Linkage of the Acetylcholine Transporter–Vesamicol Receptor to Proteoglycan in Synaptic Vesicles†

Ben A. Bahr,‡ Krystyna Noremberg, Gary A. Rogers, Barry W. Hicks, and Stanley M. Parsons*
Department of Chemistry and the Neuroscience Research Institute, University of California, Santa Barbara, California 93106
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ABSTRACT: The relationship of the acetylcholine transporter–vesamicol receptor (AcChT–VR) to proteoglycan in *Torpedo* electric organ synaptic vesicles was investigated. The cholate-solubilized VR was immunoprecipitated by a monoclonal antibody directed against the SV1 epitope located in the glycosaminoglycan portion of the proteoglycan. AcChT that was photoaffinity-labeled with a tritiated high-affinity analogue of AcCh [cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-methylisonipecotate] and then denatured in sodium dodecyl sulfate also immunoprecipitated. The labeled AcChT exhibited a M_r range of 100 000–200 000. Proteoglycan did not engage in detectable nonspecific reversible aggregation that might mask the presence of another subunit during sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In vesicles permeabilized with cholate, the enzymes keratanase and testicular hyaluronidase inactivated binding of vesamicol and destroyed the SV1 epitope without detectable proteolysis. Other glycosaminoglycan-degrading enzymes were without effect. The results demonstrate that the AcChT–VR and proteoglycan are very strongly linked and that glycosaminoglycan-like polysaccharide controls the conformation of the VR. The unexpected linkage to proteoglycan suggests that AcChT–VR in intact terminals might communicate with extracellular matrix and participate in stabilization and operation of the synapse.

Evoked release of neurotransmitters from intraterminal synaptic vesicles (SVs)[1] depends on storage that is mediated by poorly characterized transporters. Storage of acetylcholine (AcCh) is inhibited by the drug (−)-*trans*-2-(4-phenylpiperidino)cyclohexanol (vesamicol), which binds to a cytoplasmically oriented proteinaceous receptor (the VR; Bahr & Parsons, 1986a,b; Kornreich & Parsons, 1988). The VR is conserved throughout evolution (Marshall & Parsons, 1987), and it is localized to cholinergic nerve terminal regions in mammalian brain (Marien et al., 1987; Altar & Marien, 1988). The VR might participate in a novel mechanism for regulation of cholinergic nerve terminal structure and function.

Characterization of the AcCh transporter (AcChT) and the VR is of great interest. This paper is the last in a group of four that report recent progress toward this goal. The first paper presents a kinetics model for the system that posits an allosteric VR in the AcChT (Bahr et al., 1992). The second paper presents the purification of the VR without change in its ligand binding characteristics (Bahr & Parsons, 1992). The third paper presents the results of photoaffinity labeling of the AcChT (Rogers & Parsons, 1992). The latter two studies identified material that is proteoglycan-like.

Cholinergic SV proteoglycan (PG) has been characterized by several groups (Stadler & Dowe, 1982; Walker et al., 1982; Jones et al., 1982; Carlson & Kelly, 1983; Agoston et al., 1986; Kuhn et al., 1988). It is about 25% of the vesicle protein, has an apparent M_r of 100 000–200 000 in sodium dodecyl sulfate (SDS) gradient polyacrylamide gel electrophoresis (PAGE) and is present in 10–20 copies per SV. Also it is an integral protein (Carlson et al., 1986) that carries the SV1 epitope defined by the independently obtained monoclonal antibodies (mAb) tor70 and 5G1 (Kushner, 1984; Carlson, 1989). The epitope is inside the SV and probably is on the glycosaminoglycan component of the PG. The glycosaminoglycan contains glucosamine, uronic acid, and sulfate in equal molar amounts. A link between the AcChT–VR and PG would be surprising because no PGs are known to be linked to transporter activity. Nevertheless, we report here further evidence arising from immunochemical, photoaffinity labeling, SDS–PAGE, and enzymatic approaches that the AcChT–VR complex indeed is associated with the PG in SVs purified from *Torpedo* electric organ.

Materials and Methods

Materials. *Torpedo californica* electric organ VP_1 SVs were isolated as described by Yamagata and Parsons (1989). This involves differential sedimentation velocity pelleting, equilibrium buoyant density centrifugation, and size exclusion chromatography of SVs in isosmotic glycine/sucrose solutions. Vesicles were concentrated by centrifugal pelleting. [^{3}H]-vesamicol (27 Ci/mmol) was purified as described (Bahr & Parsons, 1986b). The photoaffinity AcCh analogue cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-[^{3}H]methylisonipecotate bromide ([^{3}H]AzidoAcCh, 10.8 Ci/mmol) was synthesized as described (Rogers & Parsons, 1992). Anti-SV1 mAbs were obtained as gifts from Drs. Steven Carlson (5G1, Department of Physiology, University of Washington, Seattle, WA) and Pinky Kushner (tor70, ALS Neuromuscular Research Foundation, San Francisco, CA). Unless otherwise noted, other reagents were from usual commercial sources.

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‡ Present address: Department of Psychobiology, Center for the Neurobiology of Learning and Memory, University of California, Irvine, CA 92717-3800.

* To whom correspondence should be addressed.

[1] Abbreviations: SV, synaptic vesicle; PG, proteoglycan; AcCh, acetylcholine; AcChT, acetylcholine transporter; vesamicol, (−)-*trans*-2-(4-phenylpiperidino)cyclohexanol; VR, vesamicol receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; AzidoAcCh, cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-methylisonipecotate bromide; Tris, tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PC, phosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Immunoprecipitation of the VR. A 0.8-mL slurry of 8 mg of rabbit anti-mouse Immunobeads (Bio-Rad Laboratories) in phosphate-buffered saline (PBS) was incubated with 1.3 mL of 5G1 hybridoma supernatant overnight at 4 °C with gentle agitation as described (Buckley & Kelly, 1985). The beads were washed three times by centrifugation and resuspension in 3 mL of buffer A (0.4 M KCl, 25 mM HEPES, 10 mM EDTA, 1 mM EGTA, 0.02% (w/v) NaN_3, pH 7.9 with KOH) containing 1% (w/v) Triton X-100, then three times in 3 mL of buffer A containing 1 mg of bovine serum albumin (BSA)/mL. Beads loaded with nonspecific mouse IgG1 or heat-denatured 5G1 mAb were prepared similarly. SVs (0.1 mg/mL) were solubilized in buffer A containing 1% cholic acid, 1 mg of BSA/mL, 1 mg of phosphatidylcholine (PC, Sigma type II-S)/mL, 20% (w/v) glycerol, and the following protease inhibitors per mL: 2 μg of leupeptin, 2 μg of pepstatin, 1 μg of chymostatin (type A, B, and C mixture), 1.5 μg of antipain, 5 μg of aprotinin (FBA Pharmaceuticals), 16 μg of trypsin inhibitor (type I-S), 2.5 μg of trypsin–chymotrypsin inhibitor (Bowman–Birk inhibitor), 1.5 μg of bestatin, and 0.7 μg of *N*-(*o*-rhamnopyranosyloxyhydrophosphinyl)Leu-Trp. Beads and 40 μg of solubilized SV protein were incubated together overnight at 4 °C with gentle agitation. Beads were then pelleted by centrifugation, and the supernatants each were divided into three equal portions. Each portion was divided further into triplicate samples which were assayed by [^{3}H]vesamicol binding or quantitative immunodot blot with tor70 and 5G1 mAbs as described (Bahr & Parsons, 1992).

Immunoprecipitation of Photoaffinity-Labeled AcChT. Immunobeads were loaded with 5G1 mAb or heat-inactivated 5G1 mAb as above except that they were washed the final three times in immunoprecipitation buffer (1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 0.4 M NaCl, pH 7.4). SVs (0.4 mg of protein/mL) were incubated for 1 h at 23 °C with 0.15 mM diethyl-*p*-nitrophenylphosphate (paraoxon) to block AcCh esterase, followed by 20 min at 23 °C with 400 nM [^{3}H]AzidoAcCh in the absence or presence of 10 μM vesamicol. The SV suspensions were photolyzed with a 366-nm light source (Blak-Ray Lamp, model UVL-56; UVP, Inc., San Gabriel, CA) placed about 1.5 cm above the surface for four 2-min periods, with mixing between illuminations. Following photolysis SVs were solubilized in 2% (w/v) SDS for 10 min and diluted 20-fold into immunoprecipitation buffer containing 1 mg of BSA/mL. A 100-μg portion of treated SV protein was mixed with 8.5 mg of loaded Immunobeads and incubated overnight at 4 °C with gentle agitation. The sample was centrifuged, the supernatant was discarded, and the beads were washed four times by resuspension and centrifugal pelleting in 6 mL of immunoprecipitation buffer. The beads were resuspended in 2.5% SDS and 3% 2-mercaptoethanol and incubated at 100 °C for 4 min to remove bound antigen. The beads were removed by centrifugation, and the eluted protein in the supernatant was concentrated in an Amicon Corp. Centricon-30 device and subjected to SDS gradient (3–15%) PAGE as described (Bahr & Parsons, 1992). The lanes on half of the gel were blotted to nitrocellulose paper for detection of the SV1 epitope using 5G1 mAb as described (Bahr & Parsons, 1992). For visualization of the immunoprecipitated [^{3}H]AzidoAcCh-labeled protein, the other half of the gel containing replicate lanes was fixed, stained with Coomassie Blue, treated with Entensify (Du Pont, Inc.), dried, and exposed to film (Kodak XAR-5) for 3 weeks at −80 °C.

PG Aggregation Behavior. PG was isolated in substantially purified form as a byproduct of the purification of the vesicle P-type ATPase as described (Yamagata et al., 1989). Seventy-five micrograms was subjected to SDS–PAGE using an 8.8% resolving gel under reducing conditions. The PG was stained by incubation of the gel at 4 °C in 0.33 M KCl for 10 min followed by 1 mM 2-mercaptoethanol for 1 h (Hagar & Burgess, 1980). The potassium dodecyl sulfate that precipitates in association with protein is resistant to resolubilization and serves as a very gentle and reversible stain of protein. The sample lane was cut into seven 1-cm segments extending from the top of the resolving gel toward the bottom. Each segment was homogenized in 2 mL of 25 mM Tris, 192 mM glycine, 0.1% SDS, 150 mM NaCl, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol and incubated with agitation for 4 h at 23 °C. Acrylamide was pelleted at 500*g* for 10 min. Two hundred microliters of each supernatant was subjected to SDS–PAGE and further analyzed as described in the legend to Figure 2.

Effects of Enzymes Degrading Different Classes of Glycosaminoglycans. SVs (0.8 mg of protein/mL) were suspended in 0.72 M glycine and 0.10 M HEPES adjusted to pH 7.8 with NaOH. The following protease inhibitors were present: 1 mM EGTA; 1 mM EDTA; 0.1 mM phenylmethanesulfonyl fluoride; 0.1 mM *p*-aminobenzamidine; 10 μg/mL each of leupeptin and pepstatin; 8 μg/mL each of chymostatin, antipain, bestatin and aprotinin; 20 μg/mL each of trypsin inhibitor and trypsin–chymotrypsin inhibitor; and 4 μg of phosphoramidon/mL. Intact vesicles or vesicles permeabilized by 0.4% sodium cholate were incubated at 37 °C in the absence or presence of bovine testicular hyaluronidase at a concentration of 5 milliunits/mL. Similar samples were prepared with heat-inactivated hyaluronidase. The hyaluronidase (Sigma type VI-S) first was purified by affinity chromatography on heparin–agarose to a specific activity of 52 milliunits/mg at pH 7.8 in order to reduce protease activity (Srivastava & Farooqui, 1979). One unit of hyaluronidase is the amount of enzyme producing 1 μmol of *N*-acetylglucosamine reducing equivalent/min from hyaluronate under the same conditions. At the indicated times, 10 μL of vesicle suspension was diluted into 110 μL of the same buffer at 23 °C containing 0.8 μM [^{3}H]vesamicol and no protease inhibitors, and the amount of specifically bound [^{3}H]vesamicol was determined as described (Bahr & Parsons, 1992).

For keratanase treatment, vesicles (0.5 mg protein/mL) were suspended in 0.70 M glycine containing the protease inhibitors and 50 mM Tris-HCl adjusted to pH 7.8 with maleate. Intact vesicles or vesicles permeabilized by 0.4% sodium cholate were incubated at 31 °C in the absence or presence of keratanase at a concentration of 17 units/mL (*Pseudomonas sp.* keratan sulfate endo-β-galactosidase, Seikagaku America, Inc., 20 units/mg). Similar samples were prepared with heat-inactivated keratanase. One unit of keratanase is the amount of enzyme producing 1 μmol of galactose reducing equivalent/h at 37 °C from bovine corneal keratan sulfate at pH 7.4. Incubated vesicles were assayed for binding of [^{3}H]vesamicol as above. Similarly incubated vesicle samples were subjected, after loss of vesamicol binding, to SDS–PAGE and staining with silver or to western blot analysis using 5G1 mAb visualized with horseradish peroxidase-conjugated rabbit anti-mouse IgG and 3,3′-diaminobenzidine as described (Bahr & Parsons, 1992).

Results

The VR Is Immunoprecipitated by Anti-PG mAb. Cholate-solubilized synaptic vesicles were subjected to immunoadsorption by the anti-SV1 mAb 5G1 bound to poly-

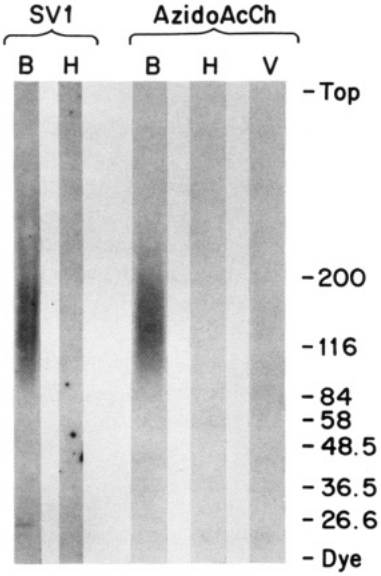

FIGURE 1: Anti-SV1 immunoprecipitation of affinity-labeled SDS-denatured synaptic vesicles. Synaptic vesicles that had been photoaffinity-labeled with [^{3}H]AzidoAcCh in the absence (B, H) or presence (V) of vesamicol were solubilized in SDS and subjected to immunoprecipitation using Immunobeads loaded with 5G1 (B) or heat-inactivated 5G1 (H) mAb. Precipitated material was dissolved in SDS under reducing conditions, subjected to SDS 3–15% gradient PAGE, and analyzed by western blot or autofluorography to detect secondary [^{125}I]anti-mouse IgG bound to 5G1 primary mAb (lanes labeled SV1) or covalently linked [^{3}H]AzidoAcCh (lanes labeled AzidoAcCh), respectively. Locations of molecular mass standards (kDa), the top of the resolving gel, and the dye front are shown to the right.

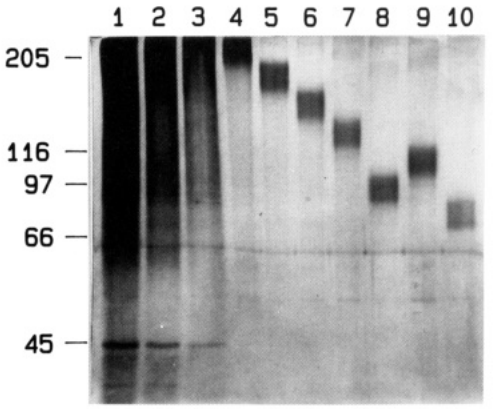

FIGURE 2: Heterogeneity of cholinergic synaptic vesicle PG. Purified PG was subjected to semipreparative SDS–PAGE, and PG in one-cm blocks extending from the top of the resolving gel was eluted as described under Materials and Methods. The separated portions of PG were subjected, along with standards, to SDS–PAGE in a similar gel that then was stained with silver. Lanes 1, 2, and 3 contained 25, 5, and 1 μg, respectively, of total PG. Lane 4 contained the PG eluted from the PAGE segment spanning 0–1 cm; lane 5, 1–2 cm; lane 6, 2–3 cm; lane 7, 3–4 cm; lane 8, 5–6 cm; lane 9, 4–5 cm; lane 10, 6–7 cm. The order of eluted segments was reversed in lanes 8 and 9 to ensure that a systematic increase in the electrophoretic mobility did not occur because of a gel defect. The locations of standard proteins (kDa) are shown to the left. Fingerprint proteins are present at about 63 kDa (Ochs, 1983). An identical gel was subjected to western blot analysis with the 5G1 mAb instead of silver staining (not shown). Lanes 1, 2, and 3 stained for the SV1 epitope from the top to about 4 cm down from the top of the resolving gel. Lanes 4–7 also stained in blocks corresponding to the silver stain whereas lanes 8–10 did not stain with the mAb. Similar results were obtained with another PG preparation.

acrylamide beads, and relevant characteristics of the immunoprecipitate and supernatant were determined. As assayed by quantitative immunodot blot using both 5G1 and tor70 mAbs, 74 ± 5% of the epitope was removed from the supernatant. The same fraction of the VR also was removed. All of the VR lost from the supernatant was recovered in the precipitate. This demonstrates that mAb 5G1 has no effect on vesamicol binding. The immunoadsorption was specific to native mAb since neither heat-denatured 5G1 mAb nor nonspecific IgG1 bound to polyacrylamide beads resulted in precipitation of the SV1 epitope nor the VR. Similar results were obtained in a total of three vesicle preparations. Thus, the SV1 epitope is associated with the VR in cholate detergent.

Heterogeneous, Photoaffinity-Labeled AcChT Is Immunoprecipitated by Anti-PG mAb. As cholate is a mild detergent that preserves native VR, the VR could be associated with the SV1 epitope through weak noncovalent interactions. The tightness of the association was probed through the linked AcCh binding site as follows. The AcCh analogue cyclohexylmethyl *cis*-*N*-(4-azidophenacyl)-*N*-[^{3}H]methylisonipecotate bromide ([^{3}H]AzidoAcCh, 10.8 Ci/mmol) is a high-affinity competitive inhibitor both of AcCh active transport and vesamicol binding to the allosteric site (Rogers & Parsons, 1992). It was used to photoaffinity label the AcCh binding site of intact vesicles. The labeled vesicles were solubilized in SDS and subjected to immunoprecipitation with anti-SV1 mAb. The immunoprecipitated material was separated by SDS gradient PAGE, and the separated proteins were assayed for the SV1 epitope by western blotting, and for covalently bound [^{3}H]AzidoAcCh by autofluorography. The SV1 epitope and a radiolabeled species were detected (Figure 1). They exhibited similar diffuse patterns at the same M_r of about 100 000–200 000. Vesicles affinity-labeled with [^{3}H]AzidoAcCh in the presence of vesamicol produced a nearly blank autofluorogram demonstrating that the detected labeling was specific for the AcChT–VR. Also, beads prepared with heat-denatured anti-SV1 mAb precipitated neither detectable SV1 epitope nor [^{3}H]AzidoAcCh-labeled protein (Figure 1). These results demonstrate that the AcChT is very tightly linked to the SV1 epitope since the association survives denaturation with SDS. Also, the result confirms the hypothesis that the VR is an allosteric site in the AcChT.

Cholinergic Synaptic Vesicle Proteoglycan Does Not Form Loose Aggregates in SDS. A potential complication in the interpretation of the above experiments lies in the possibility that the vesicle PG engages in nonspecific aggregation with itself or other proteins, even at high ionic strength and under reducing conditions in SDS. This was tested by carrying out repetitive SDS–PAGE of a substantially purified preparation of vesicle PG (Figure 2). A constant-percentage resolving gel was used rather than a gradient gel, and this resulted in smoothly decreasing intensity of PG distributed from the top of the resolving gel (>200 000 M_r) down to about 60 000 M_r. The change in electrophoretic behavior when comparing gradient to constant-percentage gels has been observed many times. The more heterogeneous mobility of the PG in the constant-percentage gel facilitated its fractionation into different M_r ranges. The severe streaking of PG in the gel is inherent in the structure of PG and does not reflect a poorly performing gel system.

SDS–PAGE on a semipreparative scale was carried out on vesicle PG. One-centimeter blocks of gel corresponding to different M_r ranges were excised, and the PG was eluted from them. The recovered PG fractions were subjected to SDS–PAGE in adjacent lanes on the same type of constant-percentage gel, along with several different amounts of unfractionated PG standard. Essentially the same electrophoretic mobilities observed in the first gel were reproduced in the

second gel as a ladder of 1-cm long blocks of protein staining (Figure 2). Moreover, a western blot demonstrated that SV1 epitope was located in the total PG from the top of the resolving gel down to about 100 000 M_r, and the 1-cm blocks exhibited the same staining pattern for the SV1 epitope (not shown). This result demonstrates that the electrophoretic behaviors of the PG and the SV1 epitope are stable toward repetitive manipulation of the sample. Moreover, delipidation of vesicles by extraction with wet acetone did not change the electrophoretic behavior of the PG (L. M. Gracz, unpublished observation). The extremely heterogeneous nature of the PG is authentic and not due to progressive dissociation of nonspecific aggregates during the electrophoresis.

Some Enzymes Degrading Glycosaminoglycans Destroy the SV1 Epitope and Inactivate the VR. It was of interest to ask whether the glycosaminoglycan portion of the PG plays an important role in the conformation of the AcChT–VR. To address this question, synaptic vesicles were permeabilized with cholate detergent and incubated in the presence of protease inhibitors with highly purified enzymes that degrade different types of glycosaminoglycan. Heparinase, heparitinase, chondroitinase ABC, and *Streptomyces* hyaluronidase had no effect on binding of vesamicol. Keratanase, which hydrolyzes keratan sulfate glycosaminoglycan and related polysaccharides (Nakazawa et al., 1989), after a lag inactivated the VR in a time-dependent manner by 50–80% in different preparations of vesicles (Figure 3). The residual binding did not decrease upon addition of more keratanase, demonstrating that the VR-inactivation plateau did not arise from inactivation of the enzyme. Testicular hyaluronidase, which hydrolyzes both hyaluronic acid and chondroitin sulfate glycosaminoglycans (Kresse & Glossl, 1987), after a lag inactivated most of the vesamicol binding (Figure 3). If vesicles were not permeabilized or if heat-denatured enzymes were used, no vesamicol binding was lost (Figure 3).

The vesicular substrate for testicular hyaluronidase likely is neither hyaluronic acid nor chondroitin sulfate because similar activity levels of the more specific enzymes *Streptomyces* hyaluronidase and chondroitinase ABC had no effect. The hydrolyzed substrate presumably is a glycosaminoglycan because the enzyme had been affinity purified. Enzymatic attack on glycosaminoglycan was confirmed for both keratanase and testicular hyaluronidase because western blot analysis of the SV1 epitope demonstrated that it was completely destroyed by keratanase and nearly completely destroyed by testicular hyaluronidase (Figure 3). SDS–PAGE of vesicles that had lost drug binding confirmed an absence of proteolysis in both cases (Figure 3).

Discussion

The results obtained here and in the preceding papers of this series provide strong evidence that the AcChT–VR is a single complex that is very tightly linked to PG. Five independent lines of evidence support this conclusion. First, it has been possible to develop a successful kinetics model based on an allosteric VR. Second, purified VR contains PG-like material that retains the AcCh binding site. Third, photoaffinity labeling of the AcChT identifies PG-like material that is protected from labeling by vesamicol. Fourth, both the VR and AcChT are associated with the SV1 epitope that is characteristic of PG. Fifth, incubation of permeabilized vesicles with certain glycosaminoglycan-degrading enzymes inactivates the VR and the SV1 epitope. Some comments on these observations follow.

Inactivation of the VR by the enzymes could occur by means of induced conformational changes in the drug binding site that are propagated from the sites of attachment of the hydrolyzed polysaccharides. The critical polysaccharide probably includes keratan sulfate–like sugar, but the substrate for testicular hyaluronidase is not clear. Both substrates are inside of the vesicles where the SV1 epitope and glycosaminoglycan are located. It seems likely that the substrates are glycosaminoglycan, but the proportion of the total glycosaminoglycan that is susceptible to keratanase or testicular hyaluronidase must be minor as little change occurred in the electrophoretic behavior of the PG after exposure to the enzymes. The vesamicol binding that is resistant to keratanase could be linked to a sulfation or branching variant of keratan sulfate that is not susceptible to attack (Nakazawa et al., 1989).

It is unlikely that the association of the AcChT–VR with PG arises as an artifact of detergent solubilization, as it persists under a wide range of conditions. No trace of a dissociated form of the AcChT was seen in SDS–PAGE of photoaffinity-labeled vesicles (Rogers & Parsons, 1992). No evidence that proteoglycan or SV1 epitope engage in nonspecific aggregation in SDS was observed in the current study. Finally, it is unlikely that a specific minor fraction of the glycosaminoglycan (namely, keratan sulfate- and hyaluronate- or chondroitin sulfate-like) would control the conformation of the VR in a nonspecific, artifactual association between PG and AcChT–VR. Conformational control by keratanase- and testicular hyaluronidase-sensitive polysaccharide suggests that linked PG plays a critical role in the function of the AcChT–VR.

At least two types of linkage to PG can be envisioned. In the first, the AcChT–VR and the PG core protein might be separate gene products that become posttranslationally associated. This could occur covalently or noncovalently, but in the latter case a noncovalent complex would have to have extraordinary stability. Some very interesting observations on electric organ SV PG are consistent with posttranslational association. Newly synthesized PG labeled with $^{35}SO_4^{2-}$ is transported to the nerve terminal in immature synaptic vesicles. As the vesicles mature, they release 80% of the label, and the M_r of the PG decreases approximately to that observed for the AcChT–VR (Kiene & Stadler, 1987; Stadler & Kiene, 1987). Moreover, the SV1 epitope is generated in the nerve terminal and not in the soma (Caroni et al., 1985). These observations demonstrate that a precursor form of the PG is processed in the nerve terminal. This processing might be coupled to formation of the link to the AcChT–VR.

In the second possible type of linkage, the AcChT–VR and the PG might be a single gene product. Possibly a transmembrane, globular domain of the AcChT–VR carries the AcCh and vesamicol binding sites. A luminal C–terminal or N–terminal tail could serve as the core protein of a PG–like domain carrying the SV1 epitope, keratan sulfate-like polysaccharide, and other glycosaminoglycan. Although no PGs previously have been found to exhibit transporter activity, many have transmembrane domains (Rouslahti, 1989). There is no apparent fundamental reason that such domains could not be elaborated to have a transport function. In this event, PG processing in the nerve terminal might generate the two immunochemically related PGs found in the electric organ vesicles (Bahr & Parsons, 1992). Another way in which closely related PGs could arise is through alternative splicing of a single pre-mRNA.

The observations on the *Torpedo* system are relevant to the mammalian synapse because immunologically related PG is conserved (Kushner, 1984; Volknandt & Zimmermann, 1986; Bjornskov et al., 1988). Moreover, the *Torpedo* AcChT–VR

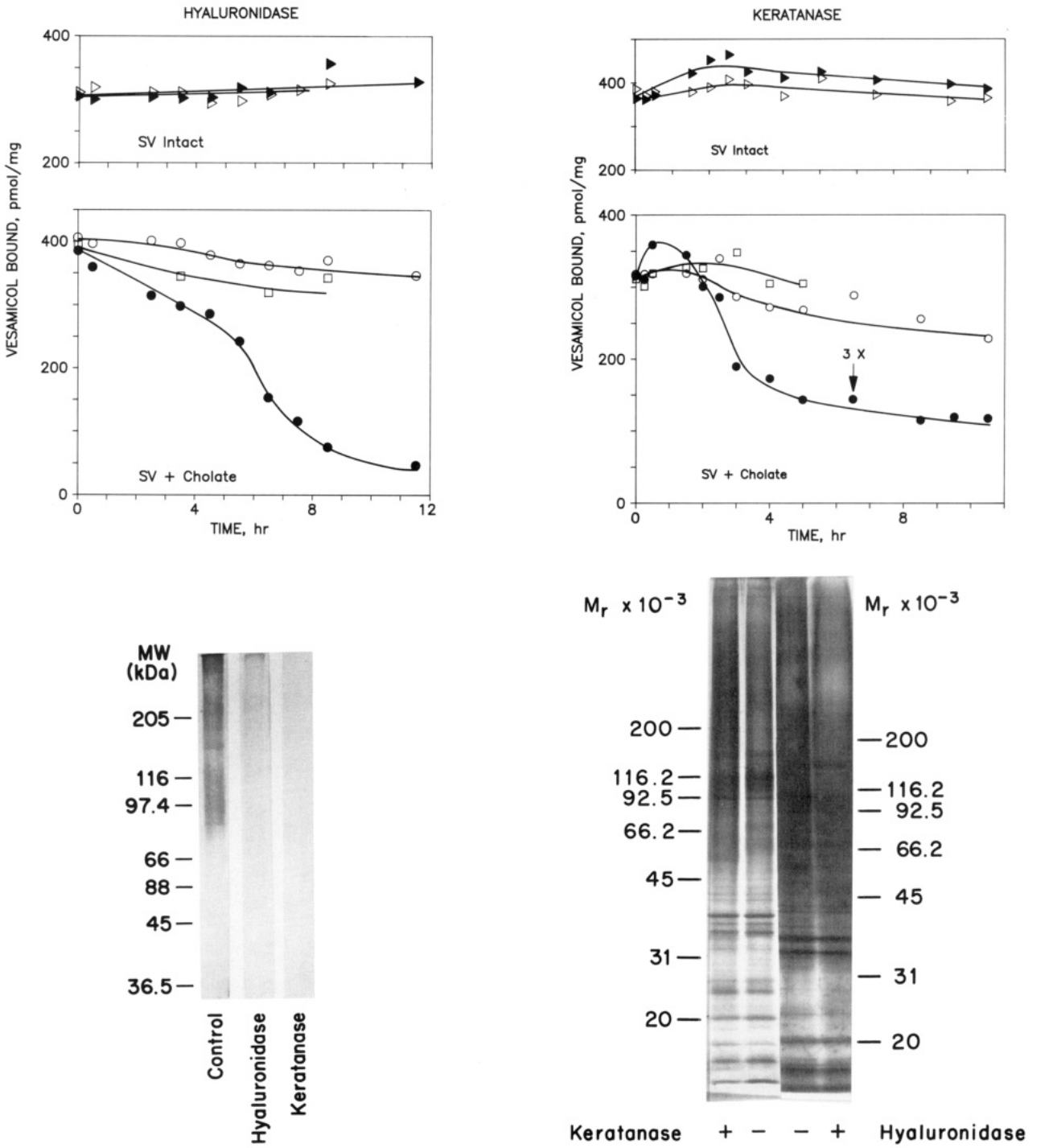

FIGURE 3: Incubation of synaptic vesicles with glycosaminoglycan-degrading enzymes. (Upper left panels) Incubation of intact (▲, △) or cholate-permeabilized (●, ○, □) vesicles in the absence (△, ○) or presence (▲, ●) of active bovine testicular hyaluronidase or heat-denatured hyaluronidase (□) was carried out as described under Materials and Methods. Binding of [^{3}H]vesamicol was determined at the indicated times. Specific binding is reported as the average of triplicate data whose relative standard deviations were less than 5%. (Upper right panels) Incubation as above in the absence or presence of active or heat-denatured keratanase was carried out as described under Materials and Methods, and binding of [^{3}H]vesamicol was determined and reported as above. The arrow indicates the time at which the keratanase concentration was increased by 3-fold. (Lower left panel) Vesicles that had been permeabilized with cholate and incubated overnight in the absence or presence of hyaluronidase or keratanase under the above conditions were analyzed by SDS–PAGE and western blot using 5G1 mAb. Positions of prestained molecular weight standards (kDa) are shown. (Lower right panel) Similarly treated vesicles were analyzed by SDS–PAGE and silver staining. The positions of molecular mass standards (kDa) are shown adjacent to each set of separately run lanes. Similar results were obtained many times in all of the experiments.

contains the SV2 epitope (Bahr & Parsons, 1992), which is found on the cytoplasmic surface of most mammalian synaptic and endocrine secretory vesicles regardless of type (Buckley & Kelly, 1985; Low et al., 1988; Floor & Feist, 1989; Volknandt & Zimmermann, 1990; Weiler et al., 1989). Linkage of the AcChT–VR to proteoglycan *and* the ubiquitous SV2 epitope suggests that the AcChT–VR plays an additional role in the economy of the nerve terminal beyond mediating AcCh storage.

Evidence that the AcChT–VR resides in the cytoplasmic membrane has been obtained. Vesamicol and other rather different pharmacological agents that block vesicular storage of AcCh also block nonquantal release of AcCh from the mototerminal (Edwards et al., 1985; Zemkova et al., 1990;

Meriney et al., 1989; Smith, 1992). Nonquantal release is thought to arise from the cytoplasm by facilitated leakage through the presynaptic membrane. Differential processing of the AcChT-VR might direct some of it to remain in the mature synaptic vesicle and some to translocate to the cytoplasmic membrane during vesicular fusion and exocytotic events, where it could mediate nonquantal release of AcCh. The latter form of the AcChT-VR would present the linked PG domain to the extracellular matrix. Buckley et al. (1983) speculated that the vesicle PG interacts with the matrix, as SV1 epitope externalized by exocytosis does not diffuse away from the synaptic gap.

These observations provide the basis for a new hypothesis regarding the connection between physiological activity of nerve terminals and synapse stabilization (Van Essen et al., 1990; Magchielse & Meeter, 1986; Rich & Lichtman, 1989; Callaway et al., 1989; Wigston, 1990). Neurotransmitter release might help establish or replenish a link between the terminal and extracellular synaptic matrix, where the link consists of the AcChT-VR complex containing an intracellular anchoring region and an extracellular PG region with synaptic matrix binding properties. Results obtained from coculture of embryonic mouse spinal cord neurons with myotube variants suggest that cholinergic synaptic vesicle protein stabilizes neurite contact with myotube surface PG (Lupa et al., 1990). This is consistent with the observation that neuronal growth cones contain the neurotransmitter release machinery (Lockerbie, 1990). The recent observation that VR levels in Alzheimer brain do not decrease, possibly in an attempt by spared neurons to compensate for the loss of cholinergic terminals (Ruberg et al., 1990; Kish et al., 1990), is consistent with a role for the AcChT-VR complex in maintenance of the synapse. Involvement of different forms of the AcChT-VR complex in both neurotransmitter storage and synapse stabilization would provide an elegantly economical, use-dependent, and coordinated contribution to formation of functional synapses. The identification of one form of SV2 protein as transporter-associated suggests that this hypothesis might also be applicable to other nerve terminal types. The unexpected macromolecular and immunochemical structure of the AcChT-VR complex elucidated here provides a novel framework of relationships for previously unconnected observations.

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Registry No. AcCh, 51-84-3; vesamicol, 22232-64-0.

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Binding of Adenine Nucleotides to the F_1-Inhibitor Protein Complex of Bovine Heart Submitochondrial Particles†

Orlando B. Martins,‡ Ione Salgado-Martins,§ Maria Angela B. Grieco,‡ A. Gômez-Puyou,‖ and M. Tuena de Gômez-Puyou*,‖

Departamento de Bioquîmica, Instituto de Ciencias Biomêdicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, Departamento de Bioquîmica, Instituto de Biologia, Universidade Estadual de Campinas, 13081 Campinas, Sao Paulo, Brazil, and Instituto de Fisiologîa Celular, Universidad Nacional Autônoma de Mêxico, Apartado Postal 70-600, Mêxico D. F., 04510 Mêxico

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ABSTRACT: The binding of ATP radiolabeled in the adenine ring or in the γ- or α-phosphate to F_1-ATPase in complex with the endogenous inhibitor protein was measured in bovine heart submitochondrial particles by filtration in Sephadex centrifuge columns or by Millipore filtration techniques. These particles had 0.44 ± 0.05 nmol of F_1 mg^{-1} as determined by the method of Ferguson et al. [(1976) *Biochem. J. 153*, 347]. By incubation of the particles with 50 μM ATP, and low magnesium concentrations (<0.1 μM MgATP), it was possible to observe that 3.5 mol of [γ-^{32}P]ATP was tightly bound per mole of F_1 before the completion of one catalytic cycle. With [γ-^{32}P]ITP, only one tight binding site was detected. Half-maximal binding of adenine nucleotides took place with about 10 μM. All the bound radioactive nucleotides were released from the enzyme after a chase with cold ATP or ADP; 1.5 sites exchanged with a rate constant of 2.8 s^{-1} and 2 with a rate constant of 0.45 s^{-1}. Only one of the tightly bound adenine nucleotides was released by 1 mM ITP; the rate constant was 3.2 s^{-1}. It was also observed that two of the bound [γ-^{32}P]ATP were slowly hydrolyzed after removal of medium ATP; when the same experiment was repeated with [α-^{32}P]ATP, all the label remained bound to F_1, suggesting that ADP remained bound after completion of ATP hydrolysis. Particles in which the natural ATPase inhibitor protein had been released bound tightly only one adenine nucleotide per enzyme. The results indicate that one of the first events that occurs during ATP hydrolysis by the F_1-inhibitor protein complex is the binding of two to three adenine nucleotides to sites that apparently are not hydrolytic. In addition, it was found that in the complex, the affinity of two to three of its adenine nucleotide binding sites is higher than in particulate enzymes devoid of the inhibitor protein.

The membranes of mitochondria, chloroplasts, and bacteria have an enzyme known as H^+-ATPase synthase or H^+-ATPase. This enzyme utilizes the energy of electrochemical H^+ gradients to catalyze the synthesis of ATP; under some conditions, the enzyme hydrolyzes ATP, and this results in the formation of electrochemical H^+ gradients (Mitchell, 1961). The enzymes have a hydrophobic portion (F_o) that transports H^+ across the membrane to a hydrophilic moiety known as F_1. The latter catalyzes the synthesis and hydrolysis of ATP [for reviews, see Cross (1981), Senior and Wise (1983); Hatefi (1985a), Futai et al. (1989), and Senior (1990)] and is formed by five different subunits with a stoichiometry α_3, β_3, γ, δ, and ϵ. In addition, the mitochondrial F_1 has a detachable low molecular weight protein, known as the natural ATPase inhibitor (Pullman & Monroy, 1963), that inhibits ATP hydrolysis, the ATP-dependent reactions of the inner mitochondrial membrane (Asami et al., 1970; Ernster et al., 1973), and the initial events of oxidative phosphorylation (Gomez-Puyou et al., 1979; Harris et al., 1979; Schwerzman et al., 1981) and favors the accumulation of ATP as driven by electron transport (Beltran et al., 1986).

Mitochondrial F_1-ATPase has six adenine nucleotide binding sites, three of which are considered catalytic; the other are

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*Correspondence should be addressed to this author.
‡Universidade Federal de Rio de Janeiro.
§Universidade Estadual de Campinas.
‖Universidad Nacional Autônoma de Mêxico.

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