Purification of the Vesamicol Receptor†

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ABSTRACT: The vesamicol receptor (VR) present in cholinergic synaptic vesicles isolated from the electric organ of *Torpedo* was solubilized in cholate detergent and stabilized with glycerol and a phospholipid mixture. The receptor was purified in 7% yield by hydroxylapatite, wheat germ lectin affinity, DEAE anion-exchange, and size exclusion chromatographies based on a [^{3}H]vesamicol binding assay. A final specific binding of 4400 pmol/mg of protein was obtained. The cholate-solubilized VR complex exhibited variable aggregation states with particle molecular masses of 210–3500 kDa in different experiments. The purified VR exhibited very heterogeneous electrophoretic mobility in sodium dodecyl sulfate–polyacrylamide gel electrophoresis with very diffuse protein staining at about 240 kDa. No "classical" polypeptide or glycopeptide band was detected. One form of the SV1 epitope, which is characteristic of cholinergic synaptic vesicle proteoglycan, copurified precisely with the VR. The SV2 epitope, which is found in most neuronal and endocrine secretory vesicles, also closely purified with the VR. Substantially purified VR retained both enantioselectivity for (−)-vesamicol and a linked AcCh-binding site. This confirms the allosteric model for the VR in the AcCh transporter. The physicochemical properties of the VR and copurification of it with the SV1 epitope strongly suggest that the VR is associated with cholinergic vesicle proteoglycan. A second proteoglycan that is not associated with the VR but which carries the SV1 and SV2 epitopes also was observed.

The preceding paper presented a kinetics model for the vesamicol receptor (VR)[1] that hypothesizes it to be an allosteric site in the acetylcholine transporter (AcChT) of cholinergic synaptic vesicles (Bahr et al., 1992a). The kinetics and substrate specificity properties (Clarkson et al., 1992) of the system are unusual. Thus, purification of the AcChT–VR and characterization of its structure are of great interest. However, our attempts to accomplish purification by utilizing an AcCh transport-reconstitution assay have not been successful. We report here success with an alternative approach that purified at least part of the system based on binding of [^{3}H]vesamicol to detergent-solubilized VR. The ligand-binding properties of the purified material were characterized and shown to be similar to those of AcChT–VR in vesicle ghosts. The purified VR exhibits unexpected chromatographic, electrophoretic, and immunochemical properties that suggest a novel structure for this transporter system.

Materials and Methods

Materials. [^{3}H]Vesamicol (27 Ci/mmol) was purified as described (Bahr & Parsons, 1986). AcCh analogue 23 was a gift from Dr. Gary A. Rogers of this institution. The monoclonal antibodies (mAbs) tor 70 and 5G1, which are directed against the SV1 epitope, were gifts from Drs. Pinky Kushner (ALS Neuromuscular Research Foundation, Pacific Medical Center, 2351 Clay Street, San Francisco, CA) and Steven Carlson (Department of Physiology, University of Washington, Seattle, WA), respectively. Hybridoma cells (10H3) producing anti-SV2 and hybridoma supernatant were a gift from Dr. Regis Kelly (Department of Biochemistry and Biophysics, University of California, San Francisco, CA). Berkeley Antibody Co., Inc. (Richmond, CA) utilized the cells to produce ascites fluid from which the mAb was obtained at approximately 50% purity (2.7 mg of protein/mL) by chromatography on DEAE Affi-Gel Blue. ^{125}I-labeled goat anti-mouse IgG or IgM was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). Rabbit anti-mouse Ig Immunobeads, nitrocellulose sheets, and Bio-Gel HTP hydroxylapatite (HTP) powder were from Bio-Rad Laboratories (Richmond, CA). Type GF/F glass-fiber filters were from Whatman Corp. (Hillsboro, OR), and 0.45-μm nitrocellulose filters (Type HAWP 025 00) were from Millipore Corp. (Bedford, MA). Ammonia-free glycine was from Calbiochem Biochemicals (La Jolla, CA) and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) was from Research Organics, Inc. (Cleveland, OH). Cholic acid was from Fluka Chemical Corp. (Ronkonkoma, NY). Wheat germ (*Triticum vulgaris*) lectin–Sepharose 4B (WG-4B; 4% cross-linked) was from Pharmacia Inc. (Piscataway, NJ). The high-performance liquid chromatography (HPLC) system used was from Beckman Instruments, Inc. (Fullerton, CA), and it employed either a Spherogel–TSK DEAE–5PW (Toyo Soda Manufacturing Co., LTD., Japan) polymer-based anion-exchange column (7.5 × 75 mm dimensions, 1000-Å pore size, 10-μm particle) or two size exclusion columns in series, a TSK gel G4000PW$_{XL}$ column (7.8 × 300 mm, 400-Å pore size, 10-μm particle, with a 7.8 × 25 mm guard column, both from Toyo

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[1] Abbreviations: AcCh, acetylcholine, AcChT, acetylcholine transporter; vesamicol, (−)-*trans*-2-(4-phenylpiperidino)cyclohexanol except where indicated as the (+)-enantiomer; VR, vesamicol receptor; mAb, monoclonal antibody; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; pAB, *p*-aminobenzamidine; SV, synaptic vesicle; PC, phosphatidylcholine; PG, proteoglycan, BSA, bovine serum albumin; HTP, hydroxylapatite; WG, wheat germ lectin; GlcNAc, *N*-acetylglucosamine; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BIGCHAP, *N*,*N*-bis(3-D-gluconamidopropyl)cholamide; M_r, relative molecular mass.

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Soda) and a SynChropak GPC500 column (7.8 × 300 mm, 500-Å pore size, 7-μm particle) from SynChrom, Inc. (Lafayette, IN). QuantiGold protein assay reagent and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) molecular weight standards were purchased from Diversified Biotech (Newton Centre, MA). The BCA protein assay reagents were from Pierce Chemical Co. (Rockford, IL). Photographic reagents including X-OMAT AR X-ray film were from Eastman Kodak Co. (Rochester, NY). Polyacrylamide solutions and liquid scintillation cocktails were from National Diagnostics (Somerville, NJ). Centricon and Centriprep centrifugal concentrator devices were from Amicon Corp. (Lexington, MA). All remaining chemicals, unless otherwise noted, were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Synaptic Vesicles. Torpedo californica (obtained locally) VP_1 synaptic vesicles (SVs) were isolated from electric organ in 800 mosM glycine and glycine/sucrose media (Yamagata & Parsons, 1989). Briefly stated, this involves differential sedimentation velocity pelleting onto a sucrose–Ficoll cushion, equilibrium buoyant density centrifugation, and Sephacryl S-1000 filtration of vesicles. Purified SVs were concentrated by centrifugal pelleting and resuspended at 4–8 mg of protein/mL in buffer A consisting of 100 mM HEPES, 10 mM EDTA, 1 mM EGTA, and 0.02% (w/v) NaN_3, adjusted to pH 7.0 with KOH. The following protease inhibitors also were present in buffer A: 100 μM phenylmethanesulfonyl fluoride (PMSF), 100 μM *p*-aminobenzamidine (pAB), 10 μg of leupeptin (hemisulfate salt)/mL, 10 μg of pepstatin/mL, 8 μg of chymostatin (type A, B, and C mixture)/mL, 8 μg of antipain/mL, 8 μg of aprotinin/mL (FBA Pharmaceuticals, New York, NY), 20 μg of trypsin inhibitor (type I-S)/mL, 20 μg of trypsin–chymotrypsin inhibitor (Bowman-Birk Inhibitor)/mL, 8 μg of bestatin/mL, and 4 μg of *N*-(*o*-rhamnopyranosyloxyhydrophosphinyl)Leu-Trp (ammonium salt)/mL. The SV suspensions were stored at 4 °C until used.

SV Solubilization in Cholate. Solubilization of SVs was achieved by bringing the above suspension to the following final concentrations in buffer A: 0.77 mg of protein/mL, 175 mM KCl, 25 mM NaCl, 20% (w/v) glycerol, 5% (w/v) cholic acid, and 5 mg of L-α-phosphatidylcholine/mL (PC; 14% purity, remaining is a mixture of phospholipids; Sigma type II-S). The solubilized SV suspension then was homogenized in an etched-glass Potter–Elvehjem tissue grinder for 30 s at high speed. After a 10-min incubation at 23 °C, the solubilized SV were diluted 5-fold to 1% cholic acid, 1 mg of PC/mL, 35 mM KCl, 5 mM NaCl, and 0.15 mg of SV protein/mL by adding buffer B consisting of 25 mM HEPES, 10 mM EDTA, 1 mM EGTA, 0.02% (w/v) NaN_3, and 20% (w/v) glycerol, pH 7.9, with KOH, and the following concentrations of each of the protease inhibitors from above: 0, 0, 2, 2, 1, 1.5, 5, 16, 2.5, 1.5, and 0.7 μg/mL, respectively. All further handling of solubilized VR was in buffer C consisting of 25 mM HEPES, 10 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 1% cholic acid, 1 mg of PC/mL, 20% (w/v) glycerol, pH 7.9, with KOH, except where noted for ion-exchange chromatography.

[^{3}H]Vesamicol Binding Assay. A sample of VR was equilibrated with 500 nM (or 20 nM for IC_{50} studies) [^{3}H]-vesamicol for 60 min at 23 °C in a total volume of 165 μL made up with buffer C. In early work, binding to solubilized VR was measured with the centrifugation–gel filtration method described by Neal and Florini (1973). The equilibrated VR was applied to a 2.0-mL bed of precentrifuged Sephadex G-50-150 in buffer C contained in a 6-mL plastic column (E&K Scientific, Commerce City, CO) at 4 °C. The column immediately was centrifuged in a swinging-bucket rotor at 250*g* for 4 min, and the excluded solution passing through the column was assayed for tritium content by liquid scintillation spectroscopy in 6 mL of aqueous counting scintillant (Hydrofluor; Amersham Corp., Lexington, MA) and for protein content. In later work, [^{3}H]vesamicol binding to solubilized VR was measured by filtering the equilibrated sample through a polyethyleneimine-coated (0.5% for 2 h and then rinsed with water) glass-fiber filter which immediately was washed with four sequential ice-cold 1-mL volumes of buffer C. Filters were prewashed immediately prior to sampling. Filter-bound tritium was determined by liquid scintillation spectroscopy. Both assay methods gave similar results. The ability of solubilized VR to bind to the filters was unexpected, but it is explicable because of the high negative charge associated with proteoglycan (PG). Nonspecific binding is defined as the amount of [^{3}H]vesamicol bound in the presence of 100-fold excess of nonradioactive vesamicol. Results are expressed as specifically bound [^{3}H]vesamicol, which equals the difference between the total bound and nonspecifically bound.

Protein Assays. Bovine serum albumin (BSA) was used as a standard. Nonsolubilized SV suspensions were assayed with either the Bradford (1976) or the BCA (Pierce Chemical Co.) methods. Solubilized SVs and chromatographed VR, except for size exclusion fractions, were assayed with the Schaffner and Weissman (1973) method where trichloroacetic acid-precipitated protein is applied to nitrocellulose filters before staining and quantification. Size exclusion chromatography fractions were assayed with the nanogram-sensitive QuantiGold method obtained from Diversified Biotech.

Receptor Purification. The VR was purified by sequential chromatographic steps. A 16-mL column bed (1.5 × 9 cm) was made from four parts HTP mixed with one part (v/v) microgranular cellulose needed to obtain a suitable flow rate. After being mixed in 10 mM potassium phosphate and 30 mM potassium chloride, pH 7.4, the column matrix was poured in 1-mL portions to minimize differential settling and then equilibrated in buffer C containing 0.3 M KCl. Immediately after they were diluted (see above), solubilized SVs (16–20 mg of protein) were applied to the column at 4 °C with a flow rate of 0.7 mL/min, after which the column was washed with three column volumes of buffer C containing 0.3 M KCl. The VR was eluted with a 50-mL linear gradient exchanging the 0.3 M KCl with 0.3 M potassium phosphate at pH 7.9. Two 25-μL portions of the 1-mL fractions were used to assay [^{3}H]vesamicol binding and protein content. The fractions containing the most VR were pooled (3–4 mL) and applied at a 0.35 mL/min flow rate to an 11-mL column of WG-4B equilibrated at 4 °C in buffer C containing 0.1 m KCl. The column was washed with three column volumes of this buffer, and the VR was eluted with 0.75 M *N*-acetylglucosamine (GlcNAc) in buffer C. The 1-mL fractions were assayed as above. The VR-rich pool (4–6 mL) was applied at a rate of 0.5 mL/min to the HPLC DEAE–5PW column equilibrated at 23 °C in buffer C containing 5 mM KCl but no PC. After washing the column with eight column volumes of equilibration buffer, a linear gradient from 5 to 400 mM KCl eluted the VR. One-half or 1-mL fractions were collected in test tubes containing 25 μL of buffer C supplemented with 19 mg of PC/mL. Fractions were assayed as before. Salt gradient profiles for the HTP and DEAE–5PW columns were determined from conductivity and standard curves. The VR-rich pool (6–8 mL) was diluted with buffer C to approximately 0.2

M KCl and concentrated about 25-fold by centrifugation in Amicon Centricon or Centriprep concentrators having an exclusion limit of 30 kDa. A 350-μL portion was applied at a rate of 0.2 mL/min to the HPLC dual size exclusion column system equilibrated at 23 °C in buffer C containing 0.1 M KCl. One-half milliliter fractions were collected, of which 60- and 20-μl portions were used for VR and protein assays, respectively. The void (V_o) and included volumes (V_i) were determined with salmon sperm DNA (slightly sheered) and tyrosine, respectively. The elution volumes (V_e) of carbonic anhydrase (29 kDa), β-amylase (200 kDa), apoferritin (450 kDa), and thyroglobulin (monomer and dimer; 670 kDa and 1.4 MDa, respectively) were determined and related to the V_o and V_i by $K = (V_e - V_o)/(V_i - V_o)$. A graph of K versus the logarithm of the M_r gave a linear relationship with a correlation coefficient of −0.996 from which the M_r's of SV proteins were obtained.

Gel Electrophoresis and Immunoblots. Samples from each chromatographic step were analyzed by SDS–PAGE (Laemmli, 1970). To reduce the concentrations of KCl and PC, samples were diluted at least 10-fold into buffer B and concentrated in Amicon Centricon-30 devices until 1–5 μg of protein per 50 μL was achieved. The samples were denatured in 2.5% (w/v) SDS, 10% (w/v) glycerol, and 3% (v/v) 2-mercaptoethanol at 100 °C for 5 min. The denatured samples were subjected to gradient PAGE (linear 3–15% acrylamide, w/v) with a 2.5% stacking gel in 0.2% SDS. The separated proteins were either silver stained or transferred electrophoretically for 20–30 h to nitrocellulose paper (immunoblot) as described by Burnette (1981). Alternatively, protein samples were applied directly to a 1.5 × 1.5 cm numbered grid pattern on nitrocellulose paper for quantitative epitope assay (immunodot blot). The nitrocellulose sheets were incubated in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 5% (w/v) BSA (Tris/BSA) for 1 h at 23 °C before the addition of tor70 or 5Gl hybridoma supernatant (diluted 1:50) or partially purified anti-SV2 mAb (diluted 1:800) for 2 h more as described (Burnette, 1981; Buckley & Kelly, 1985). The sheets were washed thoroughly, and then they were incubated with ^{125}I-labeled goat anti-mouse IgG (or anti-mouse IgM when using tor70 IgM mAb) at (0.2–1) × 10^6 dpm/mL in Tris/BSA containing 0.1% (w/v) Triton X-100. Finally, the sheets were extensively rinsed, dried, and either placed against X-ray film for 48 h at −80 °C (immunoblot) or individual squares in the grid were cut from the sheet and counted by using a γ-counter (immunodot blot). Background was determined as the number of counts per minute bound to a square of nitrocellulose treated similarly but without applied sample, and it was subtracted from all relevant data.

Immunoblot results were calibrated by use of prestained M_r standards. Stained proteins were detected with the Hoefer Scientific Instruments (San Francisco, CA) GS300 densitometer, and associated software was used to determine the M_r's.

Immunoprecipitation of Cholate-Solubilized SVs. A 0.8-mL slurry of 8 mg of rabbit anti-mouse Ig Immunobeads in phosphate-buffered saline was incubated with 1.3 mL of 10H3 hybridoma supernatant with gentle agitation overnight at 4 °C as described (Buckley & Kelly, 1985). The beads were washed three times by centrifugation and resuspension in 3 mL of buffer C containing 0.4 M KCl and 1% (w/v) Triton X-100 and three times in 3 mL of buffer C containing 0.4 M KCl and 1 mg of BSA/mL. Cholate solubilized and stabilized SVs contained 0.3–0.4 M KCl, 1 mg of BSA/mL, and 0.1 mg of SV protein/mL. This was mixed with 6 mg of antibody-loaded Immunobeads per 20–40 μg of SV protein in a final volume of about 0.4 mL for an overnight incubation with gentle agitation at 4 °C. Control beads were prepared with heat-treated (100 °C for 5 min) 10H3 hybridoma supernatant or with a nonspecific mouse myeloma IgGl. After centrifugal removal of the Immunobeads, the supernatant was assayed for VR ([^{3}H]vesamicol binding) and epitope (immunodot blot) contents. The beads, after extensive washing in buffer C, were assayed for VR binding using the glass-fiber filter assay above.

Results

Detergent Solubilization and Stabilization of the VR. Numerous detergents were screened for the ability to solubilize the VR with retention of [^{3}H]vesamicol binding. Only the steroidal detergents cholate, deoxycholate, CHAPS, and BIGCHAP were successful. Early attempts to chromatograph the VR in these detergents, though, resulted in a major loss of [^{3}H]vesamicol binding. Active VR could be preserved in cholate by including glycerol and a phospholipid mixture. An extensive cocktail of protease inhibitors also was included, and no proteolysis during the purification was observed.

The dissociation rate constant was determined for the cholate-solubilized and stabilized [^{3}H]vesamicol·VR complex by displacement with excess nonradioactive vesamicol. It was 0.37 ± 0.02 min^{-1} at 23 °C (data not shown). This is slow enough that the amount of bound [^{3}H]vesamicol can be determined reliably using usual assays.

Purification of the VR. Sixteen to 20 mg of SV protein was solubilized in 5% cholate in order to produce total membrane disruption, after which the detergent concentration was diluted to 1% to stabilize the VR. Successful purification was accomplished utilizing four chromatographic steps, namely, hydroxylapatite (HTP), wheat germ lectin affinity (WG), DEAE anion exchange (DEAE), and size exclusion (SIZ). [^{3}H]Vesamicol binding and protein profiles for fractionated material from representative chromatograms are shown in Figures 1 and 2. Protein amount, VR specific binding, the purification factor, VR binding recovery, and the VR yield at each step averaged from seven preparations are listed in Table I. Some purification details are presented next.

Solubilized, diluted vesicles were applied to the HTP column, and the VR and about half of the applied protein were eluted at a potassium phosphate concentration of 35 mM, resulting in about 1.8-fold purification of the VR (Figure 1A). The HTP step concentrated the VR and was necessary for efficient adsorption of it to the WG and DEAE columns. The pooled VR was applied to the WG column at a low flow rate to ensure full adsorption. The increase in specific binding after elution with GlcNAc was about 3.1-fold when the first fraction containing GlcNAc and protein was not pooled. Nine other lectin affinity columns exhibiting a variety of sugar specificities did not bind the VR (data not shown). The VR pool from the WG column was chromatographed on the DEAE column in the absence of phospholipid because the capacity of the column otherwise was greatly reduced. The VR eluted at 270 mM KCl about 1.9-fold purified, and it was the last protein to elute. Heterogeneity was evident because partially resolved shoulders of VR were present on the main peak. Test tubes receiving the fractions contained concentrated phospholipid dispersed in cholate in order to minimize loss of vesamicol binding. In size exclusion chromatography, the VR eluted in a moderately broad peak as a species of 3.5 MDa, with an indication that a lower M_r species was slightly resolved as a shoulder on the right-hand side of the peak (Figure 2A). The VR binding was about 1.5-fold purified, and it corresponded closely with a peak of light absorbance at 280-nm wavelength.

Table I: Purification of the Cholinergic Synaptic Vesicle VR[a]

purification step[b]	protein (μg)	specific binding (pmol/mg)	purification factor	step recovery[c] (%)	yield[d] (%)
SV	18000 ± 2100	300 ± 100	1.0	100	100
Sol SV	18000 ± 2100	270 ± 100	0.9	88 ± 8	88 ± 8
HTP	3100 ± 1100	480 ± 120	1.6	54 ± 7	34 ± 10
WG	490 ± 120	1500 ± 320	5.0	84 ± 16	24 ± 10
DEAE	150 ± 19	2900 ± 180	9.7	45 ± 15	10 ± 4
SIZ	46 ± 15	4400 ± 910	15	73 ± 18	7 ± 3

[a] The average (±1 SD) from seven VR preparations. [b] SV is the intact vesicles, Sol SV is the cholate-solubilized and stabilized vesicle solution, HTP is hydroxylapatite chromatography, WG is wheat germ lectin affinity chromatography, DEAE is anion-exchange chromatography, and SIZ is size exclusion chromatography. [c] The total VR binding recovered before losses due to assays and fraction pooling, expressed as percent of the binding applied to that step. [d] The overall yield of VR binding through each step after losses due to assays and pooling, expressed as percent of starting SV binding.

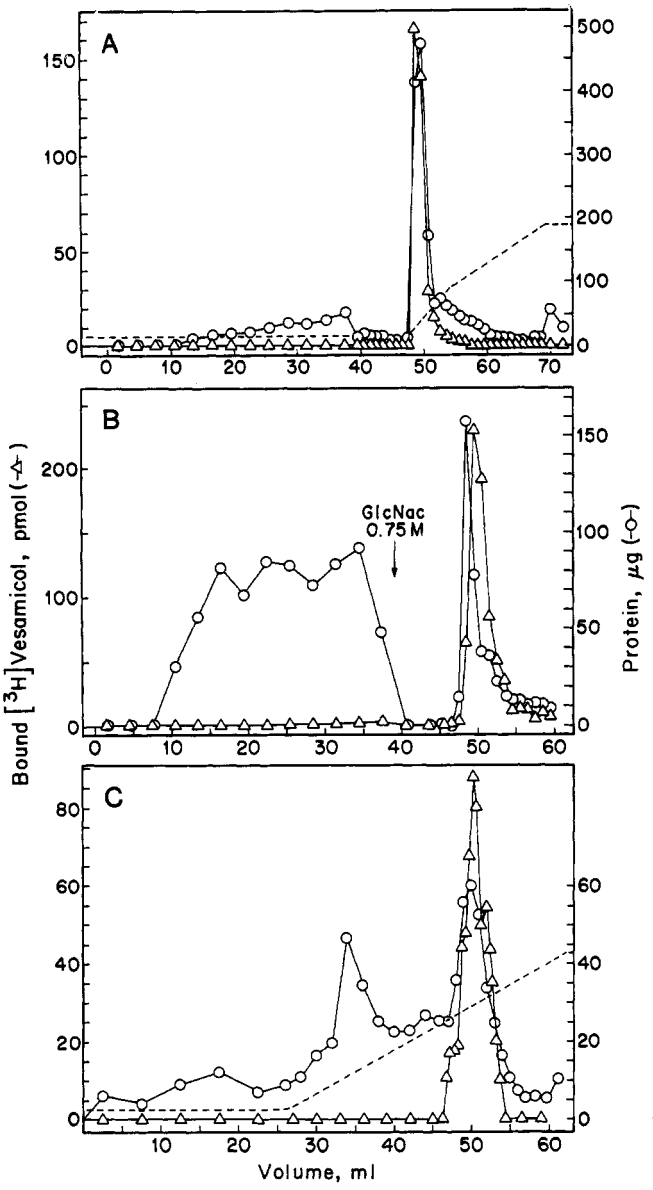

FIGURE 1: Partial purification of the VR. Each fraction was assayed for total VR binding (Δ) by centrifugation–gel filtration and for protein (O) content. (Panel A) Hydroxylapatite chromatography. Cholate-solubilized and stabilized SV protein (4.1 mg of protein) was chromatographed. The VR was eluted with a potassium phosphate gradient running from 0 to 0.3 M (---). The two fractions containing the majority of the VR activity were pooled. (Panel B) Wheat germ lectin affinity chromatography. HTP-pooled material (1.9 mg of protein) was applied to the WG column, washed, and eluted with 0.75 M GlcNAc. The five fractions containing the peak fraction and the right side of the VR profile were pooled. (Panel C) DEAE anion-exchange chromatography. WG-pooled material (650 μg of protein) was applied to the HPLC DEAE column, and VR was eluted with a KCl gradient running from 0 to 0.4 M (---). The nine fractions containing the majority of the VR activity were pooled.

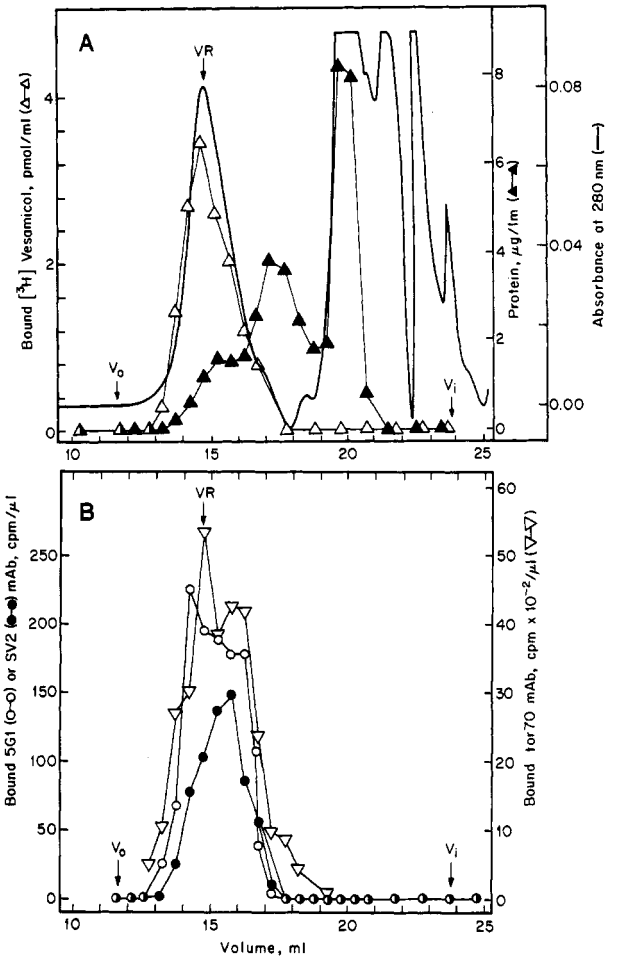

FIGURE 2: Final purification of the VR. Partially pure VR (45 μg of protein) from Figure 1C was concentrated and applied to an HPLC size exclusion column. (Panel A) the absorbance at 280 nm (—), VR binding (Δ), and protein (▲) content were measured. (Panel B) Each fraction was assayed for tor70 (anti-SV1, ▽), 5G1 (anti-SV1, O), and 10H3 (anti-SV2, ●) mAb binding.

Some of the low M_r proteins that separated from the VR were concentrated protease inhibitors. The nanogram-sensitive QuantiGold protein assay detected a species eluting one fraction after (lower M_r) the VR binding, a second species at about 460 kDa that did not absorb 280-nm light, as well as a 40-kDa species. The overall yield for the four-step VR purification was 7 ± 3% (Table I). The resulting 30–60 μg of VR protein had specific binding of 4400 ± 910 pmol of [^{3}H]vesamicol/mg of protein. Thus, the VR was purified a factor of about 15 from the SVs, corresponding to a factor of about 1400 from electric organ homogenate.

Analysis of the VR Purification by SDS–PAGE. Samples from each purification step were analyzed by SDS gradient PAGE (Figure 3). Gradient gels were required to allow all

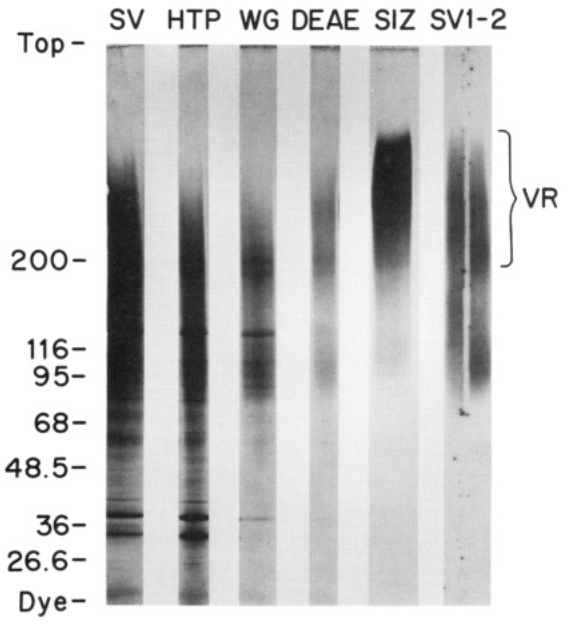

FIGURE 3: Compositions of pooled VR fractions throughout purification. The identities of pools that were subjected to SDS gradient (3–15%) PAGE are shown on top. The amounts of protein in each lane in micrograms were SV,7; HTP,4; WG,2; DEAE,0.3; and SIZ,2. The positions of the top of the resolving gel, the dye front, and the protein standards (kDa) are shown to the left. The position of the VR is bracketed on the right. An additional electrophoretic lane shown on the right contained 0.7 μg of protein from the size exclusion step. This lane was cut from the gel before silver staining of the rest of the gel, and it was electroblotted to nitrocellulose which then was cut longitudinally into two half-lanes. The left and right halves were probed with anti-SV1 (5G1) and anti-SV2 primary mAbs, respectively, and visualized with ^{125}I-labeled secondary antibody. The reassembled X-ray film pattern is shown as lane SV1-2.

of the material to enter the resolving gel. HTP chromatography did not greatly change the protein composition of the pooled VR, whereas the WG step removed a majority of the proteins smaller than 80 kDa. The DEAE pool contained two very heterogeneously migrating species as the primary components, one at about 200 kDa and a lesser amount of another at about 100 kDa. The size exclusion VR pool contained mostly a very heterogeneously migrating species centered at around 240 kDa. Also observed was a much smaller amount of another heterogeneously migrating species staining down to about 100 kDa. Except for a very faint protein band at 40 kDa, whose relative intensity differed in different VR preparations, and small amounts of P-type ATPase subunits (Yamagata et al., 1989) in some preparations, no other species was apparent. Other gels have shown no other polypeptide bands down to about 8 kDa (not shown).

The 460-kDa species detected by QuantiGold assay in Figure 2A exhibited very light, diffuse silver staining from about 20 000 to 60 000 after SDS–PAGE (data not shown). The pattern resembled that of heparin run under the same conditions. This material was not present in the purified VR.

Two other separation steps each gave a 2–3-fold increase in VR specific binding when applied to cholate-solubilized and stabilized vesicles. These were chromatography on heparin–agarose and band sedimentation velocity migration in a glycerol density gradient (data not shown). It was not necessary to include these steps to achieve a pure VR preparation, however, as a six-step procedure including both of them resulted in VR of similar specific binding and appearance in SDS–PAGE as the VR from the four-step procedure shown here. The ability of the VR to bind to heparin–agarose likely is not due to cation exchange, because the VR acts as an anion

Table II: Copurification of the VR and SV1 and SV2 Epitopes[a]

	purification factor		
purification steps	VR[b]	SV1[c]	SV2[d]
SV	1.0	1.0 ± 0.3	1.0 ± 0.3
Sol SV	1.0	1.0 ± 0.3	0.9 ± 0.3
HTP	1.4	1.4 ± 0.1	2.0 ± 0.2
WG	4.0	6.9 ± 0.3	7.7 ± 0.2
DEAE	8.3	19 ± 3	21 ± 0.7
SIZ	15	150 ± 60	100 ± 14

[a] The average (±1 SD) of purification factors from three VR preparations. [b] Starting material bound 400 pmol of [^{3}H]vesamicol/mg of protein. [c] Starting material bound 1600 cpm of secondary antibody/μg of protein. [d] Starting material bound 1100 cpm of secondary antibody/μg of protein.

in DEAE ion-exchange chromatography and it does not bind to carboxymethyl or sulfopropyl cation-exchange columns.

Immunochemical Characterization of the Purified VR. The low heterogeneous mobility of the purified VR in SDS–PAGE suggested that it might be related to the synaptic vesicle proteoglycan (PG; Carlson & Kelly, 1983). The PG carries the luminal SV1 epitope defined by the independently raised tor70 and 5G1 monoclonal antibodies (Carlson & Kelly, 1983; Kushner, 1984; Carlson, 1989). The epitope probably resides on the glycosaminoglycan portion of the PG. The purification factor for the SV1 epitope was determined for each VR pool throughout the purification. After the WG step, the SV1 epitope was purified to a greater extent than the VR (Table II). The final purification factor was about 150 for the epitope, or about 10 times higher than for vesamicol binding, despite the fact that the purification protocol was based on maximizing the latter. The SV1 epitope coeluted with the VR on size exclusion chromatography also, and a second species of SV1 epitope eluted as a somewhat smaller overlapping species of about 1.5 MDa (Figure 2B). Thus, two forms of SV PG copurified until the final size exclusion chromatography step, and one of these is associated with the VR.

Another characterized epitope in *Torpedo* synaptic vesicles is the SV2 epitope that is recognized by the 10H3 mAb. This epitope is found on the cytoplasmic surface of a glycoprotein of about 100 kDa. The epitope is not cholinergic-specific (Buckley & Kelly, 1985; Low et al., 1988). The purification factors for the SV2 epitope were determined for the same VR pools as above. This epitope also was purified to a greater extent than the VR, and, within the errors, the purification factors throughout the purification scheme were the same as for the SV1 epitope (Table II). The amounts of SV2 epitope also were measured in the separate size exclusion chromatography fractions (Figure 2B). A broad peak of SV2 epitope was found approximately overlapping both peaks of SV1 epitope. At the peak of the VR, the amount of SV2 was about 70% of its maximum.

Both epitopes were assayed by western blot analysis of the purified VR (Figure 3). Staining was heavy in the region of the VR most heavily stained with silver as well as at lower M_r where slight silver staining occurred. Careful examination of the epitope staining patterns revealed that, while they appear very similar, they actually are slightly different. SV1 staining occurred in two very diffuse regions that merged: one centered at about 240 kDa where most of the protein staining occurred and the other at about 120 kDa. SV2 staining occurred in three smaller diffuse regions that merged: one centered at about 100 kDa, another at about 200 kDa, and the third at about 300 kDa.

The similarity in behavior of the SV1 and SV2 epitopes prompted additional examination of whether the SV2 epitope

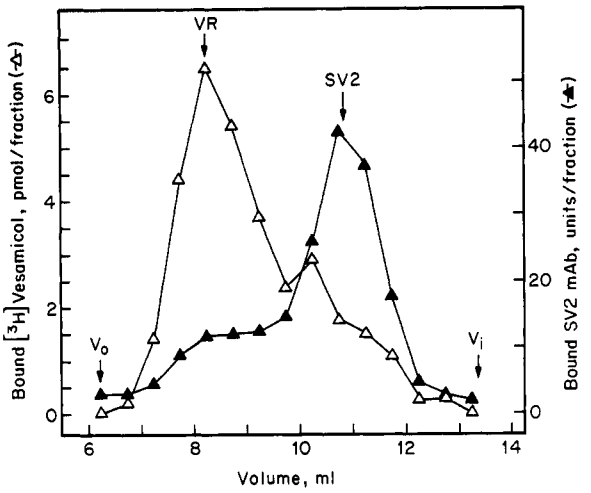

FIGURE 4: Size exclusion chromatography of the crude SV2 antigen and VR. Cholate-solubilized and stabilized SVs (270 μg of protein) in 350 μL were applied to the HPLC size exclusion column system described under Materials and Methods. Fractions (0.5 mL) were assayed for [^{3}H]vesamicol binding (△) and SV2 content, the latter by immunodot-blot analysis (▲). The latter unit of binding equals 100 cpm of bound secondary [^{125}I]antibody.

is related to the VR. Immunoprecipitation of cholate-solubilized and stabilized SVs with anti-SV2 mAb precipitated 91 ± 8% of the SV2 antigen and 29 ± 5% of the VR. No SV2 antigen or VR was precipitated by nonspecific antibody. Also, SVs that were solubilized and stabilized in cholate were subjected directly to size exclusion chromatography, and the SV2 and VR elution profiles were determined to see whether the behavior in this mixture of total SV components might differ from that in purified VR (Figure 4). The SV2 epitope eluted primarily in a broad peak of about 210 kDa, but a significant amount eluted as a large aggregate at about 3.9 MDa. Conversely, the VR eluted primarily in a broad peak of about 3.9 MDa, but a significant amount also eluted as a smaller species of about 210 kDa. In other experiments at lower protein concentration, the VR eluted primarily as the lower M_r species (not shown).

Pharmacological Characteristics of Substantially Purified VR. The ligand-binding characteristics of the VR purified through the DEAE chromatography step were assessed to determine whether purification had removed a component essential to high-affinity binding of vesamicol and AcCh analogues. The affinity for vesamicol was within the range observed for VR in vesicle ghosts, as was the selectivity for the (−)- as compared to the (+)-enantiomer (Table III; Noremberg & Parsons, 1989; Rogers et al., 1989; Kaufman et al., 1989). AcCh and AcCh analogue 23 exhibited similar potencies for inhibition of vesamicol binding as in vesicle ghosts (Table III; Bahr et al., 1992a). These results demonstrate that the VR was purified without apparent change in its ligand-binding characteristics.

Table III: Pharmacological Characteristics of Substantially Purified VR[a]

ligand	IC_{50} (μM)	ligand	IC_{50} (μM)
(−)-vesamicol	0.073 ± 0.010	AcCh	37 000 ± 9000
(+)-vesamicol	2.6 ± 0.3	analogue 23[b]	3.5 ± 0.3

[a] VR that had been purified through the DEAE chromatography step was equilibrated with 20 nM [^{3}H]vesamicol and seven different concentrations of each of the compounds in buffer C at 23 °C. The amount of specific binding of [^{3}H]vesamicol was determined with the filter assay, and hyperbolas were fitted to the data by regression analyses to estimate IC_{50} values ±1 SD for inhibition of [^{3}H]vesamicol binding. [b] AcCh analogue benzyl *cis*-*N*-benzyl-*N*-methylisonipecotate bromide.

Discussion

The VR was purified to a specific binding of 4400 pmol of vesamicol/mg of protein from *Torpedo* electric organ SVs solubilized in cholate detergent and stabilized in glycerol and a phospholipid mixture. In SDS–PAGE it is composed of very heterogeneous material of greater than 100 000 Da. No other protein component was detected in significant amounts. The apparent M_r of the VR varied in different SDS–PAGE experiments from about 120 000 to about 240 000. It is not known if this variation arises from real differences among the preparations or whether it is a consequence of the poor electrophoretic behavior of the VR, the heterogeneity of which is much greater than typically exhibited by glycoproteins. Such behavior resembles that reported for electric organ SV PG (Carlson & Kelly, 1983). Other physicochemical characteristics of the VR also suggest that it is related to PG. The VR is highly negatively charged as it is the last SV protein to elute from a DEAE column. This is so even if the DEAE column step is placed first in the purification. In cholate detergent, the VR exists in aggregation states ranging from about 210 kDa to 3.5 MDa, depending on the experimental circumstances. Moreover, the VR binds heparin through a mechanism other than ion exchange. Variable aggregation states and binding to heparin are characteristics of many nervous tissue PGs (Margolis et al., 1986; Hassel et al., 1986; Edge & Spiro, 1987).

Additional evidence that the VR is associated with PG comes from the finding that the VR is precisely correlated with one form of the SV1 epitope. This epitope resides on PG that has an estimated M_r of 100 000–200 000 and a carbohydrate content of about 28% by weight (Carlson & Kelly, 1983). Assuming that the VR is 150 kDa with the same carbohydrate content as the SV1 antigen, the VR protein is predicted to be approximately 108 kDa. A typical preparation of SVs contains 6–12 VRs per vesicle (although some preparations lie outside this range) of 20 MDa total protein molecular mass (Anderson et al., 1986). These numbers suggest that the VR constitutes 3–6% of the protein content of the SV, which correlates reasonably well with the approximately 15-fold purification required to isolate the VR. Moreover, the specific binding for purified VR corresponds to an equivalent (in the chemical sense) of 230 000 g of protein, or about two times the estimated core protein M_r of the PG. Partial denaturation of vesamicol binding during purification and use of subsaturating [^{3}H]-vesamicol to determine the specific binding could account for the discrepancy. These considerations strongly suggest that the VR is PG-like, as no other protein in purified VR is present in an amount sufficient to account for the data. Previous work demonstrated that the VR *is* protein (Kornreich & Parsons, 1988).

The VR also is associated with the SV2 epitope. The correlation is more complex than is the case for SV1 epitope, in that precise coincidence of SV2 immunoreactivity with the VR was not seen in all observations, and only about 30% of the epitope is associated with the VR. The SV1 and SV2 epitopes also are associated with another PG that copurifies with the VR PG until the final size exclusion step. The lack of precise correlation of VR with SV2 epitope in all measurements might arise because many properties of the system inherently lead to unusual difficulties in quantitation and correlation of markers. Thus, the peak of vesamicol binding in purified VR consistently eluted before the peak of protein detected by either the colloidal gold or Schaffner and Weissman (1973; data not shown) assays. This surely was

caused by inhibition of color development in the assays due to more glycosaminoglycan in the higher M_r fractions (Carlson & Kelly, 1983), because the absorbance at 280 nm, which presumably is due to aromatic amino acids, closely followed vesamicol binding. In other words, the VR PG probably carries a larger amount of glycosaminoglycan than the other PG. The observation that the purification factors for both epitopes were 7–10-fold greater than for vesamicol binding probably is an artifact of the immunodot-blot procedure that arises because the vesicle PG binds to nitrocellulose poorly (Walker et al., 1983). It seems likely that PG blotted more efficiently as it was purified, thus accounting for the excess apparent purification of the epitopes. Finally, the material that eluted at 460 kDa (Figure 2A) probably was free glycosaminoglycan because no 280-nm absorbance was present, the electrophoretic behavior in SDS–PAGE resembled that of heparin, and no classical protein subunits were detected. The hypothesized glycosaminoglycan presumably was released during purification of the VR. Instability of the PG, possible reiteration of SV1 epitope on multiple chains of glycosaminoglycan, and variable aggregation behavior in mild detergent possibly giving SV2 epitope-masking would compromise our ability to quantitatively assess the relationships among the VR and the SV1 and SV2 epitopes. Such behavior could explain the variable M_r of the VR in SDS–PAGE and the discrepancy of the previously reported M_r for the SV2-containing glycoprotein compared to the current observations. Despite these complications, the purification results present strong evidence that the VR is associated with one form of the SV PG that carries the SV1 and SV2 epitopes.

Association of the VR with the SV2 epitope is an unexpected finding. This is because the SV2 epitope is not specifically cholinergic, as one expects for an epitope in the VR. However, this could occur if a VR precursor and part of a single type of widely distributed PG carrying the SV2 epitope associated posttranslationally, thus generating two related PGs. Alternatively, related forms of PG carrying the SV2 epitope could arise by alternative splicing of a single pre-mRNA. The former mechanism seems more likely as it could generate a wider diversity of SV2-containing proteins serving different storage functions in a wide range of neurotransmitter and endocrine secretory vesicle types. Why storage-related proteins should be related to PG can only be speculated upon at present.

Our ability to purify the VR with retention of similar enantioselectivity and affinities for the vesamicol and AcCh ligand families supports the allosteric kinetics model of the AcChT–VR proposed by Bahr et al. (1992). The results demonstrate that no other protein factors are essential for ligand binding, although unidentified factors might yet play critical roles in active transport. Notwithstanding this possibility, PG appears to be associated with the core macromolecular component of the VR and probably the AcChT. The hypothesis is supported by the two following papers in this series (Rogers & Parsons, 1992; Bahr et al., 1992b).

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Registry No. AcCh, 51-84-3; vesamicol, 22232-64-0; (−)-vesamicol, 112709-59-8; (+)-vesamicol, 112709-60-1; heparin, 9005-49-6; cholate, 81-25-4; benzyl *cis*-*N*-benzyl-*N*-methylisonipecotate bromide, 124805-73-8.

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