Photoaffinity Labeling of the Acetylcholine Transporter†

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ABSTRACT: The acetylcholine (AcCh) binding site in the AcCh transporter–vesamicol receptor (AcChT–VR) present in synaptic vesicles isolated from the electric organ of *Torpedo* was characterized. A high-affinity analogue of AcCh containing an aryl azido group, namely, cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-methylisonipecotate bromide (AzidoAcCh), was synthesized in nonradioactive and highly tritiated forms. AzidoAcCh was shown to be a competitive inhibitor of [^{3}H]AcCh active transport and binding of [^{3}H]-vesamicol to the allosteric site. The [^{3}H]AzidoAcCh saturation curve was determined. In all cases the AcChT·AzidoAcCh complex exhibited an inhibition or dissociation constant of about 0.3 μM. Binding of [^{3}H]AzidoAcCh was inhibited by vesamicol and AcCh. AzidoAcCh irreversibly blocked greater than 90% of the [^{3}H]vesamicol binding sites after multiple rounds of photolysis and reequilibration with fresh ligand. Autofluorographs of synaptic vesicles photoaffinity-labeled with [^{3}H]AzidoAcCh showed specific labeling of material exhibiting a continuous distribution from 50 to 250 kDa after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The result demonstrates that the AcChT has an unexpected structure highly suggestive of the synaptic vesicle proteoglycan.

A model of the acetylcholine transporter (AcChT)[1] has been formulated that hypothesizes an allosteric binding site for the noncompetitive inhibitory drug vesamicol (Bahr et al., 1992a). AcCh binds to the transport site with the remarkably high dissociation constant of about 20–50 mM, but rapid transport produces a much lower Michaelis constant of about 0.3 mM (Bahr & Parsons, 1986). Many AcCh analogues of diverse structures also are actively transported (Clarkson et al., 1992). The kinetics and low transport specificity suggest that the AcChT has an unusual structure.

In the preceding paper, purification of the vesamicol receptor (VR) with retention of the binding pharmacology observed in vesicle ghosts was reported (Bahr & Parsons, 1992). The results were surprising. The purified material exhibited chromatographic, electrophoretic, and immunochemical properties strongly suggesting that the VR is associated with the vesicle proteoglycan. In the current paper, we took an affinity-labeling approach to identification of the coupled AcChT. An essential question was whether the AcChT is in the same macromolecule as the VR. Affinity labeling was possible because the pharmacology of the AcCh binding site has been extensively explored in a structure–activity study that revealed molecular parameters governing affinity of AcCh analogues for the transport site (Rogers & Parsons, 1989). This information has allowed rational design of a potent, highly tritiated, photoaffinity ligand for the AcChT. We report here the synthesis and chemical properties of that ligand, the reversible-binding characteristics of the ligand–AcChT interaction, and the electrophoretic nature of the target that is specifically photolabeled by the AcCh analogue.

Materials and Methods

General Methods. Melting points were determined in open capillaries on a Thomas Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Nicolet NT-300 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. Column chromatography was performed on Merck silica gel 60. HPLC analysis was carried out by reverse-phase (C_{18}) on a Altex Ultrasphere-ODS column using acetonitrile/phosphate buffer (7:3), pH 3.7, as the mobile phase. All chemicals and reagents were of the highest commercial quality.

VP_1 synaptic vesicles from *Torpedo californica* electric organ were isolated as described (Yamagata & Parsons, 1989). After the final concentrative pelleting, vesicles were resuspended at 4 °C in titration buffer (TB) containing 100 mM HEPES, 700 mM glycine, 1 mM EDTA, and 1 mM EGTA adjusted to pH 7.8 with KOH. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. All ligand binding assays were performed at 22–23 °C by filtration onto prewetted polyethylenimine-treated glass-fiber filters (Whatman GF/F, 1.3 cm) that were then washed three times with ice-cold buffer. Tritium was quantitated by liquid scintillation counting.

All calculations were performed using the commercial software MINSQ (MicroMath Scientific Software, Salt Lake City, UT). For the kinetic analysis, the linear competitive equations in hyperbolic form were fitted to the separate sets of velocities for vesamicol-inhibited transport of [^{3}H]AcCh at different inhibitor concentrations, using simultaneous nonlinear regression analysis similar to the methods of Cleland (1979). For competitive binding analysis, the hyperbolic

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[1] Abbreviations: AcCh, acetylcholine; AcChT, acetylcholine transporter; VR, vesamicol receptor, TB, titration buffer; AzidoAcCh, cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-methylisonipecotate bromide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; K_{is}, slope active transport inhibition constant; K_{IA}, equilibrium dissociation constant for AzidoAcCh binding determined by inhibition of [^{3}H]vesamicol binding; K_{DV}, equilibrium dissociation constant for [^{3}H]vesamicol binding determined by direct titration; K_{DA}, equilibrium dissociation constant for [^{3}H]AzidoAcCh binding determined by direct titration; K_{IAA}, equilibrium dissociation constant for AcCh binding determined by inhibition of [^{3}H]AzidoAcCh binding; B_{max}, the maximum amount of specifically bound ligand; FAB, fast atom bombardment; EI, electron impact; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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equations given below were fitted similarly to separate sets of values for specifically bound [^{3}H]vesamicol at different inhibitor concentrations, using simultaneous nonlinear regression analysis and a constrained competitive model. Derived parameters are quoted ±1 standard deviation (SD).

Cyclohexylmethyl cis-N-(4-Azidophenacyl)-N-methylisonipecotate Bromide (AzidoAcCh). Isonipecotic acid hydrochloride (2.1 g, 12.7 mmol) was suspended in 15 mL of thionyl chloride ($SOCl_2$) and heated to 40 °C for 12 h. Excess $SOCl_2$ was removed on a rotary evaporator with the aid of dry CH_2Cl_2. The acid chloride was dissolved in dry CH_2Cl_2 and heated in a fume hood in order to remove remaining $SOCl_2$. During several hours, aliquots of cyclohexylmethanol (a total of 3.5 equiv) were added to the stirred solution which was then refluxed overnight. The CH_2Cl_2 solution was washed with cold aqueous carbonate and dried over Na_2SO_4. Removal of the solvent resulted in an oil that was chromatographed on silica gel. Product ester was eluted with 5% EtOH/$CHCl_3$ in 65% yield as a colorless oil. The hydrochloride salt had mp = 161.5–163 °C and a parent peak of m/z = 225 by EIMS. ^{1}H NMR of the neutral ester δ 0.9–1.05 (m, 2 H), 1.1–1.35 (m, 3 H), 1.5–1.8 (m, 11 H), 1.64 (s, 1 H, NH), 1.89 (br dd, 2 H, J = 13.2, 3 Hz), 2.41 (tt, 1 H, J = 11.3, 3.9 Hz), 2.64 (td, 2 H, J = 11.9, 2.7 Hz), 3.09 (dt, 2 H, J = 12.5, 3.7 Hz), 3.88 (d, 2 H, J = 6.4 Hz).

Cyclohexylmethyl isonipecotate (350 mg, 1.55 mmol) was dissolved in 6 mL of EtOH. MeI (110 mg, 0.78 mmol) was added to the solution which was allowed to stand for 3 h. Solvent was removed on a rotary evaporator and replaced with $CDCl_3$. ^{1}H NMR indicated 20% dimethylation and therefore a maximum of 60% yield based on MeI.

To aid in the purification of the monomethylated ester, the mixture of products was treated overnight with 150 mg of succinic anhydride in $CHCl_3/CH_2Cl_2$. Addition of CCl_4 and aqueous carbonate produced an emulsion which was somewhat dispersed by the addition of a saturated NaCl solution. The lower layer was dried over Na_2SO_4/K_2CO_3 and clarified by filtration. Solvent was removed on a rotary evaporator, and the residue was taken up in CH_2Cl_2. After standing for an hour, the solution was separated from sediment by filtration, and the solvent was removed on a rotory evaporator. The yield of amber oil was 110 mg (60% yield). ^{1}H NMR δ 2.26 (s, 3 H, CH_3–N), 3.89 (d, 2 H, J = 6.6 Hz).

4-Azidophenacyl bromide (190 mg, 0.80 mmol) was added with swirling to a solution of 110 mg (0.46 mmol) of cyclohexylmethyl *N*-methylisonipecotate in 4 mL of Et_2O. Within 30 min, white crystals were observed, and, after 24 h, 200 mg was collected by filtration. Purification of AzidoAcCh by silica gel chromatography and crystallization from EtOH gave 85 mg (39%) of white crystals with mp = 181 °C (dec). The UV spectrum in TB (Figure 1) showed a maximum at 298 nm (ε = 19 000) that decreased upon exposure to UV light from an 18-W lamp (Blak-Ray, UVP, Inc., San Gabriel, CA) that emitted light with λ_{max} = 366 nm. FABMS m/z = 399 (base peak), 371 (P^+–N_2), 286 (P^+–OCH_2R). ^{1}H NMR δ 0.9–1.05 (m, 2 H), 1.1–1.35 (m, 3 H), 1.55–1.8 (m, 6 H), 2.07 (m, 2 H), 2.20 (m, 2 H), 2.96 (m, 1 H), 3.53 (s, 3 H), 3.93 (d, 2 H, J = 6.3 Hz), 4.20 (br d, 2 H, J = 12.9 Hz), 4.44 (br t, 2 H, J = 12 Hz), 6.10 (s, 2 H), 7.02 (d, 2 H, J = 8.7 Hz), 8.18 (d, 2 H, J = 8.7 Hz). The NMR spectrum clearly showed that only one of two possible geometric isomers was present. Synthesis of the same compound via an alternate pathway produced a mixture of *cis*- and *trans*-isomers. Comparison of the spectrum of the above compound with that of the mixture revealed that the product of this reaction sequence is the *cis*-isomer (Rogers & Parsons, 1989). HPLC (C_{18}) analysis under conditions that completely resolved *cis*- and *trans*-isomers showed a single peak consistent with the cis assignment.

Cyclohexylmethyl cis-N-(4-Azidophenacyl)-N-[^{3}H]-methylisonipecotate Bromide ([^{3}H]AzidoAcCh). Cyclohexylmethyl isonipecotate (9 mg, 40 μmol) and [^{3}H]CH_3I (0.65 mmol, 10.8 Ci/mmol, Amersham Corp., Arlington Heights, IL) were combined in 0.8 mL of toluene in a 1-mL screw-cap vial. After 30 h in the dark, succinic anhydride (5 mg) plus 80 μL of CCl_4 were added. The mixture was vortexed for 5 min to effect solution. After 20 h in the dark, the reaction mixture was chromatographed on silica gel. Approximately 10% of the applied tritium eluted with the reaction solvents (toluene/CCl_4), while the remainder eluted with 5% MeOH/$CHCl_3$. After the methanolic fractions were pooled and evaporated to dryness, the residue was dissolved in dry Et_2O (200 μL). 4-Azidophenacyl bromide (5 mg, 21 μmol) plus one drop of *N,N*-diisopropylethylamine were added to the ethereal solution which was stored in the dark for 2 days. Chromatography of the reaction mixture on silica gel provided product which eluted with MeOH. TLC confirmed that this compound is also the less polar, *cis*-isomer. Based on the specific activity, the overall yield was 35%. Subsequent analysis and purification by HPLC (C_{18}) gave material (75% of total radioactivity) that chromatographed coincident with the *cis*-protio isomer.

Inhibition of AcCh Active Transport by AzidoAcCh. Synaptic vesicles were diluted to 0.125 mg of protein/mL and treated with 0.15 mM paraoxon plus 2 mM $MgCl_2$ for a minimum of 30 min at 23 °C to inhibit any AcCh esterase activity present and to equilibrate the vesicles with Mg^{2+} ion. AzidoAcCh was dissolved in glass-distilled water containing 30% DMSO and then serially diluted into 0.4 M KCl. The highest final concentration of DMSO was 0.03%, which does not affect AcCh active transport. A 15 mM [^{3}H]AcCh solution (10 mCi/mmol) was prepared by diluting a sample of [^{3}H]AcCh (100 mCi/mmol; Du Pont Co., Albany, NY) with [^{1}H]AcCh (Sigma Chemical Co., St. Louis, MO) in EtOH. The EtOH was removed by evaporation, and the residue was dissolved in TB (0 °C) containing 20 mM MgATP. Vesicles were preincubated with the AzidoAcCh analogue for 15 min, after which transport at 22 °C was initiated by addition of the [^{3}H]AcCh/MgATP solution and allowed to continue for 10 min. Transport was quenched by filtration as described above. Background levels of bound tritium were assessed by measuring transport in the presence of 30 μM vesamicol. Final concentrations of protein and ATP were 0.10 mg/mL and 2 mM, respectively. This concentration of ATP was shown to be more than sufficient to promote maximal uptake of AcCh for at least 30 min.

Inhibition of [^{3}H]Vesamicol Binding by AzidoAcCh. Synaptic vesicles (60 μg of protein/mL in TB) were incubated with differing concentrations of AzidoAcCh. After 15 min, [^{3}H]vesamicol was added and allowed to equilibrate with the vesicles for 20 min. Final concentrations of AzidoAcCh and [^{3}H]vesamicol were 10 nM to 1 mM and 4.9–200 nM, respectively. Nonspecific binding of vesamicol was determined in the presence of a 100-fold excess of [^{1}H]vesamicol. Triplicate samples were filtered and washed with ice-cold buffer, as above.

Because the concentration of the VR in this experiment (29 nM) was greater than the K_{DV} for vesamicol, the concentration of free vesamicol had to be iteratively calculated at each inhibitor concentration. Hence, for each total concentration of

vesamicol, the free concentration in solution was calculated from the following two equations:

$$VF = VT - VB \quad (1)$$

$$VB = (B_{max}VF \times K_{IA})/(VF \times K_{IA} + IK_{DV} + K_{DV}K_{IA}) \quad (2)$$

where VF is the free vesamicol concentration, VT is the total vesamicol concentration, VB is the bound vesamicol concentration, K_{DV} is the dissociation constant for the vesamicol·VR complex, K_{IA} is the dissociation for the AzidoAcCh·AcChT complex, and I is the free AzidoAcCh. Mass conservation relationships for the VR and AzidoAcCh also were used.

Rate of Dissociation of [^{3}H]AzidoAcCh from Synaptic Vesicles. Synaptic vesicles (0.2 mg of protein/mL) were incubated in TB with 440 nM [^{3}H]AzidoAcCh at 23 °C for 40 min. After the solution was cooled to 0 °C, an aliquot of nonradioactive analogue was added and samples were subsequently withdrawn and analyzed for bound ligand as described above. The final concentration of [^{3}H]ligand was 400 nM and that of [^{1}H]ligand was 71 μM.

Reversible Binding of [^{3}H]AzidoAcCh to Synaptic Vesicles. Synaptic vesicles were diluted to 63 μg of protein/mL with TB containing 0.15 mM paraoxon and 3 mM $MgCl_2$. This solution was dispensed into individual tubes (80 μL each), and, after 30 min, 10 μL of either 67 mM MgATP solution or MgATP plus 50 μM vesamicol was added. After 5 min, [^{3}H]AzidoAcCh (10 μL of various stock concentrations) was added to each so that the final concentration ranged from 8 to 900 nM. Final concentrations of protein, $MgCl_2$, and MgATP were 50 μg/mL, 3 mM, and 6.7 mM, respectively. In samples used to measure nonspecific binding of [^{3}H]AzidoAcCh, the final concentration of vesamicol was 5 μM. After 20–24 min, bound tritium was determined as above.

Inhibition of [^{3}H]AzidoAcCh Binding by AcCh. Synaptic vesicles were resuspended in TB and treated with paraoxon in order to inactivate AcCh esterase. [^{3}H]AzidoAcCh was added to the vesicle suspension, which was dispensed into individual tubes and allowed to equilibrate for 15 min. AcCh from different stock solutions was added (to give final concentrations from 0.3 to 300 mM), and, after 10 min, bound [^{3}H]AzidoAcCh was determined as above. The final concentrations of vesicle protein and [^{3}H]AzidoAcCh were 0.2 mg/mL and 0.44 μM, respectively. Nonspecifically bound [^{3}H]AzidoAcCh was determined by the addition of 60 μM vesamicol to a sample that also contained 300 mM AcCh. This value was subtracted from the values for the total bound ligand to give [^{3}H]AzidoAcCh that was specifically bound. Equations of the same form as above were used to calculate K_{IAA} for AcCh.

Photoinhibition of Vesamicol Binding to Synaptic Vesicles by AzidoAcCh. To each of 10 prewetted PEI-treated glass-fiber filters (1.3 cm) was applied 4 μg of synaptic vesicle protein. In the first round of photolabeling, four of the filtered samples were bathed with 4 μM AzidoAcCh (200 μL) for 2 min, followed by 2 min of exposure to 366-nm light. A fifth sample received TB (containing no AzidoAcCh) plus light. In the second round, two samples received TB containing no AzidoAcCh and so on through succeeding rounds until each sample had been exposed to light for 8 min and to AzidoAcCh 0–4 times. All samples were washed with 0.5 mL of TB after each illumination. The remaining five vesicle samples were treated with 0–4 applications of AzidoAcCh, but no UV light. Finally, all samples except the one treated with AzidoAcCh for 2 min without light were treated with 200 nM [^{3}H]vesamicol (100 μL) for 10 min followed by 4 × 1-mL washes with ice-cold TB. The one exception received a [^{3}H]vesamicol solution that also contained 30 μM [^{1}H]vesamicol in order to determine the amount of nonspecifically bound tritium. Total bound [^{3}H]vesamicol was determined by liquid scintillation counting, and the value for nonspecifically bound [^{3}H]vesamicol was subtracted from the total bound to give specifically bound [^{3}H]vesamicol.

Photoaffinity Labeling of Synaptic Vesicles with [^{3}H]-AzidoAcCh. Synaptic vesicles that actively transported AcCh (as demonstrated by the protocol described above) were incubated with paraoxon (0.15 mM) in TB for at least 60 min prior to labeling. The vesicles were divided into six equal portions of about 100 μg of protein each. Samples B and C were controls that were not photolyzed or that contained 100 μM [^{1}H]AzidoAcCh, respectively. Samples D and E received AcCh to final concentrations of 3 and 100 mM, respectively, and sample F, 1 μM vesamicol. Sample A received only an additional aliquot of TB. Finally, all samples received [^{3}H]AzidoAcCh to 0.20 μM prior to photolysis. The final protein concentration was 0.20 mg/mL.

In order to gauge the success of photolabeling, two 20-μL aliquots were taken from sample A for a determination of bound [^{3}H]AzidoAcCh before photolysis. Both aliquots were filtered onto PEI-treated glass-fiber filters and, while one was washed three times with ice-cold TB, the other was washed repetitively with buffer at 23 °C. All six samples (A–F above) were then irradiated with 366-nm light for 8 min.[2] After photolysis, aliquots of 20 μL were taken from each sample and filtered onto PEI-treated glass-fiber filters. The filtered samples were washed repeatedly with TB (23 °C), and bound tritium was determined as before. The remaining volume of each sample was centrifuged at 160000g in a Beckman Airfuge for 60 min in order to pellet the vesicles, which were immediately analyzed by SDS–PAGE.

SDS–PAGE and Autofluorography of Photolabeled Synaptic Vesicles. Photolabeled and pelleted synaptic vesicles were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% resolving gel, a 3% stacking gel, and the discontinuous buffer system of Laemmli (1970). For this purpose, 45 μg of vesicle protein per lane was dissociated in 40 μL of a treatment buffer made by dissolving 4.8 g urea in 4 mL of 10% SDS and diluting with 5 mL of 0.75 M Tris-HCl (pH 8.8). The samples were vortexed repeatedly during a 50-min period with the addition of 6 μL of 2-mercaptoethanol to each after 20 min. Finally, all samples were heated to 90 °C for 2 min. Standard dissociation buffers not containing urea also were tried, with and without heating, but all gave virtually identical results as evidenced by protein staining and autofluorography. Alternative reducing agents such as dithiothreitol and sodium sulfite plus disodium 2-nitro-5-thiosulfobenzoate (Thannhauser et al., 1984) did not alter the electrophoretic pattern. After electrophoresis, the gel was stained with Coomassie Blue 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.

Results

Synthesis and Photolability of AzidoAcCh. One of the main objectives of our previous structure–activity study (Rogers & Parsons, 1989) was to gain insight into the spatial and electronic topography of the AcChT binding site. Active transport inhibitors based on derivatives of isonipecotic acid

[2] Under similar conditions, the half-life for photolysis of the azido group was about 1 min.

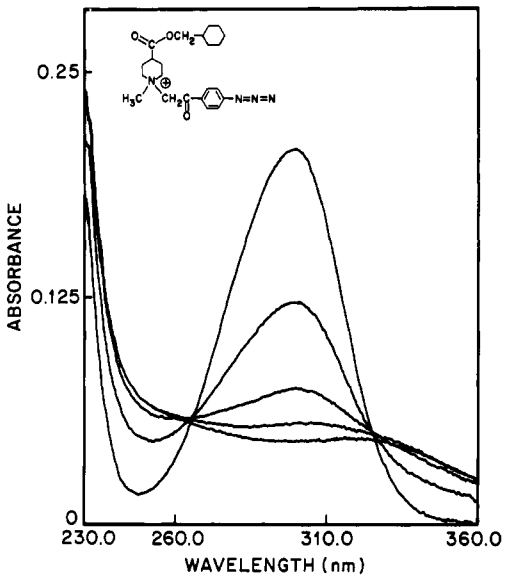

FIGURE 1: Structure and UV spectrum of AzidoAcCh. The initial spectrum (10 μM in TB) of AzidoAcCh shows a peak at λ_{max} = 295 nm that decreased in intensity when the cuvette was placed against a lamp with output centered at 366 nm. Times of illumination between consecutive spectra were 30 s, 1, 2, and 4 min so that the last spectrum was recorded after a sum of 7.5 min.

with hydrophobic groups attached to both functionalities had IC_{50} values of about 0.5 μM. On the basis of those findings, a structure was chosen that would allow facile incorporation of tritium and an arylazido group and that should retain the potency of its progenitors. This was cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-methylisonipecotate bromide (AzidoAcCh, Figure 1). The syntheses of both the nonradioactive and tritiated azido analogues are described under Materials and Methods. The overall yield for AzidoAcCh was 8%, and the incorporation of the tritium label was 26% efficient with a specific activity of 10.8 Ci/mmol.

The UV spectrum of AzidoAcCh, which has a λ_{max} = 298 nm in an aqueous buffer, is shown in Figure 1. The sample was illuminated with a lamp emitting light in a band centered at 366 nm for the times specified, and additional spectra were recorded. The half-life for photolysis of the azido group under these conditions was approximately 30 s. When the cuvette was exposed to the normal fluorescent lights in the laboratory (that has no windows), no photolysis could by detected after 8 h. Additionally, a solid sample left under the same fluorescent lamps showed no deterioration after 12 h. Thus, AzidoAcCh could be studied as a reversibly binding ligand without any special precautions required to avoid accidental photolysis.

Inhibition of AcCh Active Transport by AzidoAcCh Is Competitive. Even though AzidoAcCh was designed as an analogue of AcCh and is very similar in structure to another analogue that was shown to be a competitive inhibitor of AcCh active transport (analogue 23; Rogers & Parsons, 1989), it was thought essential to determine the kinetic mechanism of transport inhibition by this analogue. The nature of the inhibition was studied by the conventional method of varying [^{3}H]AcCh and AzidoAcCh concentrations (Cleland, 1979). The data, which are presented in Figure 2, demonstrate that AzidoAcCh is a competitive inhibitor of AcCh active transport with K_{is} = 0.38 ± 0.14 μM. This result indicates that the analogue binds to the AcCh site and not the vesamicol site, as that would have resulted in noncompetitive inhibition (Bahr et al., 1992a). No active transport of [^{3}H]AzidoAcCh could be detected in a separate experiment (data not shown).

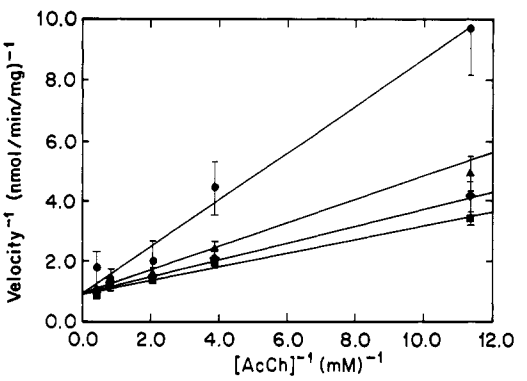

FIGURE 2: Inhibition of AcCh active transport by AzidoAcCh. Transport was measured as described under Materials and Methods. The range of concentrations of [^{3}H]AcCh was 88 μM to 2.4 mM. The concentrations of AzidoAcCh were 0 (■), 92 (◆), 276 (▲), and 920 (●) nM. Data are the mean values of triplicate observations (error bars show 1 SD) to which the hyperbolic form of the Michaelis–Menten equation was fitted using simultaneous nonlinear regression analysis of the separate sets of velocities at different inhibitor concentrations. The best lines determined by this analysis were graphed in the double-reciprocal format for presentation. The K_{is} for AzidoAcCh was 0.38 ± 0.14 μM. The V_{max} for transport was 1.08 ± 0.06 nmol/(mg·min) and the K_M for AcCh was 245 ± 60 μM, which is in excellent agreement with previously published values (Rogers & Parsons, 1989).

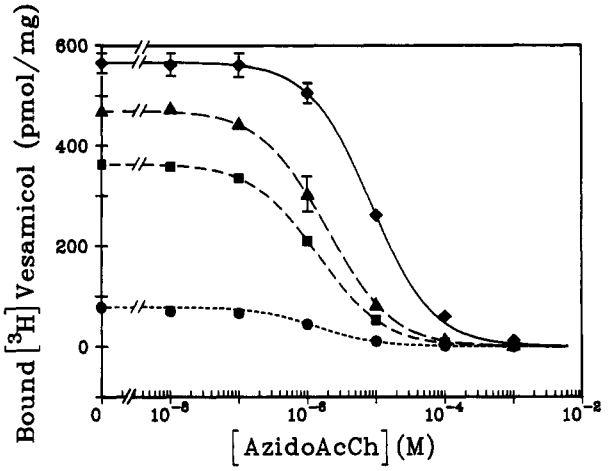

FIGURE 3: Inhibition of [^{3}H]vesamicol binding by AzidoAcCh. Specific binding was measured as described under Materials and Methods. Final concentration of vesicle protein was 50 μg/mL. Data are the mean values of triplicate observations, where error bars indicate 1 SD. Total [^{3}H]vesamicol concentrations were 4.9 (●), 28 (■), 48 (▲), and 220 (◆) nM. A competitive model that was used to fit hyperbolic curves simultaneously to all the data (using equations presented under Materials and Methods) resulted in K_{IA} = 0.24 ± 0.01 μM for AzidoAcCh and K_{DV} = 5.9 ± 0.2 nM for [^{3}H]vesamicol. B_{max} for vesamicol was 585 ± 3 pmol/mg of protein.

Inhibition of Vesamicol Binding by AzidoAcCh Is Competitive. The relationship between equilibrium binding of AzidoAcCh and vesamicol was determined by studying the effect of varying concentrations of nonlabeled AzidoAcCh on the binding of different concentrations of [^{3}H]vesamicol (Figure 3). The competitive model fit the data very well, and there was no justification for invoking the additional parameter of a noncompetitive model. The dissociation constants calculated were K_{IA} = 0.24 ± 0.01 μM for AzidoAcCh and K_{DV} = 5.9 ± 0.2 nM for [^{3}H]vesamicol. A competitive equilibrium binding relationship is predicted for AcCh analogues and vesamicol even though they bind to different sites (Bahr et al., 1992a).

Binding of [^{3}H]AzidoAcCh. The first-order rate constant for dissociation of specifically bound [^{3}H]AzidoAcCh from

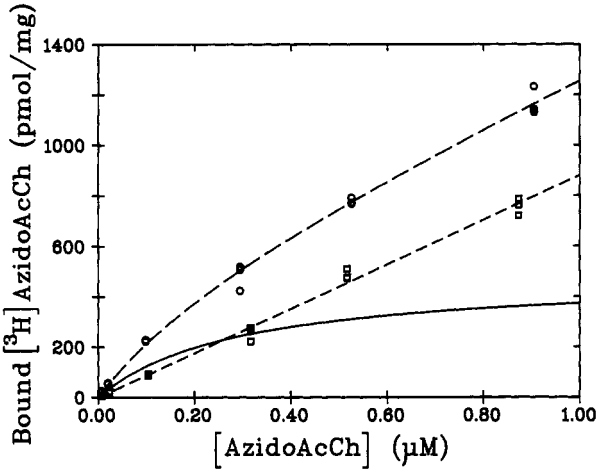

FIGURE 4: Reversible binding of [^{3}H]AzidoAcCh to synaptic vesicles. Vesicles were treated with paraoxon, $MgCl_2$, and MgATP as described under Materials and Methods. The final concentration of vesicular protein was 50 μg/mL. Bound [^{3}H]AzidoAcCh was determined in the presence (□) or absence (○) of 5 μM vesamicol. The solid line, which is derived by subtracting the former from the latter, represents specifically bound [^{3}H]AzidoAcCh (B_{max} = 480 ± 45 pmol/mg of protein) with a K_{DA} = 0.29 ± 0.06 μM.

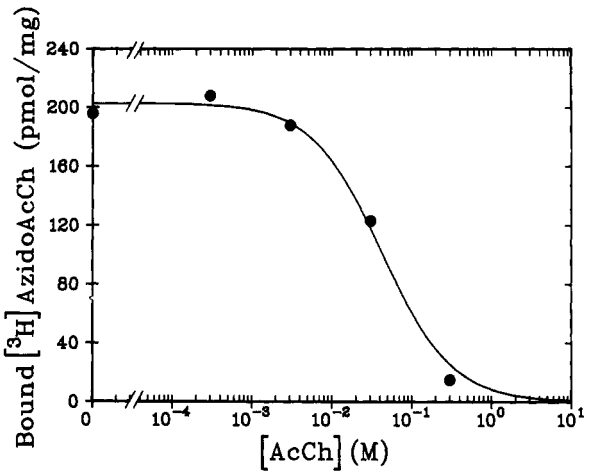

FIGURE 5: Inhibition of [^{3}H]AzidoAcCh binding by AcCh. Synaptic vesicle protein and [^{3}H]AzidoAcCh concentrations were 0.2 mg/mL and 0.44 μM, respectively. Nonspecifically bound [^{3}H]AzidoAcCh was estimated in the presence of 60 μM vesamicol (plus 0.3 M AcCh) and was subtracted from the total amount bound. The K_{IAA} for AcCh was 17 ± 3 mM as calculated using the equations described under Materials and Methods.

synaptic vesicles was determined by displacement with excess AzidoAcCh. At 0 °C, 30% of the bound tritium dissociated by a process too fast to measure by the filter assay, and about 20% by a process that fit a single-exponential decay with k_{diss} = 0.19 ± 0.02 min^{-1} (data not shown). The tritium that dissociated very rapidly or not at all was nonspecifically bound. The rapid dissociation could arise from generalized electrostatic repulsion by large amounts of nonspecifically bound nonradioactive AzidoAcCh. The tritium that dissociated by the slower process presumably was specifically bound. As the rate of dissociation corresponds to a half-life of 3.7 min, rapid filtration assays for measuring binding of [^{3}H]AzidoAcCh to synaptic vesicles should provide accurate data.

Attempted measurement of the nonspecific binding of [^{3}H]AzidoAcCh by inclusion of an excess of the protio analogue was hampered by the low aqueous solubility and high hydrophobicity of the compound. Vesamicol does not exhibit this problem, and because AzidoAcCh and vesamicol binding are competitive, a direct titration of transporter binding sites could be carried out with [^{3}H]AzidoAcCh using 5 μM vesamicol to block binding to the specific sites. The use of a different competitive ligand to determine nonspecific binding is a recommended practice (Bennett & Yamamura, 1985). Using this procedure, K_{DA} = 0.29 ± 0.06 μM and B_{max} = 480 ± 45 pmol/mg were determined for [^{3}H]AzidoAcCh (Figure 4). Specifically bound [^{3}H]vesamicol (1 μM) was 670 ± 15 pmol/mg of protein for this preparation of vesicles, which should be nearly equal to the saturation amount of binding.

Inhibition of [^{3}H]AzidoAcCh Binding by AcCh. Inhibition of [^{3}H]AzidoAcCh binding by AcCh under equilibrium conditions was assessed (Figure 5). A value for the dissociation constant of the AzidoAcCh·AcChT complex had to be assumed in order to calculate the dissociation constant for AcCh (K_{IAA}). Using the K_{DA} of 0.29 μM obtained from the direct binding experiment above, a K_{IAA} of 17 ± 3 mM for AcCh was calculated. Although saturating levels of AcCh displaced only 43% of the total bound [^{3}H]AzidoAcCh, the addition of 60 μM vesamicol produced no further decrease. Therefore, AcCh displaced all of the specifically bound azido analogue.

In addition to AcCh, other ligands known to be in vesicles were tested for the ability to affect the binding of [^{3}H]AzidoAcCh. The largest effect was seen for MgATP, which decreased nonspecific binding by as much as 30% at 10 mM. The same concentration of $MgCl_2$ had no effect, nor did 5 mM $CaCl_2$.

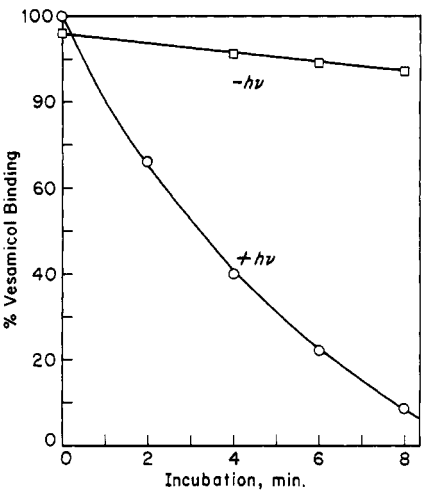

FIGURE 6: Photoinhibition of vesamicol binding by AzidoAcCh. Each datum represents a separate sample of synaptic vesicles (4 μg) applied to PEI-treated glass-fiber filters and treated as described under Materials and Methods. The data (○) on the line labeled +hν are for vesicles that were exposed to AzidoAcCh plus light prior to the assay for [^{3}H]vesamicol binding. Data (□) on the line labeled −hν were exposed to AzidoAcCh but without photolysis. Each 2-min increment represents a replacement of the photolyzed AzidoAcCh with a new portion of AzidoAcCh.

Irreversible Inhibition of Vesamicol Binding by Photolysis of the AzidoAcCh·AcChT Complex. Prior to synthesis of the radioactive form, it was desirable to know that AzidoAcCh would successfully label the AcChT. This was done with filter-mounted vesicles as follows, and the results are shown in Figure 6. In the absence of photolysis, the repetitive addition of AzidoAcCh to the vesicles, followed by a buffer wash each time, caused only a slight decrease in specific binding of [^{3}H]vesamicol that was subsequently applied to the filter. Illumination of the filter-mounted vesicles for a total of 8 min in the absence of AzidoAcCh caused no loss of vesamicol binding. However, repetitive treatment with AzidoAcCh (1 μM) and light caused the vesicles to lose greater than 90% of their [^{3}H]vesamicol-binding sites.

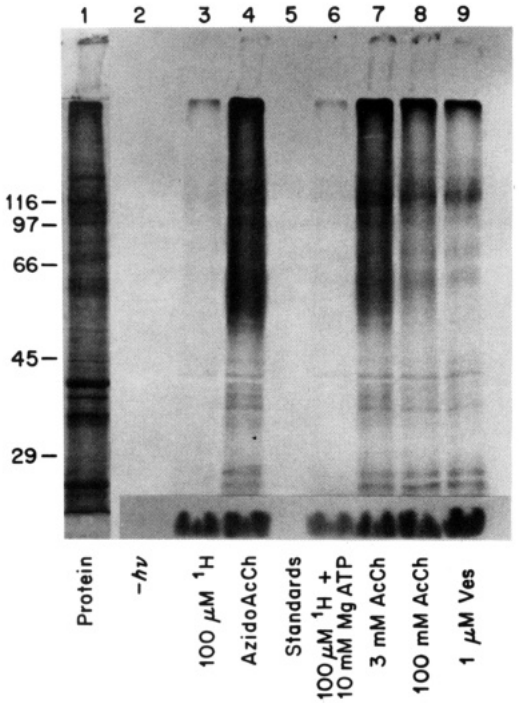

FIGURE 7: Autofluorographic analysis of synaptic vesicles photolabeled with [^{3}H]AzidoAcCh. Photolabeling in the presence or absence of the indicated reagents and the preparation of vesicles for SDS–PAGE and autofluorography were performed as described under Materials and Methods. The figure is a composite showing Coomassie staining, a short-term film exposure to the dye front region, and a long-term film exposure to the stacking and resolving regions of the gel. Lane 5 contained standard proteins (kDa) indicated on the left. Lanes 1 and 4 show the Coomassie Blue staining and autofluorographic patterns, respectively, of the same sample of labeled vesicles. Lanes 2 and 3 were controls that received no irradiation or 100 μM of [^{1}H]AzidoAcCh, respectively. Lanes 7 and 8 contained 3 or 100 mM AcCh, respectively, and lane 9 contained 1 μM vesamicol. Lane 6 contained 100 μM [^{1}H]AzidoAcCh and 10 mM MgATP. Similar results were obtained for three other preparations of vesicles.

Photolabeling of Synaptic Vesicles with [^{3}H]AzidoAcCh. The results above demonstrated that AzidoAcCh could be used to photoaffinity label the AcCh binding site with high efficiency and that synthesis and use of [^{3}H]AzidoAcCh could lead to identification of the AcChT. Due to the high level of nonspecific binding demonstrated above, [^{3}H]AzidoAcCh concentrations near the K_{DA} value were used in labeling experiments. This concentration yields an optimal compromise between maximal specific and excessive nonspecific labeling. Vesicle preparations were assayed for AcCh active transport competency prior to photolabeling experiments in order to ensure that a functional vesicle preparation was utilized. AcCh esterase was inhibited with paraoxon prior to the addition of [^{3}H]AzidoAcCh so that protection by AcCh could be tested. After a short incubation, suspended vesicles mixed with [^{3}H]AzidoAcCh were irradiated. In different samples, specificity of labeling was assessed by the coaddition of [^{1}H]-AzidoAcCh, AcCh, vesamicol, or the more potent vesamicol analogue 4-aminobenzovesamicol (analogue 72; Rogers & Parsons, 1990). Prior and subsequent to irradiation, binding of [^{3}H]AzidoAcCh to vesicles was determined by filter assay and liquid scintillation counting. Vesicles then were pelleted to remove most of the nonbound radioactivity and analyzed further by SDS–PAGE followed by Coomassie Blue staining and autofluorography.

As analyzed on SDS–PAGE (10%) (Figure 7), a continuous region from about 50 kDa to the top of the resolving gel was heavily labeled by photolyzed [^{3}H]AzidoAcCh (lane 4). The protein stain (lane 1) of the same gel lane demonstrated that the diffuseness of the fluorogram did not arise from poorly focused proteins. However, no protein was stained that corresponded to the diffuse radiolabeling pattern. An absence of nonspecific aggregation was indicated by the lack of protein staining or radioactivity in the stacking gel. Lane 2 demonstrates that photolysis was required for labeling, and lane 3 shows that excess [^{1}H]AzidoAcCh blocked labeling of virtually all proteins. Lanes 7 and 8 demonstrate increasing levels of protection from labeling by 3 and 100 mM AcCh, respectively. Lane 9 shows that vesamicol at only 1 μM nearly completely blocked the diffuse labeling seen in lane 4. An autofluorogram that was developed after a shorter exposure time did not reveal any discrete labeling that might have been obscured by the intense, diffuse pattern shown in lane 4.

Heavy labeling of material that ran with the dye front also occurred, but it was not blocked by any of the ligands that bind to the AcChT or VR. A filter assay of the vesicles shown in Figure 7 prior to pelleting showed that the excess protio analogue blocked only about 30% of the incorporated label and that 10 mM MgATP increased this protection to 60%. This latter effect was accompanied by a significant decrease in the labeling of the material that migrated with the dye front (lane 6). Thus, material that does not behave as a classical protein during SDS–PAGE was labeled in a manner that was partially inhibited by MgATP. The basis of the effect is not known, but possibly it is caused by activation of the two vesicle ATPases with concomitant perturbation in the transmembrane electrical or pH gradient (Yamagata & Parsons, 1989).

If similarly labeled vesicles were subjected to electrophoresis on 5–15% gradient gels and subsequently silver stained, rather uniform, diffuse staining of 50–250-kDa material was observed (data not shown). After destaining, the range and extent of radiolabeling as detected by autofluorography were coincident with the silver-stained image. Without producing any change in the appearance of the proteins as visualized by silver staining, vesamicol (10 μM), 4-aminobenzovesamicol (200 nM) and, to somewhat of a lesser extent, AcCh (100 mM) blocked the incorporation of [^{3}H]AzidoAcCh into the diffusely labeled material. When 200-fold excess [^{1}H]AzidoAcCh was included, about 35–40% of the incorporated tritium was blocked (as determined by filter-based binding assays) and virtually no proteins were labeled as judged from the fluorogram. In this gel, specifically labeled proteins as small as 14 kDa would have been detected. Supernatant from the vesicle pelleting procedure was also examined by SDS–PAGE, and a few light bands were revealed by Coomassie staining, but no tritium by autofluorography. If vesicles were not treated with paraoxon prior to photolabeling, a protein band at about 66 kDa also was heavily labeled. This labeling was blocked by AcCh (100 mM), but not by vesamicol (1 μM).

The preparation of vesicles that was used to determine the reversible binding parameters for the AzidoAcCh site was also subjected to photoaffinity labeling and quantitation using the normal filter assay. Vesamicol (5 μM) and 4-aminobenzovesamicol (1 μM) each blocked 45 pmol of label incorporation/mg of protein. Thus, about 9% of the specific binding sites for [^{3}H]AzidoAcCh was photolabeled in these experiments.

Discussion

The AcCh analogue that was used here to photoaffinity label the AcChT exhibits several desirable photochemical properties. The wavelength of maximum absorption is sufficiently near the visible region that light of wavelength longer than that absorbed by typical proteins can be used for the photolysis.

This minimizes photodamage to vesicular components. No damage to the irradiated VR was detected by the [^{3}H]vesamicol-binding assay or to other proteins at a level that could be detected by SDS–PAGE. On the other hand, ordinary fluorescent lights caused no detectable photolysis. Therefore, it is straightforward to perform "dark" experiments with AzidoAcCh in which binding is reversible. An undesirable property of the compound is its significant hydrophobicity. Despite being a quaternary ammonium ion, organic solvents are required to achieve millimolar aqueous concentrations. The combination of charge plus hydrophobicity possibly produces detergent-like behavior and probably is the cause of the inability to use high concentrations of the protio ligand to assess nonspecific binding of the tritio ligand to the vesicles. The AcCh structure–activity data (Rogers & Parsons, 1989) suggest that it would be difficult to eliminate this problem while maintaining high affinity for the AcCh binding site. Nevertheless, by avoiding high concentrations, the analogue can be utilized successfully.

AzidoAcCh binds to a saturable site in the vesicles, and it is a competitive inhibitor of the active transport of AcCh and of the binding of vesamicol. It is not actively transported. All of this behavior is consistent with the kinetic model for the AcChT–VR, in which vesamicol is a noncompetitive inhibitor of AcCh active transport but a competitive inhibitor of AcCh binding at equilibrium (Bahr et al., 1992a). The model predicts that the K_{DA} for saturation of the specific binding site for AzidoAcCh (0.29 ± 0.06 μM) will be the same as the K_{IA} for inhibition of the binding of vesamicol (0.24 ± 0.01 μM). This was found. Moreover, the model predicts that the K_{is} for inhibition of active transport by nontransported AzidoAcCh (0.38 ± 0.14 μM) will be the same or lower. The result is consistent with a similar value. The model also predicts that the equilibrium dissociation constant for AcCh is much greater than the transport K_M value of 0.3 mM. The K_{IAA} estimate for the inhibition of AzidoAcCh binding by AcCh (17 ± 3 mM) is consistent with this expectation. Thus, there is good internal consistency in the reversible binding characteristics of AzidoAcCh, as monitored from complementary perspectives, and it is clear that the analogue binds to the AcChT transport site as required.

The only discrepancy of note in the reversible binding studies is that B_{max} for AzidoAcCh (480 ± 45 pmol/mg) was about 70% of the B_{max} for vesamicol (at least 670 ± 15 pmol/mg) in the same preparation of vesicles. This could occur if the binding sites reside in separate subunits of the AcChT–VR that are present in unequal numbers. The concentration of binding sites for AzidoAcCh corresponds to about 10 per vesicle and for vesamicol to about 14 per vesicle (Anderson et al., 1986). Alternatively, the binding sites might be in the same subunit but be affected differently by damage during purification of synaptic vesicles or by physiological regulation. Regardless, the result constitutes further evidence that AcCh and vesamicol bind to separate sites.

Preliminary assurance that AzidoAcCh would irreversibly label that AcChT with good efficiency was obtained by use of filter-mounted vesicles. Such assurance was felt to be necessary before undertaking synthesis of radioactive analogue because the photogenerated nitrene undergoes competing reactions. The reaction with water often predominates and produces a hydroxylamine derivative that in this case likely would be a competitive inhibitor of AzidoAcCh binding to the AcChT (Bayley, 1983). This phenomenon reduces the efficiency of labeling, particularly at high azido analogue concentrations, and it is common that only a moderate fraction of the specific binding sites can be labeled (less than 10%; Haugland, 1987). By mounting vesicles on a glass-fiber filter where photolyzed AzidoAcCh could be washed away and replaced by fresh ligand, several rounds of photolysis could be carried out on the same vesicles. The status of the AcChT was assessed after each photolysis step by quantitating the available sites for [^{3}H]vesamicol binding after washing away the nonbound photolysis products. The results demonstrate that AzidoAcCh does photolabel the AcChT with good efficiency and that use of [^{3}H]AzidoAcCh could lead to identification of the AcChT. It was not practical, however, to use filter-mounted vesicles for the purpose of identifying the AcChT. Instead, a suspension of vesicles suitable for subsequent SDS–PAGE was photolabeled once at a moderate concentration of [^{3}H]AzidoAcCh in order to maximize the specificity of labeling. The extent of specific labeling obtained (9% of the AcChT sites) is excellent considering that a subsaturating concentration of label was utilized.

The results of this affinity-labeling study as analyzed by SDS–PAGE are very surprising. Highly labeled material exhibited a continuous distribution from about 50 to about 250 kDa, and this labeling was blocked efficiently by vesamicol, 4-aminobenzovesamicol and [^{1}H]AzidoAcCh. The rather modest amount of protection obtained with 3 mM AcCh might seem inconsistent with the K_M value for AcCh of 0.3 mM, but it is consistent both with the K_{IAA} value of 17 mM for AcCh (measured by displacement of reversibly-bound AzidoAcCh) and with the kinetic model for the AcChT–VR, which posits very weak binding of AcCh (Bahr et al., 1992a). As AzidoAcCh is much more tightly bound, it will protect the AcChT at concentrations above 0.3 μM. In summary, the protection pattern seen in Figure 7 is fully consistent with specific labeling of the AcChT.

The electrophoretic characteristics of the specifically labeled material could not be changed by manipulation of the conditions of sample dissolution in SDS before PAGE. The presence or absence of 8 M urea, as well as use of different temperature regimens and reducing agents, did not affect the outcome. This suggests that nonspecific aggregation does not cause the diffuse streaking behavior of the labeled material. Moreover, we know that our protocol *does* produce predictable labeling of the AcCh esterase that contaminates the vesicle preparation. If vesicles were not first incubated with paraoxon to block the AcCh esterase binding site, a protein of the same M_r as esterase (Lee et al., 1982) was photolabeled in a manner blocked by AcCh but not vesamicol. Thus, we must conclude that the labeled material is inherently diffuse by SDS–PAGE.

This diffuse appearance is very similar to that of the vesicle proteoglycan, which does not stain well with Coomassie Blue (Carlson & Kelly, 1983). Such severe streaking of PG in SDS–PAGE is inherent in the structure of PG and does not reflect a poorly performing gel. Moreover, the VR has been purified, as reported in the preceding paper, and it has been found to copurify with one form of the vesicle proteoglycan. No other protein species was evident. In the fourth paper in this series, Bahr et al. (1992b) demonstrate that even after SDS denaturation the photolabeled AcChT remains associated with proteoglycan, that the VR is immunoprecipitated by an antibody directed against the proteoglycan, and that enzymes that degrade certain glycosaminoglycan components of proteoglycans inhibit vesamicol binding. It seems clear that involvement of proteoglycan provides the explanation for the surprising results observed in this photoaffinity-labeling study.

The nature and origin of the association of the AcChT–VR with proteoglycan is not certain. Binding of [^{3}H]AzidoAcCh

to proteoglycan, which is highly negatively charged, surely did not occur by an ion-exchange mechanism because labeling was blocked by two distinct classes of ligands acting in concentration ranges (as low as 200 nM) appropriate to the affinities for their specific binding sites in the AcChT–VR. It is conceivable that [^{3}H]AzidoAcCh bound to the AcChT was oriented so that the photoderived nitrene pointed toward adjacent proteoglycan in the intact vesicle, thus achieving specific labeling of proteoglycan that really has no role in the function of the AcChT. The AcChT, in this case, could be a classical globular, integral protein that remains unidentified. However, the phenylazido group is important to the affinity of the analogue, which implies that it makes favorable contacts with some component of the AcChT. In this regard, it has been reported recently (Dougherty & Stauffer, 1990) that AcCh binding to model receptor sites can be mediated by interaction between the quaternary ammonium group and the π electrons of aromatic rings such as those found in tyrosine and tryptophan. The same type of interaction has been hypothesized to be important to the "aromatic guidance" mechanism for binding of AcCh by AcCh esterase (Sussman et al., 1991). Such an interaction could explain the large increase in potency of analogues such as AzidoAcCh that bear an aromatic substituent on the quaternary nitrogen. Likely, the photogenerated nitrene is in intimate contact with the AcChT, and this results in the good efficiency of photolabeling that is observed. Because the VR is an allosteric site in the AcChT, the observation that the VR is associated with proteoglycan (Bahr & Parsons, 1992) is consistent with the conclusions of this study.

Registry No. AcCh, 51-84-3; MeI, 74-88-4; AzidoAcCh, 141376-32-1; [^{3}H]AzidoAcCh, 141376-33-2; vesamicol, 22232-64-0; isonipecotic acid hydrochloride, 5984-56-5; cyclohexylmethyl isonipecotate, 141376-34-3; cyclohexylmethanol, 100-49-2; 4-azidophenacyl bromide, 57018-46-9; cyclohexylmethyl *N*-methylisonipecotate, 124805-93-2.

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