (ABV), a tritiated, arylazido ligand (azidoABV) of the vesamicol receptor (VR) in cholinergic synaptic vesicles was synthesized. azidoABV is an inhibitor of acetylcholine (AcCh) active transport, and it binds to the VR with higher affinity than vesamicol. The rate of dissociation of azidoABV from synaptic vesicles is 0.058 ± 0.003 min^{-1} at 20 °C (about 3-fold slower than that of vesamicol), and the equilibrium dissociation constant is 2 nM (about 4-fold lower than that of vesamicol). Photolysis of [^{3}H]azidoABV in the presence of a stoichiometric excess of the VR led to incorporation of 28% of the radiolabel, of which 57% was blocked by 50 μM vesamicol. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the labeled vesicles revealed, after autofluorography, specific labeling over a broad molecular weight range that extended from about 50 to 200 kDa. This labeling pattern was essentially the same as that obtained with an azido analog of AcCh that was used to label the AcCh transporter (Rogers, G. A., & Parsons, S. M. (1992) *Biochemistry 31,* 5770–5777). In addition, about 6% of the radioligand that was specifically incorporated into proteins with M_r greater than 12 kDa labeled four polypeptides that corresponded to bands in the Coomassie image at M_r = 23, 33, 35, and 38 kDa. The results suggest that the VR exists as part of a complex system of subunits.

We recently reported the results of a successful effort to photoaffinity label the acetylcholine transporter (AcChT)[1] of cholinergic synaptic vesicles using a radiolabeled arylazido derivative of an acetylcholine analog [azidoAcCh (Rogers & Parsons, 1992)]. That ligand covalently labeled in a pharmacologically relevant manner a vesicular component with diffuse electrophoretic migration very similar to that of the vesicular proteoglycan (VPG). The AcChT is reversibly inhibited by a large family of drugs based on the vesamicol structure [(−)-*trans*-2-(4-phenylpiperidino)cyclohexanol (Rogers et al., 1989)] that binds at a site (the vesamicol receptor, VR) allosteric to the AcCh transport site (Bahr et al., 1992a).

The VR has been purified in a detergent-solubilized form, and it also exhibits diffuse electrophoretic migration similar to that of the VPG (Bahr & Parsons, 1992). The VR copurifies with two epitopes, namely SV1 and SV2 (Bahr & Parsons, 1992), and exists in a very tight linkage with the VPG as confirmed by immunochemical and enzymatic techniques (Bahr et al., 1992b). The SV1 epitope is luminal and resides on a keratan sulfate-like (Scranton et al., 1993; Bahr et al., 1992b) portion of the VPG. The SV2 epitope is cytoplasmic and is found on secretory vesicles in essentially all neuronal and endocrine cell types (Buckley & Kelly, 1985) and, therefore, is not cholinergic. These are surprising results that prompted us to further secure the identification of the VR by photoaffinity labeling. We report confirming results in this paper, as well as the labeling of other polypeptides that are possibly associated with the VR.

† Supported by Grant NS15047 from the National Institute of Neurological Disorders and Stroke and a grant from the Muscular Dystrophy Association of the USA.

[1] Abbreviations: AcCh, acetylcholine; AcChT, acetylcholine transporter; vesamicol, (−)-*trans*-2-(4-phenylpiperidino)cyclohexanol; VR, vesamicol receptor; VPG, vesicular proteoglycan; DTT, dithiothreitol; ABV, 4-aminobenzovesamicol; GlyABV, *N*-glycyl-4-aminobenzovesamicol; azidoABV, *N*-(azidobenzoylglycyl)-4-aminobenzovesamicol; azidoAcCh, photoaffinity acetylcholine analog; k_ψ, pseudo-first-order rate constant.

MATERIALS AND METHODS

General Methods. ^{1}H NMR spectra were recorded on a General Electric GN500 with tetramethylsilane as internal standard. UV spectra were recorded on a Shimadzu UV265 spectrophotometer. Mass spectra were determined on a VG Analytical 70–250 HF mass spectrometer in the FAB or EI mode. Autofluorographs were scanned and quantitated using an LKB Ultroscan XL. Column chromatography was performed on Merck silica gel 60. All chemicals and reagents were of the highest commercial quality. All calculations were performed using the commercial software MINSQ (MicroMath Scientific Software, Salt Lake City, UT) and the appropriate equations. Derived parameters are quoted ±1 standard deviation.

Resting synaptic vesicles (VP_1) were isolated from the electric organ of *Torpedo californica* as previously described (Yamagata & Parsons, 1989), with the modification that concentration of the vesicles after size exclusion chromatography on Sephacryl 1000 was carried out using Centriprep-30 centrifugal ultrafiltration concentrators (Amicon Corp.) at 4 °C, unless stated otherwise. After concentration of the vesicles, the pH of the isolation buffer (7.0) was adjusted to 7.8 by addition of the required amount of buffer A at pH 8.2 (adjusted with KOH) that was made up of 100 mM HEPES, 700 mM glycine, 1 mM EDTA, and 1 mM EGTA. All subsequent experiments in buffer A were at pH 7.8. All ligand-binding assays were performed by vacuum-assisted filtration onto prewetted polyethylenimine-treated glass-fiber filters (Whatman GF/F, 1.3 cm) that were then washed three times with ice-cold buffer unless stated otherwise. Tritium was quantitated by liquid scintillation spectroscopy. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

N-(4′-Azidobenzoylglycyl)-4-aminobenzovesamicol (azidoABV). Succinimidyl 4-azidobenzoate (Pierce) (95 mg, 365 μmol) was partially dissolved in 2 mL of dry CH_2Cl_2. Racemic *N*-glycyl-4-aminobenzovesamicol [GlyABV (Rogers et al.,

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1993)] (150 mg, 396 μmol) was added to the solution, which subsequently became homogeneous. The solution was left in the dark for 24 h, after which time thin-layer chromatography (silica gel; 3% ethanol/$CHCl_3$) indicated that the reaction was complete. Solvent was removed on a rotary evaporator, and the residue was refluxed with methanol for 15 min in an attempt to destroy any ester formed by the acylation of the alcohol moiety of the vesamicol ring. Notwithstanding this effort, silica gel chromatography ($CHCl_3$) provided two distinct fractions, the first of which was diacylated GlyABV (FABMS base peak at m/z = 670 for P + 1). Diacylation was confirmed by ^1H NMR. The second chromatography fraction (97 mg) was identified as the desired product by analysis: FABMS, base peak at m/z = 525 for P + 1 and 497 for loss of N_2; UV, loss of absorbance at λ_{max} = 268 nm upon exposure to a lamp that emits light at 254 nm; IR, strong absorbances at 1688 (coincident with GlyABV) and 1645 cm^{-1} for amide I bands and 2108 cm^{-1} for the azido group.

Tritiated (–)-N-(4'-Azidobenzoylglycyl)-4-aminobenzovesamicol ([^3H]azidoABV). *N*-[*benzoate*-3,5-$^3H_2$]Succinimidyl 4-azidobenzoate (5.09 nmol, 49.10 Ci/mmol, Du Pont NEN) was combined with 10.7 μg (28.2 nmol) of (–)-GlyABV (Rogers et al., 1993) in 100 μL of 2-propanol and placed in the dark. After 4 days the sample was diluted with 100 μL of ethanol and kept at –80 °C until subsequent workup.

Analysis of the crude reaction mixture by C_{18} reversed-phase HPLC (20% 25 mM, pH 3.2 phosphate buffer in acetonitrile) demonstrated that 93% of the radioactivity eluted coincident with an authentic sample of nonlabeled azidoABV. Purification of the product on a semiprep Chiralpak AD (Daicel Chemical Ind.) column (25% ethanol/hexane at 4 mL/min) afforded 160 μCi (64% radiochemical yield) of material that eluted in the region of the chromatogram coincident with the retention time of (–)-azidoABV (26.3 min). The exact elution time of the product could not be recorded because the UV detector was turned off as soon as elution of the photosensitive azidoABV began. Unreacted (–)-GlyABV was observed to elute at 15.7 min.

Inhibition of AcCh Active Transport by azidoABV. Active transport of AcCh was constituted as described (Rogers et al., 1993) at a synaptic vesicle concentration of 0.20 mg of protein/mL in buffer A. A 30 mM stock solution of azidoABV was made by dissolving 5.6 mg in 50% DMSO/0.11 M HCl. Serial dilutions were made into 11% DMSO/H_2O and subsequently into 0.4 M KCl so that the final concentrations in the transport assay solutions were 0.30 nM to 30 μM azidoABV and a maximum of 0.16% DMSO.

Inhibition of [^3H]Vesamicol Binding by azidoABV. Synaptic vesicles (0.20 mg of protein/mL in buffer A) were incubated with differing concentrations of azidoABV (0.30 nM to 30 μM) and 2 nM [^3H]vesamicol. Bound [^3H]vesamicol was assayed by vacuum-assisted filtration through PEI-treated glass-fiber filters as described (Rogers & Parsons, 1992). Nonspecific binding of [^3H]vesamicol to vesicles was determined in the presence of a 100-fold excess of [^1H]vesamicol.

Binding of [^3H]azidoABV to Container Surfaces. A 50 nM solution of [^3H]azidoABV in buffer A was placed in a polypropylene tube. The tritium concentration was monitored as a function of time and vortexing. In a similar manner, a 100 nM solution was placed in standard borosilicate glass tubes that were either untreated or treated with Sigmacote (Sigma Chemical Co.) and assayed as a function of time. Sigmacote produces a coating of silicone on container surfaces.

Rate of Dissociation of [^3H]azidoABV from Synaptic Vesicles. Synaptic vesicles (20 μg of protein/mL in buffer A that contained 3 mM $MgCl_2$) were incubated with 4.8 nM [^3H]azidoABV (in glass treated with Sigmacote) at 20 °C for 60 min. Dissociation of [^3H]azidoABV was initiated by the addition of a vesamicol solution so that the final concentrations were 4.62 nM and 30 μM, respectively. [^3H]azidoABV bound to vesicles was assayed by filtration at various times as described above.

Rate of Association of [^3H]azidoABV with Synaptic Vesicles. Synaptic vesicles (about 0.6 μg protein/mL in buffer A at 20 °C) were mixed with a small volume of [^3H]azidoABV (in glass treated with Sigmacote) to yield a final concentration of 3.7 nM. After 15–20 s of vigorous vortexing, 200-μL aliquots were taken for the determination of bound radioligand by the filter assay described above.

Reversible Binding of [^3H]azidoABV to Synaptic Vesicles. Synaptic vesicles from the fraction represented by the center of the chromatography peak from the size exclusion chromatography step of purification (as described above) were used without concentration. The pH of the solution was adjusted to 7.8 by addition of buffer A at pH 8.2. Final vesicle protein concentration was about 30 μg/mL. The VR concentration was determined by the addition of 60 μL of vesicles to 300 μL of [^3H]vesamicol (about 120 nM). After 30 min of incubation, 100-μL aliquots were assayed for bound tritium and 10-μL aliquots for the total [^3H]vesamicol in solution. The same procedure was performed with the addition of [^1H]vesamicol to a final concentration of 28 μM in order to assess nonspecific binding.

On the basis of the concentration of the VR determined above, vesicles were diluted about 60-fold for the binding experiments with [^3H]azidoABV. All solutions of [^3H]-azidoABV were made in glass that had been treated with Sigmacote as described above. Duplicate samples of vesicles were diluted with one-tenth volumes of various stock concentrations of [^3H]azidoABV to give final concentrations of ligand that ranged from 59 pM to 8 nM. After an 11-h incubation period, bound tritium was determined as above. Actual final total concentrations of radioligand in each sample were determined by liquid scintillation spectroscopy at the completion of the incubation period. In samples used to measure nonspecific binding of [^3H]azidoABV, (–)-4-aminobenzovesamicol (ABV) was added to yield a final concentration in 100-fold excess of the radioligand.

Photoaffinity Labeling of Synaptic Vesicles with [^3H]-azidoABV. Volumes of 156, 31, and 6.25 μL of an ethanolic solution of [^3H]azidoABV were evaporated under a stream of N_2 in three glass test tubes that had been treated with Sigmacote. To the first test tube was added 2.4 mL of synaptic vesicles that were 0.65 mg of protein/mL in buffer A. To the second was added 8.33 μL of a 3 mM solution of vesamicol in buffer A followed by 500 μL of the same vesicle suspension. The final concentration of vesamicol was 50 μM. To the third tube was added 6.25 μL of a 27 μM ethanolic solution of racemic ABV, which was also evaporated before the addition of 100 μL of vesicle suspension. The final concentration of ABV was 1 μM. A fourth tube was constituted by incubating 100 μL of the vesicle suspension with 1 μM ABV for 1 h prior to introduction of the [^3H]azidoABV as above. All four solutions were incubated for 10 min and then illuminated with an 18-W UV lamp (Ultra-Violet Products Inc., San Gabriel, CA) that emits light with intensity centered at 254 nm. For 10 min the tubes were alternatively illuminated and vortexed to yield a total illumination time of about 5 min each. Following the photolysis, 5 μL of the solution was removed from each tube and diluted into 145 μL of buffer A

containing 60 μM vesamicol. From each of the diluted samples, 100 μL was vacuum-filtered through PEI-treated glass-fiber filters and washed with 1 mL of buffer A. Each filter was incubated two times with 1-mL volumes of buffer A (containing 60 μM vesamicol) for 10 min at 23 °C. Finally, each was washed with three 1-mL volumes of buffer A, and the bound tritium was determined as before.

SDS-PAGE and Autofluorography of Photolabeled Synaptic Vesicles. Volumes equivalent to 30 μg of protein were removed from each sample of photolabeled vesicles from above and diluted to 150 μL with buffer A. Remaining vesicles were set aside and frozen for potential later analysis. The diluted vesicles were pelleted in an Airfuge (Beckman Instrument Co.) at 73 000 rpm for 70 min. The supernate was carefully removed, and the pellets were covered with 20 μL of treatment buffer that contained 10% sodium dodecyl sulfate (SDS) and 20% glycerol in 0.125 M Tris-HCl (pH 6.8). The samples were vortexed occasionally over a 6-h period at 23 °C. Finally, each was diluted with 20 μL of a 10% (w/v) solution of dithiothreitol (DTT) and heated in a 90 °C bath for 3 min. Molecular weight standards were treated with the same solutions but were given only a brief incubation prior to addition of the DTT. The dissociated samples were subjected to SDS-PAGE using a 5–15% linear gradient resolving gel, a 3% stacking gel, and the discontinuous buffer system of Laemmli (1970). Electrophoresis was halted when the tracking dye was about 1 cm from the bottom of the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R in order to visualize proteins and then treated with Intensify (Du Pont) before being dried onto filter paper under vacuum. The dried gel was autofluorographed with XAR-5 film (Kodak) at −80 °C for 5 days.

RESULTS

Synthesis and Photolability of azidoABV. From previous studies (Rogers et al., 1993) it was apparent that 4-aminobenzovesamicol (ABV) with a dissociation constant of 6.5 pM might provide the basis for the synthesis of a successful photoaffinity reagent for the VR. Toward this goal, the chemical reactivity of ABV was increased by the addition of a glycyl moiety [GlyABV (Rogers et al., 1993)] to yield an aliphatic primary amine of about 5 orders of magnitude greater basicity than the anilino nitrogen. However, the secondary alcohol of ABV, which is an essential pharmacophore, has much greater reactivity in acylation reactions than expected, perhaps due to the proximity of the tertiary amine. The hyperreactivity caused facile diacylation of GlyABV, and because of this the yield of racemic azidoABV was only 51%. GlyABV was resolved by chiral chromatography and acylated by tritiated succinimidyl 4-azidobenzoate, which provided enantiomerically pure (−)-[^{3}H]azidoABV in 64% radiochemical yield of 49.1 Ci/mmol specific activity.

Because the absorption maximum of azidoABV occurs at 268 nm, it was necessary to use a short-wavelength lamp that emits light with intensity centered at 254 nm (18 W). azidoABV in aqueous solution decomposed with a half-life of about 1 min in the configuration utilized here. It is stable under ordinary fluorescent illumination and therefore can be used conveniently in reversible binding experiments.

Inhibition of [^{3}H]AcCh Active Transport and [^{3}H]Vesamicol Binding by azidoABV. As an analog of vesamicol, azidoABV should be a potent inhibitor of AcCh active transport in synaptic vesicles. When tested for transport inhibition under conditions where the VR concentration was about 60 nM, (±)-azidoABV inhibited active transport with an apparent IC_{50} value of 150 ± 60 nM (Figure 1). In the same preparation of vesicles, azidoAcCh exhibited an IC_{50} value more than 5 times greater (data not shown). In competition with 2 nM [^{3}H]vesamicol bound to synaptic vesicles with a VR concentration of 67 nM, (±)-azidoABV exhibited an apparent IC_{50} value of 600 ± 200 nM and displaced 98% of the specifically bound vesamicol (Figure 1). The IC_{50} values for the inhibition of AcCh active transport and vesamicol binding are denoted as apparent because subsequent experiments with radiolabeled azidoABV demonstrated that there is a time-dependent loss of azidoABV to glass surfaces, as discussed below. Therefore, the concentrations of unlabeled ligand were significantly lower than anticipated, which produced artifactually high values of IC_{50}. However, the data demonstrate that azidoABV is a potent inhibitor of AcCh active transport, as expected for a vesamicol analog, and it is significantly more potent than azidoAcCh.

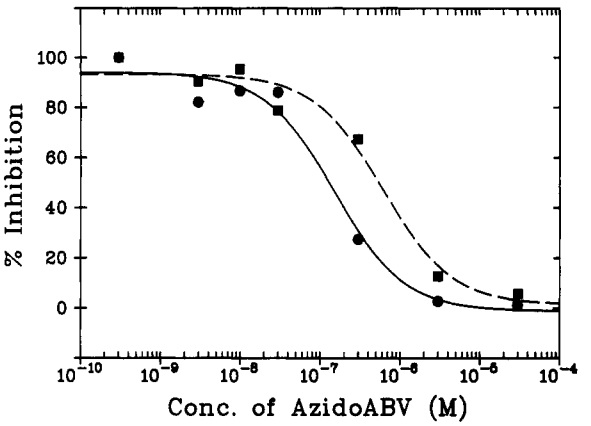

FIGURE 1: Inhibition of AcCh active transport and [^{3}H]vesamicol binding by azidoABV. AcCh active transport was measured at 22 °C as previously described (Rogers et al., 1989). Synaptic vesicle protein and [^{3}H]AcCh concentrations were 0.20 mg/mL and 50 μM, respectively. The best hyperbolic fit to the data (–●–) using nonlinear regression analysis gave an IC_{50} value of 150 ± 60 nM. Inhibition of [^{3}H]vesamicol binding to vesicles was measured at 22 °C as described in Materials and Methods. VR and [^{3}H]vesamicol concentrations were 67 and 2 nM, respectively. The best hyperbolic fit to the data (- - ■ - -) gave an IC_{50} value of 600 ± 200 nM. About 98% of the specifically bound [^{3}H]vesamicol was displaced.

Binding of azidoABV to Containers. Initial kinetic experiments on the binding of [^{3}H]azidoABV to vesicles suggested complex behavior. Analysis of the solubility properties of the ligand led to the discovery that [^{3}H]azidoABV deposits on glass surfaces in a time-dependent manner that is likely dependent on the surface-to-volume ratio. In an untreated borosilicate test tube, 67% of the [^{3}H]azidoABV in 1 mL of a 100 nM solution deposited on the glass with a half-life of about 35 min (data not shown). Polyethylene also adsorbs the ligand, but mixing appeared to cause partial redistribution from the plastic surface back into solution. Because the affinity for glass could be blocked substantially by treatment with Sigmacote, all reported experiments involving [^{3}H]azidoABV were conducted in treated glass.

Binding Kinetics of [^{3}H]azidoABV. The rate constant for dissociation of bound [^{3}H]azidoABV from synaptic vesicles was determined by displacement with 30 μM vesamicol (Figure 2A). At 20 °C the process was well-described by a single exponential decay with k_{-1} = 0.058 ± 0.003 min^{-1} ($t_{1/2}$ = 12 min).

The rate of association of [^{3}H]azidoABV with vesicles was determined at 20 °C under pseudo-first-order conditions (i.e., the concentration of free ligand remained essentially constant during the process) (Figure 2B). Under these conditions, k_ψ

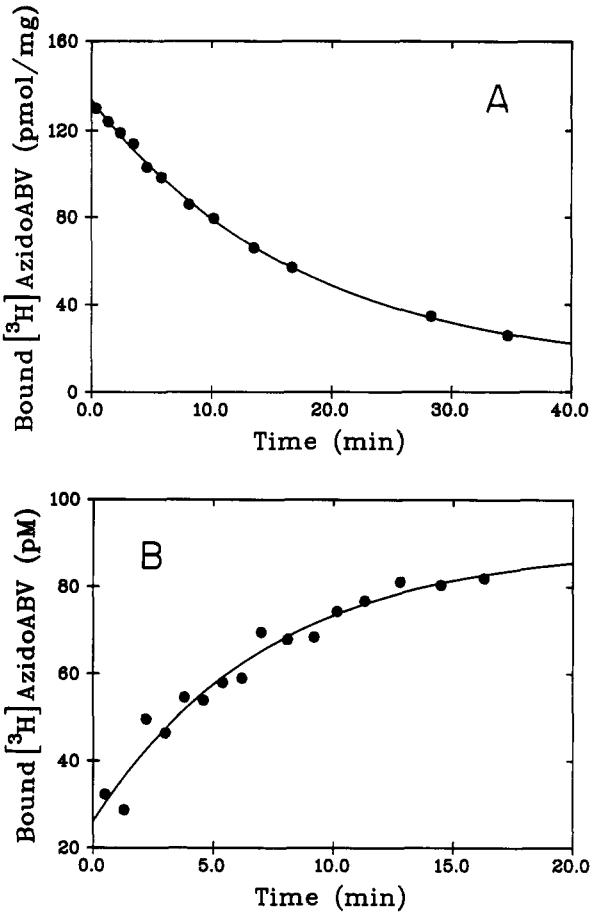

FIGURE 2: Kinetics of interaction of [^{3}H]azidoABV with synaptic vesicles. (A) Dissociation of 4.6 nM [^{3}H]azidoABV from vesicles (20 μg of protein/mL) was promoted by 30 μM vesamicol at 20 °C. About 92% of the total bound tritium dissociated by a process that is described by a single exponential equation with $k = 0.058 \pm 0.003$ min^{-1}. Similar results were obtained with two other preparations of vesicles. (B) Association of 3.7 nM [^{3}H]azidoABV with synaptic vesicles (about 0.6 μg of protein/mL) at 20 °C. The VR concentration was about 0.18 nM as measured by [^{3}H]vesamicol binding, and at equilibrium only 0.1 nM drug was bound to the VR. The association process was well-described by an equation with a single exponential and a pseudo-first-order rate constant (k_ψ) = 0.14 ± 0.03 min^{-1}.

= 0.14 ± 0.03 min^{-1}, and as the ligand concentration was 3.7 nM, the second-order rate constant (k_ψ/[azidoABV]) = 3.8 × 10^7 M^{-1} min^{-1}. Therefore, the value of K_D determined from the rate processes ($k_{-1}/(k_\psi/[\text{azidoABV}])$) = 1.5 nM.

Equilibrium Binding of [^{3}H]azidoABV to Synaptic Vesicles. The reversible binding of [^{3}H]azidoABV to vesicles was assessed after an 11-h incubation period at 23 °C (Figure 3). Based on the concentrations employed and the rate constants determined above, the approach to equilibrium should have been greater than 90% completed at the lowest concentrations of the ligand that were used. Under these conditions, values for the parameters K_D and B_{max} were determined to be 2.1 ± 0.4 and 0.29 ± 0.03 nM (or about 580 ± 60 pmol/mg at about 0.5 μg of protein/mL), respectively.

Photolabeling of Synaptic Vesicles with [^{3}H]azidoABV. The synaptic vesicles that were used for the photolabeling experiment were assayed by [^{3}H]vesamicol (250 nM) and found to possess 690 pmol of VR/mg of protein. At the protein concentration of 0.65 mg/mL, therefore, the VR concentration was 450 nM. The measured concentrations of [^{3}H]azidoABV in solution in the four samples are listed in Table I.

The amount of photolabeling was analyzed by two methods. The first method was by filter assay following a 30-fold dilution into a 60 μM vesamicol solution. In addition, two 10-min incubations on the filters with 60 μM vesamicol were used in order to promote dissociation of specifically bound but not covalently incorporated ligand. Kinetic data presented in Figure 2A demonstrate that the high concentration of vesamicol should have promoted nearly complete dissociation of reversibly bound [^{3}H]azidoABV from the VR. The amounts of tritium that remained bound to the filters and were presumably covalently incorporated into the vesicles are listed in Table I. The values for the percent incorporation are also listed. These data suggest that over 50% of the total labeling was blocked by a large excess of vesamicol but that much less was blocked by a small excess of the much more potent ligand ABV.

The second method of analysis was by gradient SDS-PAGE (5–15%) followed by staining with Coomasie Blue and autofluorography (Figure 4). Protein staining revealed well-focused bands throughout the molecular weight range of 12–200 kDa. Autofluorography of the unprotected sample (sample 1) demonstrated heavy labeling of a continuous region from about 50 to 200 kDa. Quantitation of the absorbance indicated that 94% of the total labeling of proteins (exclusive of the labeling at the dye front) was in this region. In addition, four polypeptides with M_r's of about 22.6, 33, 35.1, and 37.5 kDa were specifically labeled. The 33-kDa band was somewhat diffuse, but correlation with the Coomassie image suggests that the diffuseness arises as the result of a doublet

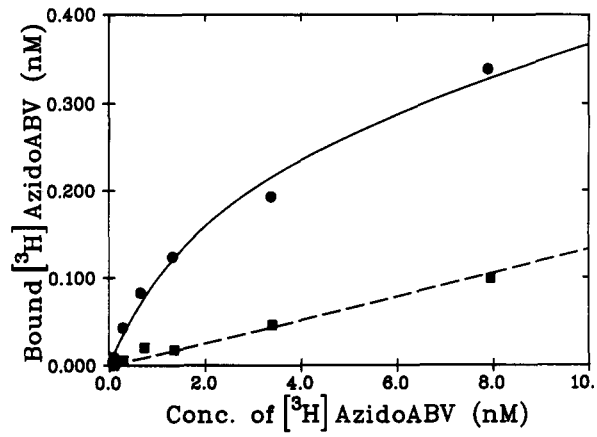

FIGURE 3: Reversible binding of [^{3}H]azidoABV to synaptic vesicles. Vesicles were constituted as described in Materials and Methods. Bound [^{3}H]azidoABV was determined in the presence (- - ■ - -) or absence (– ● –) of a 100-fold excess of ABV to yield curves representing the amount of nonspecific and total bound, respectively. A simultaneous fit of a linear equation (for nonspecific binding) and an equation combining the linear equation with a hyperbolic term (for total binding) to the separate data sets yielded values of K_D and B_{max} of 2.1 ± 0.4 and 0.29 ● 0.03 nM (about 580 ± 60 pmol/mg of protein), respectively.

Table I: Analysis of Photolabeling by Filter Assay[a]

sample no.	additions	concn of [^{3}H]azidoABV (nM)[b]	bound [^{3}H]azidoABV[c] (pmol/mg)	(%)[d]
1	none	128	65	28
2	50 μM vesamicol	154	31	12
3	1 μM (±)-ABV	151	71	26
4	1 μM (±)-ABV[e]	136	54	23

[a] The same vesicles were also analyzed by SDS-PAGE and autofluorography, and the results are shown in Figure 4. [b] Concentrations varied due to adsorption to the glass test tube. [c] Tritium bound to vesicles that could not be removed by exhaustive washing. [d] Bound [^{3}H]azidoABV divided by the total amount of [^{3}H]azidoABV that was present. [e] Added 1 h prior to the addition of [^{3}H]azidoABV.

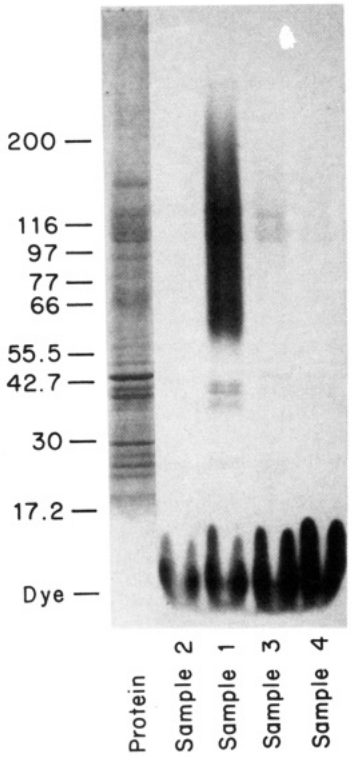

FIGURE 4: Autofluorographic analysis of synaptic vesicles photolabeled with [^{3}H]azidoABV. The amount of tritium covalently incorporated into vesicles was also analyzed by the filter assay, and the results are presented in Table I. Photolabeling in the presence or absence of competing ligands and the preparation of vesicles for SDS-PAGE and autofluorography were performed as described under Materials and Methods. The figure is a composite showing Coomassie Blue staining and a 3-day film exposure at −80 °C. The first lane, labeled Protein, shows the Coomassie staining pattern, which was identical for all four samples. Sample 1 shows the autofluorographic pattern for nonprotected vesicles. Samples 2 and 3 show labeling when 50 μM vesamicol or 1 μM (±)-ABV was added simultaneously with the addition of [^{3}H]azidoABV, respectively, and Sample 4 shows labeling when 1 μM (±)-ABV was added 1 h prior to the addition of [^{3}H]azidoABV. The M_r's in kilodaltons and positions of standard proteins are indicated to the left. Labeled bands at 22.6, 33, 35.1, and 37.5 kDa in Sample 1 were visible in the original photograph but may not be in the published reproduction.

of closely-spaced bands at 32.4 and 33.8 kDa. Addition of 50 μM vesamicol coincident with [^{3}H]azidoABV completely blocked incorporation of the photoaffinity ligand into components with M_r greater than 12 kDa and somewhat diminished the labeling of components at the bottom of the gel at the dye front (sample 2). At the lower concentration of 1 μM, (±)-ABV protected nearly all sites above 12 kDa from being labeled but actually increased incorporation of label into the small components near the dye front, whether it was applied coincident (sample 3) or prior (sample 4) to exposure of the vesicles to the radioligand. Neither vesamicol nor ABV produced any visible change in the protein staining pattern (data not shown).

DISCUSSION

Structure–activity studies demonstrated that the benzovesamicol series of analogs has much higher affinity for the VR than does vesamicol (Rogers et al., 1993). Furthermore, earlier studies showed that attachment of a large polar moiety such as biotin to the 4-position of ABV via a tether does not greatly diminish the potency of the analog compared to vesamicol (Rogers et al., 1989). It was reasonable to expect, therefore, than an arylazido moiety attached to GlyABV would constitute a high-affinity photolabile probe (azidoABV) suitable for specifically labeling the VR.

azidoABV was synthesized as reported above and shown to possess inhibitory properties consistent with those of a vesamicol analog, as both AcCh active transport and [^{3}H]-vesamicol binding were inhibited. One of the most useful parameters that characterizes ligand receptor interactions is the half-life for dissociation of the complex. This we determined for the [^{3}H]azidoABV–VR complex to be 12 min at 20 °C, or about 3 times longer than for vesamicol (Rogers et al., 1993). Therefore, azidoABV can be used for reversible binding experiments. The association rate constant of about 4×10^7 M^{-1} min^{-1} is approximately 5- and 2.5-fold lower than those of vesamicol and ABV, respectively (Rogers et al., 1993). The dissociation constant K_D that can be calculated as the ratio of the dissociation and association rate constants is 1.5 nM. The value for K_D determined from the equilibrium binding curve is in good agreement at about 2 nM. These data provide evidence that [^{3}H]azidoABV has highly desirable kinetic and equilibrium properties for a photoaffinity ligand. The relatively slow dissociation of the ligand from the binding site should allow ample time for the photogenerated nitrene to insert into nearby receptor chemical bonds.

A simple and rapid analysis of the photolabeling experiment by filter assay suggested that vesamicol but not ABV blocked labeling of the VR by [^{3}H]azidoABV. That this is not the case can be seen clearly in the analysis of the same vesicles by SDS-PAGE followed by autofluorography. Photolysis of the unprotected sample of vesicles led to intensive labeling of a species that extends from about 50 to 200 kDa. This result is essentially the same as that obtained by labeling vesicles with [^{3}H]azidoAcCh. Thus we conclude that this diffuse, heavily-labeled species is the VR. At first glance the differences in labeling patterns among the protected samples might seem difficult to reconcile with each other and with the filter analysis of the same samples. However, it should noted that the conditions of the experiment (receptor concentration about 3-fold greater than that of [^{3}H]azidoABV) were devised to minimize nonspecific labeling in the unprotected sample and make maximum use of the radioligand. Thus, at a [^{3}H]-azidoABV concentration of about 130–150 nM in the presence of 450 nM VR, nearly all of the ligand will bind to the receptor and very little will remain free in solution. A 300-fold excess of vesamicol over the [^{3}H]azidoABV concentration, added at the same time as [^{3}H]azidoABV, would be expected to abolish all labeling of the VR, as was observed. However, a less than 7-fold excess of the high-affinity analog ABV (K_D = 6.5 pM), added to the vesicles at the same time as [^{3}H]azidoABV, will allow some specific labeling to occur because the ligands have similar association rate constants and photolysis was performed before equilibration of the ligands with the VR was established. In contrast, ABV that is added before [^{3}H]azidoABV will occupy all of the VR and more completely abolish labeling, as was observed. It is noteworthy that the concentration of the more potent enantiomer, (−)-ABV, that was used to block specific labeling was 500 nM, or only in slight excess over the concentration of the VR, and so very little (−)-ABV remained free in solution.

The next point that deserves comment concerns the intensive labeling of vesicular components near the bottom of the gel. As virtually all of the [^{3}H]azidoABV was bound to the VR in the unprotected sample, the photogenerated nitrene must have labeled lipid or other small components in the surrounding membrane even while bound to the VR. More labeling of the small components was produced by the presence of a relatively

low concentration of ABV. This demonstrates that the increased free (or solution) concentration of [^{3}H]azidoABV, which results as a consequence of displacement from the VR, produces apparently nonspecific labeling of similar small components. Labeling of this material also occurred in the presence of a large excess of vesamicol (300-fold higher concentration than [^{3}H]azidoABV). Even though the free or solution concentrations of [^{3}H]azidoABV in the ABV- and vesamicol-protected samples were equal, the decreased nonspecific labeling of small components in the presence of vesamicol could result from electrostatic screening of the membrane by the relatively high concentration of vesamicol. Vesamicol, ABV, and azidoABV are all positively charged at the pH of the photolabeling experiments. If the labeled low M_r components are lipids, the intensive labeling of these components in the unprotected sample indicates that specifically bound [^{3}H]azidoABV is in contact with the membrane bilayer.

About 6% of the specific labeling above 12 kDa was on four or five peptides of about 23, 33 (possibly a doublet), 35, and 38 kDa. At least three mechanisms for producing this labeling can be envisioned. By the first, the specifically labeled peptides might arise from proteolytic breakdown of the VR. Although vesamicol binding is extremely resistant to proteolysis (Kornreich & Parsons, 1988), it is possible that proteolysis could produce smaller fragments still able to bind vesamicol. However, in three photolabeling experiments with azidoAcCh, there was no evidence for the presence of proteolytic fragments of the AcChT–VR (Rogers & Parsons, 1992). By the second mechanism, it is conceivable that [^{3}H]-azidoABV bound to the VR was oriented so that the nitrene could covalently react with other peptides that the VR randomly collided with as a result of lateral diffusion in the membrane bilayer. If this mechanism obtains, the result suggests that only a few of the many vesicular proteins randomly collided with the VR during the several minutes required for photolabeling. By the third mechanism, the specifically labeled peptides of 23–36 kDa might represent proteins that are associated with the AcChT–VR. As it was present on a flexible tether extending from the tightly bound ABV core, the photogenerated nitrene could range over a region of space adjacent to the vesamicol binding site. This could lead to specific labeling of accessory proteins if they are present in a heterooligomeric complex with the VR. This interpretation is consistent with localization of the vesamicol binding site on the surface of the VR near the bilayer. In contrast, the AcCh binding site has been suggested to be inside of the AcChT in a channel-like structure (Bahr et al., 1992b) where the nitrene derived from azidoAcCh would not be expected to be able to reach accessory proteins.

In summary, it is clear that azidoAcCh and azidoABV specifically label the same vesicular component, which exhibits extremely heterogeneous electrophoretic behavior. The vesamicol analog yields evidence of interaction with other protein subunits, which suggests that the AcChT–VR is a complex system.

ACKNOWLEDGMENT

We thank Daniel Oros and Wendy Connelly for the preparation of synaptic vesicles.

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