Cholinergic Synaptic Vesicle Heterogeneity: Evidence for Regulation of Acetylcholine Transport†

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ABSTRACT: Crude cholinergic synaptic vesicles from a homogenate of the electric organ of *Torpedo californica* were centrifuged to equilibrium in an isosmotic sucrose density gradient. The classical VP_1 synaptic vesicles banding at 1.055 g/mL actively transported [^{3}H]acetylcholine (AcCh). An organelle banding at about 1.071 g/mL transported even more [^{3}H]AcCh. Transport by both organelles was inhibited by the known AcCh storage blockers *trans*-2-(4-phenylpiperidino)cyclohexanol (vesamicol, formerly AH5183) and nigericin. Relative to VP_1 vesicles the denser organelle was slightly smaller as shown by size-exclusion chromatography. It is concluded that the denser organelle corresponds to the recycling VP_2 synaptic vesicle originally described in intact *Torpedo marmorata* electric organ [Zimmermann, H., & Denston, C. R. (1977) *Neuroscience (Oxford) 2*, 695–714; Zimmermann, H., & Denston, C. R. (1977) *Neuroscience (Oxford) 2*, 715–730]. The properties of the receptor for vesamicol were studied by measuring binding of [^{3}H]vesamicol, and the amount of SV2 antigen characteristic of secretory vesicles was assayed with a monoclonal antibody directed against it. Relative to VP_1 vesicles the VP_2 vesicles had a ratio of [^{3}H]AcCh transport activity to vesamicol receptor concentration that typically was 4–7-fold higher, whereas the ratio of SV2 antigen concentration to vesamicol receptor concentration was about 2-fold higher. Based on an antibody standardization, in a typical preparation the VP_1 vesicles contained 237 ± 15 pmol of receptor/mg of protein whereas VP_2 vesicles contained 102 ± 3 pmol of receptor/mg of protein, and VP_2 vesicles transported AcCh 2–3-fold more actively than VP_1 vesicles. The Hill coefficients α_H and equilibrium dissociation constants K for vesamicol binding to VP_1 and VP_2 vesicles were essentially the same, with $\alpha_H = 2.0$ and $K = 19$ nM. The positive Hill coefficient suggests that the vesamicol receptor exists as a homotropic oligomeric complex. The results demonstrate that VP_1 and VP_2 synaptic vesicles exhibit functional differences in the AcCh transport system, presumably as a result of regulatory phenomena.

It has long been known that newly synthesized acetylcholine (AcCh)[1] is the first released by neural stimulation (MacIntosh & Collier, 1976). Preexisting "old" AcCh is released primarily after sustained stimulation. Since most workers think that evoked release occurs by exocytosis of synaptic vesicle contents, this observation implies that there are at least two types of vesicular stores of AcCh. This has been elegantly demonstrated in *Torpedo* electric organ tissue blocks by Zimmermann and Denston (1977a,b), in rat brain by von Schwarzenfeld (1979), and in guinea pig myenteric plexus by Agoston et al. (1985). Suszkiw et al. (1978) formulated a model of synaptic vesicle action to explain these observations. Ordinarily, a small pool of mobilized vesicles (termed VP_2) undergoes repetitive exocytosis, endocytosis, and reloading with newly synthesized AcCh while a larger pool of resting vesicles (termed VP_1) resupplies the metabolically active VP_2 pool as it becomes demobilized for unknown reasons. Other behavior consistent with the model has been observed by Luz et al. (1985), who showed pharmacologically that new and old AcCh are released from *Torpedo* synaptosomes by separate vesicular pathways which converge. Thus, it seems clear that at least two types of cholinergic vesicles exist.

The gross physical properties of VP_1 and VP_2 vesicles differ enough to allow their separation and study biochemically (Whittaker, 1984; Kelly & Hooper, 1982; Whittaker & Roed, 1982; Morris, 1980). VP_1 vesicles contain high concentrations of AcCh and ATP whereas VP_2 vesicles contain little of these components, and VP_1 vesicles are larger and less dense. Since VP_2 vesicles have been only partially purified (Giompres et al., 1981b), little is known about the structural basis for these differences.

The mechanism of AcCh storage by synaptic vesicles has been studied with the readily available VP_1 vesicles purified from *Torpedo* electric organ (Parsons et al., 1982; Marshall & Parsons, 1987). The current model of the transport system is as follows. A vesicle-associated ATPase probably establishes an internally acidic proton gradient, which drives secondary active transport of AcCh through a hypothesized separate AcCh transporter. The system exhibits a unique pharmacology (Anderson et al., 1983a,b). The compound *trans*-2-(4-phenylpiperidino)cyclohexanol (vesamicol, formerly AH5183) inhibits active transport of AcCh noncompetitively by binding to a cytoplasmically oriented protein receptor that is not the ATPase (Kornreich & Parsons, 1988). It is clear that the vesamicol binding site is separate from the AcCh binding site in the transporter, but in other respects the spatial relationship between the two sites is unknown (Bahr & Parsons, 1986a,b). It is possible that the vesamicol receptor is a separate protein, or it might represent an allosteric site in the AcCh transporter.

As with mechanistic studies of the AcCh transport system, most other biochemical work with cholinergic synaptic vesicles has been conducted with VP_1 vesicles. This is a natural

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[1] Abbreviations: AcCh, acetylcholine; vesamicol, *trans*-2-(4-phenylpiperidino)cyclohexanol (formerly AH5183); nAcChR, nicotinic acetylcholine receptor; α-BuTx, α-bungarotoxin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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consequence of their majority status, ease of isolation, and very high content of AcCh, which served as the original characteristic marker of cholinergic vesicles. Nevertheless, VP_2 vesicles are of potentially greater interest, particularly for mechanistic studies of the AcCh transport system, since they are the more active in vivo. For example, comparative studies of VP_1 and VP_2 vesicles might reveal what regulatory features account for the ability of VP_2 vesicles to preferentially take up newly synthesized AcCh in vivo. Such studies would be greatly facilitated if the difference observed for intact tissues was preserved after nerve terminal disruption. Accordingly, this study compared the AcCh transport and vesamicol receptor characteristics of partially purified VP_1 and VP_2 synaptic vesicles.

Materials and Methods

Live *Torpedo californica* were obtained locally. The *Torpedo* were placed in filtered sea water tanks and allowed to recover from handling for a period of 3 days. Fish were stunned by a blow to the back of the head, and the electric organs were excised, quick frozen in liquid nitrogen, and stored at −87 °C until used.

[^{3}H]Vesamicol (32.7 Ci/mmol) was synthesized as described (Bahr & Parsons, 1986b). It is the optically pure *l* isomer. Nonradioactive vesamicol was synthesized by condensation of 4-phenylpiperidine with cyclohexene oxide in refluxing ethanol. It is a *dl* racemate. *N*-[^{3}H]Propionyl-α-bungarotoxin (58 Ci/mmol), [^{14}C]AcCh chloride (58 mCi/mmol), and [^{3}H]AcCh chloride (2.5 Ci/mmol) were from Amersham. [^{3}H]AcCh was diluted with nonradioactive AcCh chloride to give [^{3}H]AcCh of 50 mCi/mmol specific activity before use. Diethyl *p*-nitrophenyl phosphate (Paraoxon), diaminobenzidine tetrahydrochloride, potassium phosphoenolpyruvate and pyruvate kinase (type III) were from Sigma Chemical Co. Controlled-pore glass beads (3000-Å pore size) were from Electronucleonics Corp. Nigericin was a gift from W. E. Scott of Hoffman-La Roche, Inc. Sucrose was a gift from Union Sugar Corp. 2-[3-Acetamido-5-(*N*-methylacetamido)-2,4,6-triiodobenzamido]-2-deoxy-D-glucose (Metrizamide) was from Accurate Chemical and Scientific Corp. Nitrocellulose acetate filters (0.22-μm pore size) were from MicronSep Corp. Rabbit anti-*T. californica* affinity-purified, sodium dodecyl sulfate denatured, nicotinic acetylcholine receptor (nAcChR) antiserum was obtained from Dr. James W. Patrick of the Salk Institute, San Diego, CA. Anti-SV2 monoclonal antibody 10H3 was obtained from Dr. Regis B. Kelly, University of California, San Francisco. Affinity-purified ^{125}I-labeled sheep anti-mouse Ig whole antibody (18 μCi/μg) was from Amersham. Biotinylated goat anti-rabbit IgG antiserum was obtained from Cappel or Vector Laboratories. Avidin–biotinylated peroxidase complex (Vectastain ABC reagents) was from Vector Laboratories. All reagents required for acrylamide gel electrophoresis were from Bio-Rad Laboratories. Nitrocellulose paper (BA85, 0.45-μm pore size) was from Schleicher and Schuell. All other chemicals were from usual commercial sources.

Crude synaptic vesicles were separated as follows. Approximately 400 g of frozen electric organ was mechanically shaved and allowed to warm slowly with occasional stirring. When the temperature reached 0 °C, 400 mL of ice-cold 0.76 M glycine containing 0.05 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), 0.01 M ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.02% NaN_3, 2 μM 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT), and 2 mg/L phenylmethanesulfonyl fluoride (PMSF) adjusted to pH 7.2 with 0.8 M NaOH was added. The suspension was vigorously blended for approximately 15 s and then homogenized in portions in a 350-mL glass tube and spinning Teflon pestle. The homogenate was centrifuged at 17700*g* for 3 h to remove membrane debris. The resulting supernatant was centrifuged at 48500*g* for 18 h at 4 °C to yield a crude synaptic vesicle pellet. Isosmotic density gradient solutions containing 0, 0.4, 0.5, or 0.65 M sucrose were prepared with buffer (0.01 M Hepes, 0.001 M EDTA, 0.001 M EGTA, and 0.02% NaN_3, adjusted to pH 7.0 with 0.8 M KOH) and enough glycine to achieve an osmotic pressure of approximately 800 mosM (measured on a Wescor Corp. 5100C vapor pressure osmometer). The crude synaptic vesicle pellet was resuspended in 15 mL of the 0.5 M sucrose solution and gently homogenized. A typical density gradient was formed from 0 M sucrose (7 mL), 0.4 M sucrose (8 mL), 0.5 M sucrose containing resuspended vesicles (14 mL), and 0.65 M sucrose (11 mL). The solutions were applied to the bottom of a 1 × 3.5 in. dome-top polyallomer quick-seal tube (Beckman Instruments, Inc.) in the order stated with a cannulated syringe. After tube sealing the gradient was centrifuged at 196200*g* for 3.5 h at 4 °C in a balanced Beckman VTi 50 vertical rotor, which resulted in an equilibrium distribution of vesicles. Typically, 20 fractions of 1.5 mL each were obtained from the gradient by bottom puncture and displacement with Fluorinert (Sigma Chemical Co.).

The density of each fraction was estimated from the refractive index (Sheeler, 1981). The ATP concentration of each gradient fraction was determined by using luciferase similarly as described (Kimmich et al., 1975). The protein concentration of each gradient fraction was determined as described with a bovine serum albumin standard (Bradford, 1976). Active transport of AcCh was carried out for 30 min at 23 °C with a Millipore filter assay as described (Bahr & Parsons, 1986a). Typically 60 μL of the gradient fraction was mixed with 20 μL of buffer A (0.70 M glycine, 0.10 M Hepes, 1 mM EDTA, and 1 mM EGTA adjusted to pH 7.80 with 0.80 M KOH). In the transport sample per se there were in addition final concentrations of 50 μM [^{3}H]AcCh, 10 mM MgATP, 10 mM phosphoenolpyruvate, 2 mM $MgCl_2$, and 30 μg of pyruvate kinase/mL. Duplicate samples were prepared in which one also contained 10 μM *dl*-vesamicol. Only uptake of [^{3}H]AcCh that was blocked by vesamicol is graphed. Equilibrium binding of [^{3}H]vesamicol to membranes was measured similarly to the method of Bahr & Parsons (1986b), except that an assay based on glass fiber filters (Whatman, 2.4 cm GF/F) coated for 2 h with 0.5% poly(ethylenimine) was used. Typically, 120 μL of vesicles incubated under the stated conditions and concentration of [^{3}H]vesamicol was filtered through immediately prerinsed filters by vacuum suction and rapidly washed in like manner 4 times with 1-mL portions of ice-cold buffer A. Radioactivity was determined in 10 mL of Hydrofluor cocktail (National Diagnostics Corp.) by liquid scintillation spectroscopy.

Results

AcCh Is Transported by Two Organelles. A crude synaptic vesicle pellet from the homogenized *T. californica* electric organ was resuspended and centrifuged to equilibrium in an isosmotic sucrose density gradient. The gradient was fractionated and analyzed for a number of parameters of interest. The estimated density, protein, and ATP contents of each fraction are shown in Figure 1B. The large peak of ATP that banded around fraction 6 at 1.055 g/mL corresponds to VP_1 synaptic vesicles. The vesamicol-inhibitable [^{3}H]AcCh active transport activity of each fraction was determined. As shown

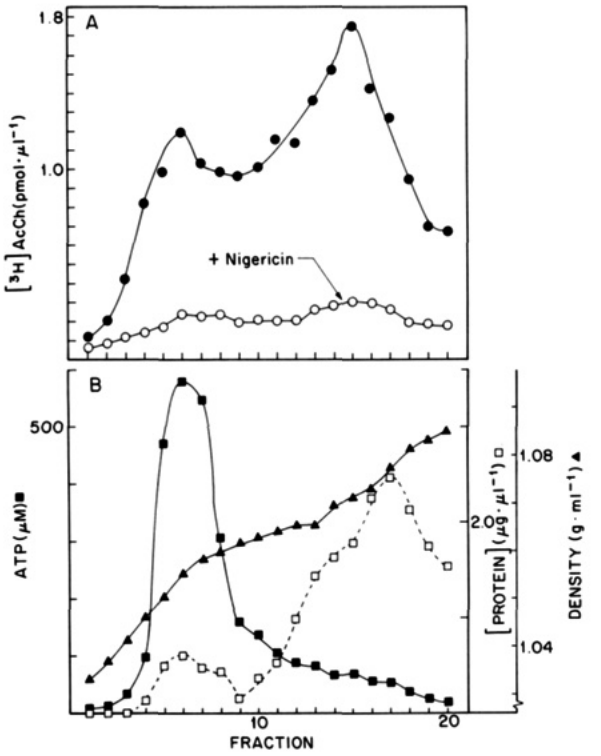

FIGURE 1: AcCh transport across the density gradient fractions. (A) Effect of an uncoupler. Vesamicol-sensitive active transport of [^{3}H]AcCh by membranes was measured for each fraction of a sucrose density gradient as outlined under Materials and Methods (●). Uptake of [^{3}H]AcCh in the presence of 10 μM *dl*-vesamicol was less than 10% of the vesamicol-sensitive uptake in all fractions. Uptake is reported per microliter of gradient rather than per milligram of protein to avoid distortion of peak shapes. Vesamicol-sensitive uptake also was determined in the presence of 1 μM nigericin (○). (B) Physical characteristics of the gradient fractions. Estimated density (▲), ATP concentration (■), and protein concentration (□) for the fractions in frame A are shown. The peak of ATP corresponds to classical VP_1 synaptic vesicles.

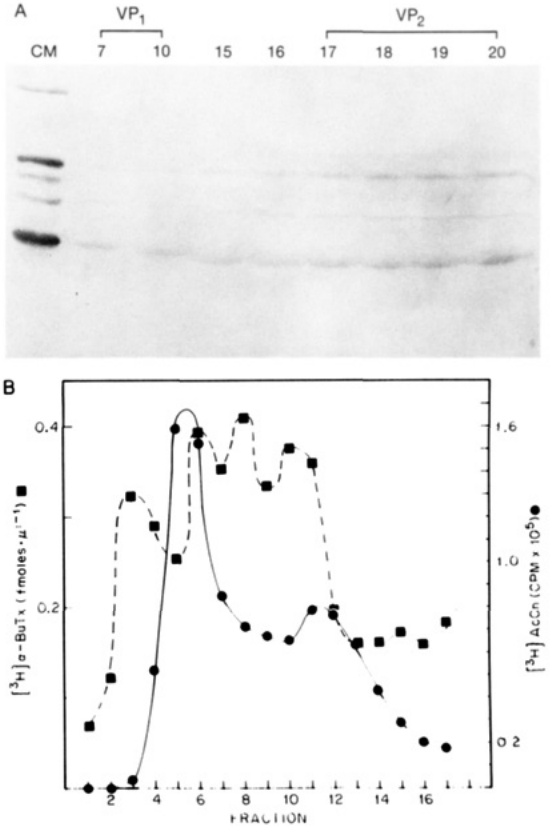

FIGURE 2: Absence of nAcChR in density gradient fractions. (A) Western immunoblot of selected density gradient fractions. A different gradient from that shown in Figure 1 was examined. Fraction numbers are given across the top. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fractions was performed as described (Laemmli, 1970). Separated proteins were transferred to nitrocellulose paper by diffusion. The immunoblot was developed similarly to the method of Towbin et al. (1979). Briefly, gelatin (0.25%) was used to deactivate the paper, rabbit anti-*T. californica* nAcChR antiserum diluted 1 to 1000 was used as primary antibody, and biotinylated goat anti-rabbit IgG antiserum diluted 1 to 400 was used as secondary antibody. Immune complexes were visualized by incubation with horseradish peroxidase conjugated avidin diluted 1 to 200, followed by incubation with diaminobenzidine–hydrogen peroxide. The paper was washed well with neutral buffers after each treatment before proceeding to the next treatment. Thirty micrograms of crude membrane fragments (CM) was used as a control to ensure staining of the four nAcChR subunits. An undissociated aggregate of the nAcChR appears at high molecular weight. Lane 7 (80 μg of protein) and lane 10 (120 μg of protein) were from the VP_1 region. Lanes 17–20 (200–300 μg of protein each fraction) were from the VP_2 region as defined by the [^{3}H]AcCh uptake profile for this gradient. (B) α-Bungarotoxin binding in gradient fractions. A separate gradient was studied. The amount of nAcChR in each fraction was determined with *N*-[^{3}H]-propionyl-α-bungarotoxin as described (Schmidt & Raftery, 1973) (■). AcCh active transport also was measured as in Figure 1 (●).

in Figure 1A, two peaks of transport activity were found. One at low density occurred coincident with VP_1 vesicles. Another very broad peak banded around fraction 15 at ~1.071 g/mL. On the basis of an estimate of the peak areas the amount of [^{3}H]AcCh transported by the denser fractions was about 2.6-fold more than that transported by the VP_1 fractions, or about 72% of the total. An isosmotic gradient achieving much higher densities by inclusion of Metrizamide gave no evidence that even denser AcCh-transporting organelles were present. Transport in all of the fractions was inhibited substantially and to similar extents by the uncoupler nigericin, which collapses transmembrane proton gradients (Figure 1A). Thus, the measured accumulations of [^{3}H]AcCh in all fractions responded to vesamicol and nigericin in the manner expected for authentic synaptic vesicle transport (Anderson et al., 1982).

Absence of Significant nAcChR and Cross-Reactive Species. Electric organ is rich in nicotinic AcCh receptor (nAcChR). The assay for AcCh transport detects tight binding of [^{3}H]-AcCh to any membranous particle. Thus, binding of AcCh to nAcChR-rich membrane fragments could contribute to the apparent [^{3}H]AcCh transport shown in Figure 1. This possibility was tested by assaying similar density gradient fractions for the presence of nAcChR.

In Figure 2A a Western immunoblot of fractions subjected to sodium dodecyl sulfate electrophoresis is shown. The primary antiserum was raised against sodium dodecyl sulfate denatured highly purified *T. californica* nAcChR. A control lane contained a small amount of total crude electric organ membranes and exhibited the characteristic α, β, γ, δ pattern of the nAcChR, with apparent M_rs from 40 000 to 65 000, plus a high molecular weight aggregate of the nAcChR receptor subunits. The other lanes contained much larger amounts of the density gradient fractions, which correlated as shown with apparent AcCh transport activities (as suggested by the figure, the heavier organelle is identified later as the VP_2 synaptic vesicle). Faint blotting patterns characteristic of the nAcChR can be seen in every fraction. Although we did not quantitatively determine how much nAcChR was stained in these fractions, it was evident that only a trace amount was present that did not correlate with AcCh transport activity. In the denser fractions very slight staining also occurred at M_r ~ 75 000. This was of unknown origin, but in any case this species also did not correlate with AcCh transport activity.

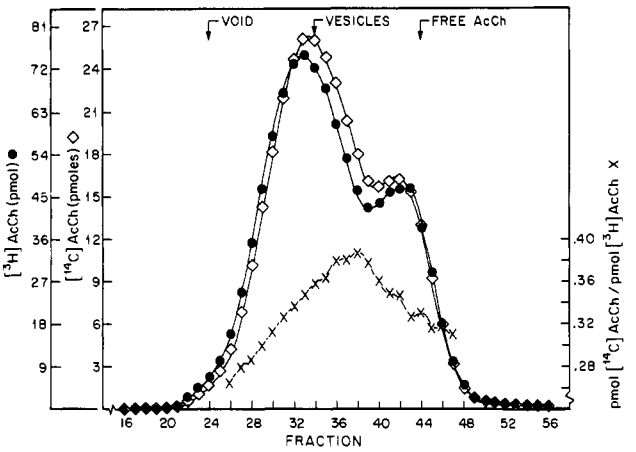

FIGURE 3: Size-exclusion chromatography of AcCh-transporting organelles. VP_1 vesicle fractions were identified in a density gradient by ATP content, while the denser organelle fractions in the same gradient were identified by AcCh uptake. [^{3}H]AcCh was then used to label the peak fraction of VP_1 by active transport, and [^{14}C]AcCh was used to label the peak fraction of the denser organelle (900 μL each population) similarly to Figure 1, except that transport was terminated by addition of 100 μL of ice-cold buffer A containing 100 μM *dl*-vesamicol. Free AcCh was removed by the technique of centrifugation–gel filtration (Neal & Florini, 1973) in columns equilibrated with buffer A at 4 °C. Equal volumes (675 μL) of each sample were then mixed and loaded onto a previously calibrated 100 cm × 0.9 cm controlled-pore glass bead (3000-Å pore size) column equilibrated in buffer A. Elution at 8 mL/h was carried out with buffer A, and 1.5-mL fractions were collected. Aliquots (850 μL) of each fraction were analyzed by double-channel liquid scintillation spectroscopy to determine ^{3}H and ^{14}C disintegrations per minute (Hendee, 1973). These values were converted to picomoles [^{3}H]AcCh (●) and [^{14}C]AcCh (◇) per 850 μL of the fraction. The ratio of values in each fraction (×) is also shown.

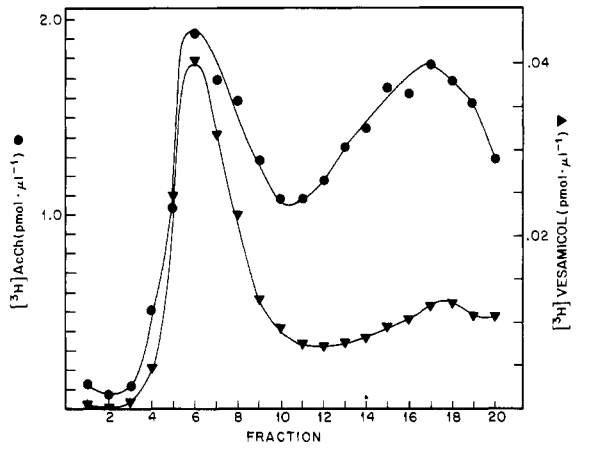

FIGURE 4: Vesamicol binding across the density gradient. Higher density is to the right. Gradient fractions made up to 10 mM MgATP, 2 mM $MgCl_2$, 10 mM potassium phosphoenolpyruvate, and 30 μg of pyruvate kinase/mL (190 μL) were incubated for 100 min at 22 °C with 500 nM [^{3}H]vesamicol in the absence or presence of 100 μM *dl*-vesamicol before determining the amount of bound ^{3}H by filtration as described under Materials and Methods. The amount of specifically bound [^{3}H]vesamicol (total minus amount in the presence of 100 μM *dl*-vesamicol, which was less than 10%) per microliter of fraction is shown (▼). [^{3}H]AcCh active transport by the same fractions is also shown (●).

α-Bungarotoxin (α-BuTx) binds irreversibly to nAcChR and thus is an excellent tool for quantitative analysis of the nAcChR. In Figure 2B the results from [^{3}H]-α-BuTx binding and AcCh transport assays are shown. Again, there was no correlation of the nAcChR with AcCh transport activity. Also, the maximal amount of bound [^{3}H]-α-BuTx that was found in any fraction (about 0.4 fmol/μL) is negligible compared with the amount of [^{3}H]AcCh typically accumulated by vesicles (1–2 pmol/μL). Thus, binding of AcCh to nAcChR cannot contribute significantly to the apparent transport of AcCh that was observed in any of the density gradient fractions.

Relative Sizes of AcCh Transporting Organelles. VP_1 vesicles and denser organelles, which had been made to transport [^{3}H]AcCh and [^{14}C]AcCh, respectively, were mixed after removal of most of the external AcCh and subjected to size-exclusion chromatography on a controlled-pore glass bead column calibrated with large membrane fragments, purified VP_1 vesicles, and free AcCh. The bound [^{3}H]AcCh eluted from the column slightly before the bound [^{14}C]AcCh, as shown in Figure 3. This can be seen most clearly in the ratios of [^{14}C]AcCh to [^{3}H]AcCh across the synaptic vesicle peak region. At the leading edge the ratio started at about 0.27. The ratio rose to about 0.37 toward the trailing edge before it fell in the fractions dominated by residual free AcCh. The result demonstrates that the denser organelle which transports AcCh is slightly smaller than the VP_1 vesicle.

Transport of AcCh Relative to Vesamicol Receptor Levels. Each fraction from another density gradient was assayed for active transport of [^{3}H]AcCh and for specific binding of [^{3}H]vesamicol at an essentially saturating concentration of the drug (see below). As shown in Figure 4, the receptor for vesamicol was present in all of the fractions of the gradient where [^{3}H]AcCh transport activity was present. More important is the observation that in the denser fractions the ratio of [^{3}H]AcCh transport activity to the apparent concentration of the vesamicol receptor was about 4.0 times higher than at the peak of the VP_1 fractions. This increase in ratio suggests that in the denser organelle either the AcCh transporter is more active, or there is less apparent vesamicol receptor per equally active transporter, or both changes have occurred as compared with VP_1 vesicles.

SV2 Antigen Levels Relative to the Vesamicol Receptor. We cannot tell from the above data which alternative is correct because the gradient fractions are impure. To make the decision, we need an independent measure of synaptic vesicle concentrations. A marker for synaptic vesicle membrane is available in the SV2 antigen, which is recognized by a monoclonal antibody raised against electric organ synaptic vesicles by Buckley and Kelly (1985). This antigen is located on the cytoplasmic surface of a M_r 100 000 transmembrane glycoprotein of unknown function.

Each fraction from a density gradient was assayed for the concentrations of the SV2 antigen and vesamicol receptor. As shown in Figure 5 the SV2 antigen was present in all of the fractions of the gradient where [^{3}H]vesamicol binding was present. However, in the denser fractions the ratio of the SV2 antigen concentration to apparent vesamicol receptor concentration was about 2.2-fold higher than for the VP_1 peak fractions. Thus, the denser organelle exhibits less vesamicol receptor per amount of vesicle membrane as compared with VP_1 vesicles. Also, in this same gradient the ratio of AcCh transport activity to apparent vesamicol receptor concentration was 7.0-fold higher for the denser organelle, which was even greater than the ratio increase for the denser organelle in the experiment shown in Figure 4.

By standardizing the ^{125}I antibody binding assay with known amounts of highly purified VP_1 vesicles, we could calculate the actual amounts of vesamicol receptor present per milligram of vesicle protein in the density gradient fractions. At the peak fractions this was 237 ± 15 pmol/mg for VP_1 vesicles and 102 ± 3 pmol/mg of the denser organelle. These numbers correspond to 4.8 receptors per single VP_1 vesicle and 2.0 receptors per single dense organelle, assuming the same protein content

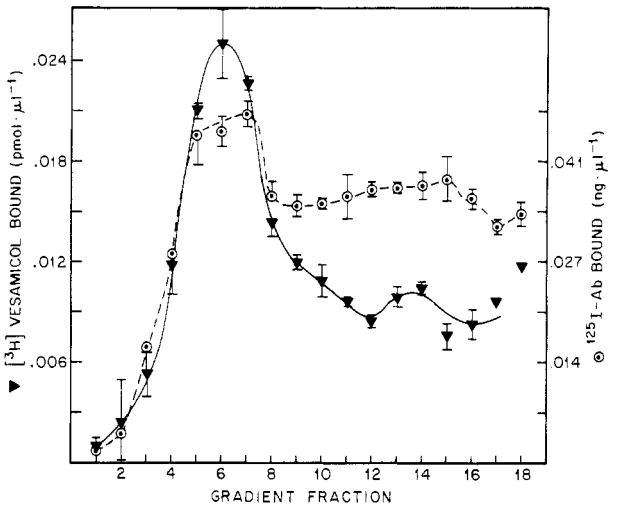

FIGURE 5: SV2 antigen distribution across the density gradient. Higher density is to the right. A standard curve for 10H3 anti-SV2 monoclonal antibody binding to highly purified VP_1 vesicles was generated with a quantitative dot-immunobinding assay on nitrocellulose paper (Jahn et al., 1984) using ^{125}I-labeled sheep anti-mouse Ig as the secondary antibody and γ counting. The standard curve of bound ^{125}I was linear up to 2 μg of VP_1 protein (corresponding to 4900 cpm of ^{125}I). The amount of ^{125}I antibodies bound by 10 μL of each fraction of a density gradient was determined in triplicate. The mean ±1 SE is shown (⊙). The largest amount of bound ^{125}I fell within the linear range of the standard curve. The background value for bound ^{125}I was less than 7% of the values for the VP_1 and denser organelle peak fractions. Also shown are the amounts of [^{3}H]vesamicol specifically bound to membranes in the same fractions, determined as in Figure 4 in triplicate ± 1 SE (▼).

in VP_1 vesicles and denser organelles and a vesicle protein content of 20 000 000 Da per vesicle (Giompres et al., 1981c; Giompres & Whittaker, 1984; Anderson et al., 1982, 1986). Reduced to stoichiometric numbers, then, the numbers of vesamicol receptors per single organelle are reasonable.

Vesamicol Binding Saturation Curves. The previous results demonstrate a difference in the apparent vesamicol receptor levels in the two density gradient organelles, but they do not tell us whether the receptors are otherwise identical. To examine this, full binding titrations were carried out with [^{3}H]vesamicol (Figure 6). The ratio of total to nonspecific binding was excellent, even for these impure fractions. The saturation curves demonstrate that the concentration of [^{3}H]vesamicol used to study the receptor distributions in Figures 4 and 5 was essentially saturating.

The Scatchard plots for the specific binding to both density gradient organelles are curvilinear in a concave-down manner, indicating positive cooperativity (not shown).[2] This demands that interacting binding sites for vesamicol exist. In view of our ignorance about the oligomeric structure of the vesamicol receptor, we have used the Hill equation to describe the cooperativity (Cantor & Schimmel, 1980). This macroscopic analysis assumes nothing about the microscopic equilibria involved. For VP_1 vesicles the estimated concentration of binding sites was 152 ± 9 pmol/mg of vesicle protein and the estimated equilibrium dissociation constant was 19 ± 2 nM. The Hill coefficient was estimated to be 2.0 ± 0.2. For the denser organelle, the estimated concentration of binding sites was 64 ± 4 pmol/mg of protein and the estimated equilibrium dissociation constant was 19 ± 2 nM. The Hill coefficient was estimated to be 2.0 ± 0.3. Thus, while less vesamicol receptor appears to be present in the denser organelle, its binding properties are indistinguishable from those of the receptor in VP_1 vesicles.

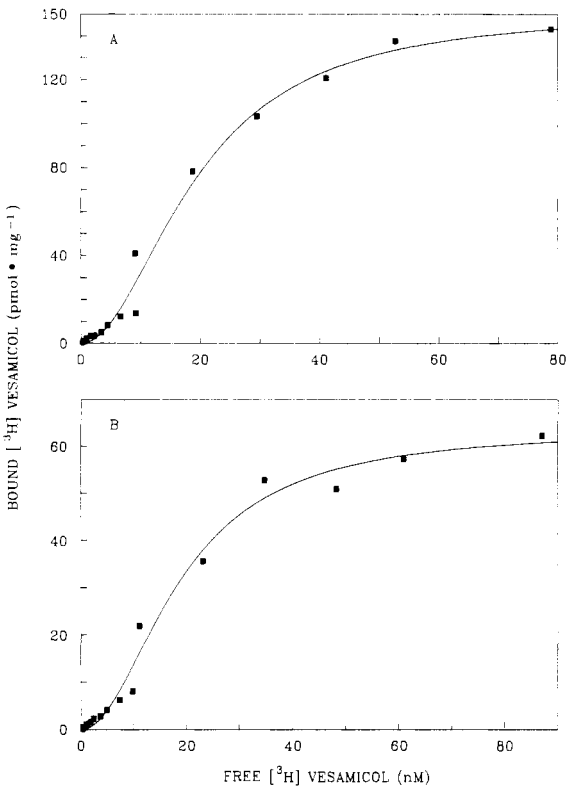

FIGURE 6: Vesamicol binding saturation curves. VP_1 vesicles and the denser species were located in a density gradient from the ATP content and the AcCh transport activity of each fraction. The three peak fractions of each species were separately pooled, and the SV2 antigen concentrations were determined as in Figure 5. Both samples were diluted with buffer A to contain 0.1 μg of vesicle protein/μL as determined by a standard curve constructed with highly purified VP_1 vesicles and 10 mM MgATP, 2 mM $MgCl_2$, 10 mM potassium phosphoenolpyruvate, and 30 μg of pyruvate kinase/mL. Portions (64 μL) of these samples were incubated in duplicate for 30 min at 22 °C in the presence of different concentrations of [^{3}H]vesamicol. The total amount of bound [^{3}H]vesamicol was determined by filtration as described under Materials and Methods. The concentration of free [^{3}H]vesamicol for each incubation sample was determined by subtracting the amount of bound [^{3}H]vesamicol from the total added. A similar set of data was obtained for each species incubated in the presence of a 200-fold excess of *dl*-vesamicol in order to obtain the amount of nonspecifically bound [^{3}H]vesamicol. This amount was typically 1–2% of the total amount bound for both species at a [^{3}H]vesamicol concentration of 40 nM. The amount of specifically bound [^{3}H]vesamicol at its corresponding free [^{3}H]vesamicol concentration was determined as the difference between a datum for total binding and the linear regression line through the nonspecific binding data, and the duplicates were averaged (■). The Hill equation, namely, $R_b = R_t([V]^{\alpha_H}/K^{\alpha_H})/(1 + [V]^{\alpha_H}/K^{\alpha_H})$, where R_b is the amount of specifically bound [^{3}H]vesamicol, R_t is the total concentration of receptor binding sites, V is the free [^{3}H]vesamicol concentration, K is the equilibrium dissociation constant, and α_H is the Hill coefficient, was fit to the data by using nonlinear regression analysis based on the Gauss–Newton method. The standard error is quoted. (A) Binding of [^{3}H]vesamicol to VP_1 vesicles. The Hill equation parameters estimated were R_t = 152 ± 9 pmol/mg of vesicle protein, K = 19 ± 2 nM, and α_H = 2.0 ± 0.2. (B) Binding of [^{3}H]vesamicol to the denser organelle. The Hill equation parameters estimated were R_t = 64 ± 4 pmol/mg of protein, K = 19 ± 2 nM, and α_H = 2.0 ± 0.3.

DISCUSSION

The typical isolation protocol for cholinergic VP_1 synaptic vesicles involves electric organ homogenization, differential

[2] These binding studies were done much more carefully than a previously published study that, even though exhibiting curvilinearity, was analyzed by a linear fit to the Scatchard plot since a more complicated binding model was not justified by early observations (Bahr & Parsons, 1986b).

sedimentation velocity pelleting, equilibrium banding in a density gradient, and size-exclusion chromatography. VP_1 vesicles can be detected at each step by assaying for particulate-bound AcCh or ATP. Because the ATP assay is so much more rapid, it usually is preferred for routine detection of VP_1 vesicles.

Detection of VP_2 vesicles has been difficult in the past since they contain very little AcCh or ATP. Accordingly, they have not been purified to homogeneity, and they typically are discarded at the density gradient step in the purification of VP_1 vesicles. This appears to be a major reason VP_2 vesicles have been little studied except for the work cited in the introduction. That work involved labeling of VP_2 vesicles in intact neuronal preparations with newly synthesized radioactive AcCh. This approach cannot be applied to the large quantities of tissue required for repetitive preparative isolation of synaptic vesicles. Thus, alternative markers of VP_2 vesicles are a prerequisite for their isolation to homogeneity and extensive characterization.

The development of an in vitro biochemical assay for AcCh transport provides a new characteristic marker of cholinergic synaptic vesicles. This assay monitors authentic transport by synaptic vesicles even in crude preparations since the observed AcCh uptake was blocked by uncouplers, which suggests involvement of a transmembrane gradient. All of the AcCh-sequestering organelles have the size of synaptic vesicles, and the observed AcCh uptake was blocked by vesamicol, which is known to block AcCh transport in highly purified VP_1 vesicles and in intact neuronal preparations. No other organelle is known to transport AcCh, and after the density gradient step there is very little of the only other species likely to bind a significant amount of AcCh, namely, the nAcChR. In addition, the recent development of the vesamicol binding assay provides yet another characteristic marker of the cholinergic vesicle membrane. This marker, however, might not always be specific for synaptic vesicles per se, since Edwards et al. (1985) presented evidence that the vesamicol receptor also resides in the presynaptic cytoplasmic membrane, perhaps as a result of the vesicle exocytosis–endocytosis cycle. Thus, nonvesicular nerve terminal membrane fragments in homogenates might contain this receptor.

Use of the AcCh transport and vesamicol binding assays in this study has confirmed the conclusion of Whittaker, Zimmermann, and their colleagues that the physical properties of cholinergic synaptic vesicles are very heterogeneous. The current work shows that AcCh is transported in vitro by organelles ranging in density from 1.055 to 1.071 g/mL. The more dense organelles are slightly smaller than VP_1 vesicles, as observed by the previous workers. Since the parallelism with their work is good, the denser organelles in this work will be called VP_2 synaptic vesicles. However, the VP_2 vesicles from *T. californica* are significantly more dense than the VP_2 vesicles from *Torpedo marmorata*, which have a density of 1.065 g/mL. The VP_1 vesicles from both species have nearly equal densities (Zimmerman & Denston, 1977b). This fortuitously makes separation of the two vesicle types from *T. californica* easier.

About 35 density gradients were examined in this work. While the results presented in this paper are characteristic and representative, the relative amounts of [^{3}H]AcCh transported by VP_1 and VP_2 vesicles sometimes were greatly different from the results presented here. The origin of this variation was probed. Reassay of a particular gradient a day later reproduced the original result, which demonstrated that the VP_1–VP_2 AcCh transport pattern for a given gradient is stable. Also, a second fully separate homogenization of the tissue and preparation of another gradient with the same electric organ reproduced a similar VP_1–VP_2 pattern of [^{3}H]AcCh transport activity. Thus, while the origin of the variation among the experiments is not known, it appears to be traceable to the status of the electric organ itself. Possibly this arises from differences in the extent of stimulation of the *Torpedos* when they are sacrificed. This was not closely controlled because it is difficult when working repetitively on a large scale. Zimmermann and Denston (1977a,b) report that fully rested *Torpedo* electric organ contains a few percent of VP_2 vesicles, whereas extensively stimulated organ contains up to 74%. Our organs usually appear to contain an intermediate percentage of VP_2 vesicles, which suggests that the fish were not rested enough or were significantly stimulated during sacrifice. This too appears to be a fortuitous circumstance, since it facilitated these studies by providing larger than expected amounts of VP_2 vesicles.

It is highly likely that the AcCh transport system in VP_2 vesicles is modified relative to that of VP_1 vesicles. The ratio of AcCh transport activity to vesamicol receptor level was 4–7-fold higher in VP_2 vesicles. The relatively larger amount of receptor in the VP_1 fractions is not likely to be due to the presence of contaminating nonvesicular membrane fragments, since lysed (and therefore nontransporting) vesicles have a much greater density (Giompres et al., 1981a) and all of the vesamicol receptor in these fractions migrated with vesicle ATP, ATPase, and AcCh transport activity during size-exclusion chromatography (not shown).

If we assume that the SV2 antigen levels per vesicle do not differ between VP_1 and VP_2 vesicles (there is no evidence that they do), then we estimate that VP_2 vesicles exhibited about 2–3-fold stimulation of the AcCh transport activity and about a 2-fold decrease in the apparent amount of the vesamicol receptor. That is, *both* components of the AcCh transport system monitored here are altered in VP_2 vesicles in a roughly reciprocal manner. In view of the uncertainty about the behavior of the SV2 antigen, however, this last conclusion must be considered tentative. Certitude will require isolation of VP_2 vesicles to homogeneity. Nevertheless, no matter what the behavior of the SV2 antigen, *at least* one of the two components of the AcCh transport system is altered in VP_2 vesicles.

Even though there is some uncertainty, it is instructive to continue the discussion about these observations on the assumption that they are not misleading. The increase in the AcCh transport activity of VP_2 vesicles is consistent with the hypothesized role of VP_2 vesicles in vivo. On the other hand, the apparent decrease in the amount of the vesamicol receptor in VP_2 vesicles poses a dilemma. Because the VP_1–VP_2 interconversion is reversible (Agoston et al., 1986), it seems implausible that some of the vesamicol receptor, which is an integral protein (B. A. Bahr, unpublished observation), is actually removed from the membrane. Rather, the interesting possibility arises that some of the receptors found in a VP_1 vesicle become masked or cryptic when the vesicle is recruited to VP_2 status. Crypticity could contribute to the substantial variation in the apparent amount of vesamicol receptor per milligram of protein found in different preparations of VP_1 vesicles (Bahr & Parsons, 1986b).

The meaning of the particular values of the Hill coefficients and the equilibrium dissociation constants determined for vesamicol binding cannot be interpreted at this time. While they were essentially identical for VP_1 and VP_2 vesicles, other ongoing work demonstrates that the values for a given preparation of vesicles readily can be made to vary. Thus, we defer

detailed consideration of them. Nevertheless, while particulars are uncertain, it is clear that positively cooperative vesamicol binding exists. This suggests that two or more binding sites interact conformationally with each other in an oligomeric complex. The observed behavior is not likely to arise from an artifact such as damage to some vesicles during homogenization since nonspecific effects generally produce site heterogeneity that appears negatively cooperative. Thus, we infer that complex molecular machinery often associated with regulatory phenomena is part of the AcCh transport system.

Whether the AcCh transporter is related by evolution to other AcCh binding proteins is of fundamental interest. In addition to detecting little nAcChR in the density gradient fractions, we detected no cholinergic synaptic vesicle protein immunologically related to the nAcChR. Other experiments not shown utilized very large amounts of highly purified VP_1 vesicles, which had been solubilized in Triton X-100 and slowly passed through an agarose column containing covalently bound α-BuTx to remove most of the contaminating nAcChR before sodium dodecyl sulfate gel electrophoresis. Even then no cross-reactivity attributable to a vesicle protein was detected with either rabbit or rat anti-nAcChR antiserum (not shown).

In summary, this paper demonstrates that AcCh transport and vesamicol binding assays are useful new markers for multiple forms of the cholinergic synaptic vesicle. The AcCh transport system is complex, and a functional difference in the AcCh transport systems of VP_1 and VP_2 vesicles is preserved after electric organ disruption and partial vesicle purification. This suggests that long-lived physiological regulation of the transport system occurs. It would be premature to speculate in detail about potential regulatory mechanisms. It can be said, though, that the persistence of VP_2-like AcCh transport behavior after partial purification indicates that the preferential storage of newly synthesized AcCh by VP_2 vesicles in intact cholinergic terminals is not simply the result of their juxtaposition to the presynaptic membrane and the high-affinity choline uptake system that supplies choline for AcCh synthesis.

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